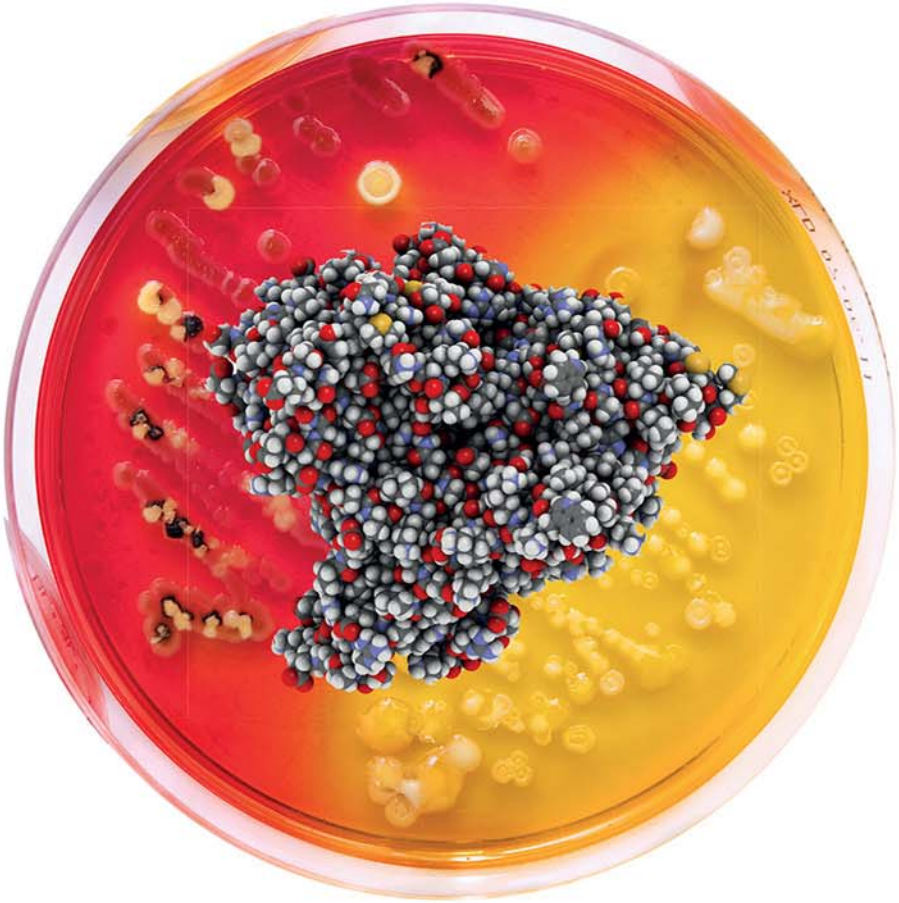
ENZYMES IN HUMAN AND ANIMAL NUTRITION

Principles and Perspectives



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Enzymes in Human and Animal Nutrition

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Preface

In modern-day nutrition research, there is a distinct division between the nutrition of humans and animals. Even within animal nutrition, there are clear demarcations between those involved in nutrition research for pigs, poultry, ruminants, fish, and companion animals (dogs, cats, and horses). This discrimination between species may seem logical from a practical point of view as there are distinct physiological and biochemical differences between humans and terrestrial animals, as well as between animals. However, there are far more aspects of nutrition which all species have in common. In academia, research, and even industry, animal and human nutrition appear as two separate worlds, each with its own scientific approaches and foci. Human and animal nutrition departments exist side by side in many organizations (e.g., universities, companies), more often than not, living within their own world with cooperation between these departments being, unfortunately, rather the exception than the norm. Over recent years, the concept of OneHealth has been (re)introduced. Within this concept, a multiple-discipline approach is taken to provide the best health for people, animals, and our environment.

A recent example of a OneNutrition approach can be found in the area of protein quality evaluation of human foods. Whereas in the past the protein digestibility corrected amino acid score (PDCAAS) system was used for the evaluation of protein quality of human foods, the recent FAO approach has been to capitalize on the decades of methodology development in pig nutrition (ileal digestibility) and adopt the digestible indispensable amino acids score (DIAAS) system. Where the previous PDCAAS values were hindered by (often major) inaccuracies from a correction by rat fecal nitrogen digestibility values, the new DIAAS system utilizes state-of-the-art standardized ileal digestibility values of individual amino acids of an animal species more similar in digestive physiology to humans. Although ultimately such measurements should be conducted on the species of interest, in this case humans, until more accurate methodologies are developed, a OneNutrition approach will provide more accurate data.

The OneNutrition concept also provides an excellent approach in our understanding of the nutrition of individual species. Differences (and similarities) in anatomy and digestive physiology as well as specializations (or adaptations) of species in their metabolism of nutrients as a result of diet-induced evolutionary adaptations, can provide insights into species-specific nutrition. For example, the spatial localization of alanine:glyoxylate aminotransferase 1 (AGT1), responsible for the removal of glyoxylate which is involved in hyperoxaluria, seems to be species dependent. In carnivores and insectivores, AGT1 is mainly present in mitochondria of liver cells, while in humans, Old World monkeys (macaques, baboons), rabbits, and guinea-pigs, AGT1 is almost exclusively located in the peroxisome. The mitochondrial localization of AGT1 is seen in carnivorous and insectivorous species of different genera (mammals, birds, reptiles), indicating

that AGT1 localization in the mitochondrion might be required when consuming high-protein, low-carbohydrate diets. In rodents (rats, mice, hamsters) and marmosets (New World monkey), AGT1 is distributed approximately equally between both organelles. These species differences in intracellular localization of hepatic AGT1 provide clear indications of dietary selection pressure during evolution and hence guidance to the nutrition of individual species.

An area in which there appears to be little overlap between human and animal nutrition, research is in the application of enzymes. The use of enzymes in human food production dates back to 6000 BC or earlier, first as a product of microbial fermentation, for example in the production of beer, wine, cheese, and yogurt. For decades, industrially produced, more or less pure, enzymes have been used in the production of bread and lactose-free milk, to mention a few, to improve quality (bread) or make nutritious milk accessible to lactose-intolerant people. Most enzymes used in human nutrition are, however, used during food production/processing. Very few enzymes are used as such, meaning they are consumed (as a small pill or a few drops) to act within the gastrointestinal tract where they should degrade specific unwanted factors. Current examples are enzymes to degrade lactose and gluten for lactose- and gluten-intolerant people, respectively.

In contrast, apart from enzymes produced during fermentation processes as in the production of silage, enzyme application in animal feeds is only a relatively recent phenomenon. In the 1980s, the first enzyme products gaining commercial importance entered the feed market. These first enzymes mainly degraded fiber; xylanase and β-glucanase. In 1991, the first commercial viable phytase entered the feed market, which rapidly changed the entire landscape. Nowadays, most poultry and pigs feeds contain specific enzyme products. They are active in the stomach and intestines and degrade antinutritional factors or improve the nutritional value of the feed for the animal.

Obviously, the OneNutrition approach can also be applied to enzymes. Unwanted (antinutritional) factors in animal feeds are very likely also unwanted in the diet of humans, and vice versa. What are such common unwanted factors? Which compounds can be broken down to improve the nutritional value for animals and could also improve the nutritional value for humans? This could be either by degradation of unwanted factors (similar to lactose and gluten), or by increasing the digestibility and availability of (relatively) scarce nutrients. Examples are increasing the availability of some amino acids for athletes, sick, or elderly people, and of specific minerals in populations of people with deficiencies. Improving the nutritional value of foods in general is a clear objective for feeding undernourished people. And, vice versa, can enzymes that are presently used in food production be applied to improve the nutritional value of animal feeds?

This book reintroduces the once (pre-1970) common approach to nutrition, that of OneNutrition by posing these questions and thoughts. The editors, Prof. Dr. Carlos Simões Nunes and Assistant Professor Dr. Vikas Kumar, realized that enzymes are important in both animal and human nutrition and that by bringing our current knowledge in these, hitherto, two separate areas into one book, the

reader is able to develop new insights for applications of enzymes in foods and feeds. The authors of the various chapters have a wealth of knowledge in various aspects of enzymes for feed and food. After chapters dealing with more general aspects of enzymes, phytase is discussed in detail. The direct application of phytase has been extensively investigated in pigs and poultry, but has also been investigated for use in humans. Although for humans the improved digestibility of phosphorus may be less important than for animals, its effect on micro-minerals such as iron and zinc may be of great importance for many people.

In 10 chapters, depolimarizating enzymes are discussed, both from a food and feed perspective. The multitude of applications of such enzymes is amazing, and we are only at the beginning of our understanding of how these can be used more effectively. Increased understanding of vegetable cell wall composition and morphology, and of their effect within the gastrointestinal tract will ultimately result in the development of more specific enzymes to break down or modify these complex structures. In combination with currently used enzymes such as xylanases and cellulases, they will not only enable the more complete use of potential food/feed energy, but also induce specific effects on the gut wall and the gut microflora, resulting in improved gut health (for both humans and animals). Lately, the importance of the microbiota in human diseases such as obesity has been described. The production of prebiotics by a specific (combination of) enzyme(s) may promote a favorable microbial balance, and thus promote health.

In the third part of the book a number of different enzymes and some direct microbials are discussed. I was especially triggered by the chapter on chitinases. Given the future predicted protein shortage and our focus on the use of insects in feed and food, this enzyme may prove to be of great importance. Protein digestibility of many insects is limited because of the presence of chitin, but may be greatly improved by effective application of chitinases.

The final area of focus of the book deals with important technological issues related to enzyme use and production: formulation and analysis, the continued discussion of regulatory aspects, and the overall questions regarding economy. The final chapter of the book contains a great review regarding the potential of enzymes for both humans and animals, discusses general perspectives, and provides conclusions.

The editors should be complemented on bringing together many experts in the field of enzyme use in feeds and foods and achieving the OneNutrition approach in the use of enzymes in nutrition. We have to look into each other's kitchens and silos more often! This book allows the reader to look into those silos and kitchens, and be able to develop new insights and understanding of the application of enzymes in food and feed.

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First of all, we would like to thank Elsevier for accepting our proposal for this book more than 2 years ago. The challenge has been handled professionally and also in a friendly manner, particularly by Jaclyn Truesdell and Megan Ball.

We believe in the interest of the present book, taking into account that humans and animals are targets for dietary supplements of microbial enzymes. There are plenty of published works on industrial applications and, more rarely, on direct human or animal applications. Thus far, extensive reviews on both aspects have not been frequently available. Also, subjects such as the basis and the state-of-the-art regarding the use of enzymes in therapy, decontamination, and remediation, as well as intellectual property, represent quite a new development in the present work.

Obviously we would like to express our deep recognition of the difficult, but fantastic work performed by all of the co-authors. One of us, Carlos Simões Nunes, has cooperated, for a longtime, with many of them in a very stimulating, constructive, and productive manner; they have become, after the start of shared work more than 30 years ago, very good friends. On this matter, we would like to mention the following in a very similar rank: Kjell Malmlöf, Glenn Monastersky, Petra Phillips, Guido Rychen, and Kurt Vogel.

We miss the contribution of two very important colleagues, for reasons that are independent of their personal choice. Both are highly personally respected colleagues and furthermore friends, with a stimulating capacity for discussions. They share a broad knowledge and experience of nutritional physiology and food and feed additives. We have always had, and continue have interesting exchanges with them, particularly on several perspectives of the development of new enzymes and the prospect of enzyme applications.

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Introduction

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INTRODUCTION

Enzymes are pivotal for the mechanisms which maintain all forms of life. To name just a few processes, photosynthesis, respiration, and homeostasis could not occur with necessary speed, without enzymes. Enzymes are proteins, and their importance in nature is indicated in human medicine by the fact that the mutation of one single base pair, leading to the disruption of the expression of just one protein-enzyme, can result in disabling metabolic disorders, or even death of the neonate. The power of these natural, chemical catalysts can be harnessed for industrial purposes; in the context of this chapter, most specifically for improving the speed and outcome of hydrolytic, digestive processes.

The definition of catalysis is to enable a reaction to proceed at an increased speed than it would otherwise. All processes catalyzed by enzymes would occur in their absence but at a much reduced rate. These activities may be synthetic, hydrolytic, or transformative, but at such a slow rate that it would not be of any value for the process under catalysis, whether it be to sustain life or for industrial conversion purposes. From the beginning of the reaction to the end, there is energy released which drives the reaction forwards, but in order to initiate the reaction a certain amount of energy has to be provided, the activation energy, in order to move the substrate from its current and probably stable state to a transition state. At this point, the forward reaction produces the product and the reverse takes it back to the substrate. The rate at which a reaction would proceed in the absence of the enzyme is dependent on the energy released in the conversion of the substrate to the product, and the activation energy needed to reach the transition state. Some reactions are incredibly slow due to a very high activation energy, which can also be related to how stable the substrate is, and a marginal release in energy in the whole reaction. In such cases, enzymes can speed up the reaction by an almost unimaginable rate. For example, the decarboxylation of orotidine-5′-phosphate to uridine monophosphate (a step in the pyrimidine synthetic pathway) would normally take millions of years, but in the presence of orotidine-5′-phosphate decarboxylase this takes place in milliseconds. This is the most extreme example of what enzymes are capable of, as it accelerates the uncatalyzed process by a factor of 10^{17}.

To put this into perspective, this is greater than the number of seconds that the universe has been in existence. Clearly most enzymes are not responsible for such extreme degrees of acceleration of a reaction, and indeed such feats would be

problematic for most synthetic pathways where more than one enzyme is involved.

All enzymes in nature do not work in isolation, but as part of a co-ordinated process, or pathway, such that the product of one enzyme may become the substrate of another. Evolution has resulted in pathways that employ multiple enzymes in the transformation, synthesis, or hydrolysis of compounds into the desired outcome, and the successful integration of many if not all of the individual pathways involved in the process of life means that each process needs to be aware of the overall status and needs of the cell and indeed the whole being, whether it is a microbe or mammal. Thus each enzymatic pathway, which may involve tens of individual enzymes, has to be controlled in its overall rate and be able to change its speed if circumstances change and alter the requirements for its product. As a result, the enzymes which have evolved in nature are adapted to catalyze a reaction under the specific circumstances/conditions under which the organism lives, and as stated above, the rate at which it catalyzes the reaction will depend on the needs of the organism. Consequently, maximum speed of the reaction may not be the specific priority of a given enzyme if its involvement in a pathway is not a critical step. Most if not all enzymes are up- or downregulated by compounds which may or may not be related to the reaction it catalyzes in order to enable co-ordination of the whole pathway into all other pathways in the cell. This means that such enzymes may be optimally adapted to a specific set of conditions—temperature, pH, or ionic strength, for example, which is optimal for the organism. Such conditions may be significantly divergent from those in which industry currently employs enzymes. The first and most obvious difference is that most enzymes employed in industry are used in single-step processes, and as a result there is no need for integration of the enzyme into up- or downstream enzymatic processes. Secondly, the conditions under which industrial enzymes are employed are often hugely divergent from those from whence the enzyme originated, thus there is often significant room for improvement in their catalytic properties. Much of the development of enzymes in the feed or food industry has in fact focused on adapting them to function optimally under the conditions of the industrial process.

Enzymes can be categorized into six classes, as defined by the International Union of Biochemistry and Molecular Biology (Table 1). This is useful for nomenclature but also to describe the types of reactions catalyzed by enzymes.

As modern day industry evolved, it was noted that specific reactions may be better suited to include, or indeed be based upon, an enzymological rather than physicochemical process, and as a result the search for candidates began. In the beginning, microbial or organ-based extraction methods of the enzyme of interest were entirely dependent on the enzyme of interest being present in sufficient quantities to be of economic interest. Evolutionary pressures and selection techniques used in microbial fermentation processes were rudimentary, but nevertheless progress was made in evolving candidate enzyme characteristics to that they suited the industrial process needs more so than those of the organism.

Table 1 Enzyme Classes With Examples

EC Number	Title	Type of Reaction	Example
EC 1	Oxidoreductases	Oxidation/reduction	Catalase (EC 1.1.11.6); the decomposition of hydrogen peroxide to water and oxygen
EC 2	Transferases	The transfer of a functional group (e.g., a methyl or phosphate group)	Hexokinase (EC 2.7.1.1); transfers an inorganic phosphate group from ATP to glucose (i.e., in the first step of glycolysis)
EC 3	Hydrolases	The hydrolysis of various bonds	α-Amylase (EC 3.2.1.1); hydrolyzes starch to maltose
EC 4	Lyases	Cleaving of various bonds by means other than hydrolysis and oxidation	Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39); catalyzes the reaction of ribulose-1,5-bisphosphate and carbon dioxide to form an unstable intermediary (i.e., during carbon fixation by plants)
EC 5	Isomerases	Changes a molecule from one isomer to another	UDP-glucose 4-epimerase (EC5.1.3.2); the conversion of UDP-galactose to UDP-glucose (i.e. glucose synthesis)
EC 6	Ligases	Joining of two molecules with covalent bonds	Pyruvate carboxylase (EC 6.4.1.1); carboxylation of pyruvate to oxaloacetate (i.e., during the citric acid cycle)

Expression rates were also targeted such that the organisms selected for industrial production produced more and more of the enzyme(s) of interest, meaning the organisms became less and less like the wild-type organism. The history of this process is described briefly below.

HISTORY

Some of the earliest experiments investigating enzymes have been attributed to French scientist René-Antoine Ferchault de Réaumur. In 1752, interested in how his falcon could digest meat, he fed the bird small pieces of meat contained in metal capsules which had holes. After a period of time he retrieved the capsules to find the meat was partly degraded and concluded that it could not be due to mechanical action as previously thought, since the metal capsule had protected

the meat. Individual enzymes were not named until 1833 when two sugar chemists, Anselm Payen and Jean Francoise Persoz described diastase. This agent was seen to separate starch from the barley seed and convert it to soluble sugars. The term "diastase" came from the Greek word for separation and the suffix "-ase" is still used to name enzymes today. However, the term "enzyme" was not itself used until it was coined in 1876 by Wilhelm Kuhne, more famous for his work on understanding the physiology of the eye. The word "enzyme" was derived from the Greek word meaning leavened or "in yeast." It was known that something in yeast was involved in the leavening of dough. However, at this stage there was still confusion as to the difference between the fermentation process of yeast and those agents, such as trypsin, secreted by larger organisms.

The understanding of the true nature of enzymes developed following the work of Eduard Buchner in the late 19th century. Buchner was able to resolve the debate between Louis Pasteur and Justus Liebig (and others) who argued, respectively, that fermentation was internalized within a yeast cell, or that it was a separate chemical that was responsible for the process. Buchner published the paper "On alcoholic fermentation without yeast cells" which suggested that an intact yeast cell was not necessary for fermentation to occur. This finding implicated chemical agents, independent of the intact cell, as being responsible for the fermentative process: enzymes, and that they were produced by the intact cell. Thus, the intact cell was necessary for production of the enzyme but not the fermentation process per se. Buchner was awarded the 1907 Nobel Prize for chemistry for this and other work.

Sometime after the discovery of diastase by Payen and Persoz, Jokichi Takamine, a Japanese chemist, described takadiastase and was granted a US patent in 1894. This was an important step for the industrial production of enzymes because Takamine's patent described the production of takadiastase from *Aspergillus oryzae*, which is still an enzyme source today. It was the first microbial enzyme-related patent. Probably the first application of cell-free enzymes was the use of rennin isolated from calves' or lambs' stomachs in cheese making. Rennin is an aspartic protease (see *Mechanisms of Enzyme Action*) which coagulates milk protein and has been used for hundreds of years by cheesemakers. However, Röhm in Germany prepared the first commercial enzyme preparation in 1914. This trypsin enzyme, isolated from animals, degraded proteins, and was used as a detergent. It proved to be so powerful compared with traditional washing powders that German housewives' suspicions were aroused by the small size of the original package, so the product had to be reformulated and sold in larger packages.

However, although enzymes were now in commercial production, the true chemical nature was not fully understood. In 1926, James Sumner, a chemist working at Cornell University in New York, showed that urease could be extracted in pure form, and crystallized, from jack bean. Later, he was able to isolate catalase (see Table 1). In 1946, he went on to share the Nobel Prize for Chemistry with John Howard Northrop and Wendell Meredith Stanley for this

and other work. In 1929, Northrop had isolated and crystallized pepsin and went on to describe other proteases.

The real breakthrough for commercial enzymes occurred with the introduction of microbial proteases into washing powders. The first commercial bacterial *Bacillus* protease was marketed in 1959. It became big business when Novozymes in Denmark began manufacture, and other major detergent manufacturers started using it around 1965. In 1930, in addition to cheese manufacture, enzymes were already being used in the food industry in the manufacture of fruit juice. These enzymes, known as pectinases, clarify the juice and contain numerous different enzyme activities. The major usage of microbial enzymes in the food industry started in the 1960s in the starch industry. The traditional acid hydrolysis of starch was completely replaced by alpha-amylases and glucoamylases that could almost entirely convert starch to glucose. The starch industry became the second-largest user of enzymes after detergent manufacture.

One of the major production platforms in the enzyme industry today is the filamentous fungi, *Trichoderma reesei*. It was initially discovered during the Second World War in the Solomon Islands by Mandels and Reese (the latter donating his surname to the organism) as the destructive organism responsible for rotting away the cotton on US Army tents in a matter of several months. Mandels and Reese screened over 14,000 molds and found one particular candidate that had an incredible ability to digest native crystalline cellulose. This candidate, designated QM6a, is the strain from which all the mutants used in industry today are derived.

DEVELOPMENT OF THE PRODUCTS AND INDUSTRY

At the outset of the enzyme industry the products made were enzymes produced in an organism that could be cultured. Bacterial production platforms included *Bacillus* and yeast and fungi systems provided for by *Aspergillus* and *Trichoderma* spp. If the organism could produce the enzyme of interest then evolution techniques, which included UV and chemical mutagenesis, were applied in order to improve, not only the characteristics of the enzyme, but also the production rates and hence costs of production. Regardless, the starting point was the selection of the organism that already produced the enzyme of interest and the focus was simply to apply evolutionary techniques to this organism. The big step forward came with the advent of molecular tools and techniques which allowed the transfer of genes encoding an enzyme of interest from one organism into another. Thus, enzymes which did not occur or were not expressed adequately in the limited production organisms available could be cloned from an organism that may not culture particularly well, and thus would never be a viable production candidate, into a viable production organism. Heterologous fermentation was born and is now the mainstay of the enzyme industry. Nevertheless these advancements

are quite rudimentary in comparison to where we are today. For example, the identification of the enzymes of interest was undertaken by fermentation and expression methods. Selection of a new protease for example would be based on screening hundreds if not thousands of isolates, grown in a medium, for activities of potential interest. The enzyme would then have to be purified, identified, and cloned, and produced in the production organism of interest. This was time consuming and slow, but given the state-of-the-art of the industry, it still led to significant advances.

Advances in robotics and in particular that of an understanding of the genetic sequences of the enzymes of interest and the sequencing of genomes markedly accelerated discovery rates. Genes encoding enzymes of interest could be "seen" in the sequence of any organism and similarities and differences in the gene sequence of different enzymes of a similar class used as an indicator of functional differences. Evolutionary techniques evolved from uncontrolled irradiation or chemical mutagenesis all the way to single point mutations such that changes in a single amino acid in and enzyme could be undertaken to test whether such interventions improved the characteristics of the enzyme in the process of interest. The understanding of structure/function evolved and more rational design and evolutionary techniques with it. Needless to say in 10 years' time we will look back and consider the current state-of-the-art rudimentary as well. Despite our major advances in the understanding of the reasons why a particular change to an enzyme may change its thermostability or pH optimum we still have no clue as to how to design an enzyme for a given process. In short, if we want an enzyme to convert X to Y, our current state of knowledge is such that we would not know where to start. Our starting point is always to find an enzyme in nature that performs the activity of interest and then screen for the best possible candidates and evolve it to fit our needs more optimally.

ANIMAL FEED APPLICATION

NON-STARCH POLYSACCHARIDE DEGRADING ENZYMES

The two main classes of enzymes used in animal nutrition are non-starch polysaccharide degrading enzymes (NSPases) and phytases, with significant but lesser use of amylases and proteases. Phytases, for example, are almost ubiquitous in the diets of non-ruminant product species.

Although Sumner was yet to describe the true nature of an enzyme, the use of additive enzymes in poultry feeding was first suggested in the mid-1920s. In 1925 [1], described the use of Protozyme, an enzyme which he suggested had multiple amylolytic and proteolytic activities, which improved leghorn performance. In 1926, the same year as Sumner published his work on the nature of enzymes, Clickner and Follwell [2] described Protozyme as having multiple activities, with the major activity appearing to be an amylase with a broad pH optimum.

The early development of xylanases for animal feeding is described in Masey et al. [3] and readers are directed there. In short, by the 1950s and 1960s, the area of enzymes in animal nutrition had become a hot topic. Teams in the United States showed that adding an amylolytic enzyme preparation to barley-based broiler diets could reduce sticky droppings and improve growth. It was not until the late 1960s that the specific activities and substrates were described. Bell and Burnett [4] attributed the effect of an amylolytic product to a contaminating side activity—endo-β-glucanase—which shortened soluble glucan chains, resulting in reductions in viscosity and improved nutrient digestibility. Association of a response from an enzyme preparation to the wrong activity is an issue even today, despite the use of recombinant "mono-component" enzymes. Even relatively small amounts of a contaminating activity may be responsible in part or in total for the response observed, and unfortunately journals are ineffective in demanding a wide ranging analysis of all potential activities in a product claiming to be a single activity.

The first commercial application of feed enzymes was in Finland in 1984 when a feed company took advantage of the fact that it happened to have a sister company which produced β-glucanases for use in the brewing industry. The application of the β-glucanases in diets based principally on barley was very successful, reducing viscous droppings which resulted in far drier litter and better animal performance, but it still took 5 years for this practice to expand outwards to other EU countries. Concurrently, work showed the negative effects of rye on digestibility of various nutrients and animal performance [5]. This was attributed, similarly, to the viscosity induced by soluble pentosans and as such attenuated by the specific use of pentosanases for broilers [6] and pigs [7]. Australian work later showed that the negative effects of adding extracted pentosans from wheat to broiler diets [8,9] could be overcome by inclusion of a pentosanase, subsequently improving apparent metabolizable energy and nutrient digestibilities [10]. Expanding on the successful use of β-glucanases in barley-based rations, the feed enzyme industry which had consolidated in Northern Europe as a result of the use of barley in these regions, turned its focus to wheat and xylanases to address similar, albeit lesser, viscosity problems of poor performance and litter quality. It was fortunate for the emergent European feed enzyme industry that the varieties of wheat endemic at the time tended to be relatively high viscosity, hence the problems were quite apparent and easily seen to be solved. The almost miraculous curing of wet litter was probably the biggest selling point of these enzymes at this point in time.

In the late 1990s and early 2000s, the use of NSPases became more and more common and work began to understand the mechanism of action in animals and also to further develop the products themselves. The viscosity effect was well understood and it was certainly clear that a diet containing high levels of soluble NSP reduced performance and this was ameliorated with the use of an NSPase. The function of NSP in cereal grains is in the formation and integrity of the cell wall. It follows that they are implicated in encapsulating the nutrients within the

cell. In a cereal endosperm cell this is starch and protein. As such, since animals do not possess any NSPase activity, there is potential for nutrients to be completely inaccessible without exogenous enzymes. Thus, the "cell-wall" theory is conceivable. In fact, using microscopy, it has been shown that cereal cell walls appear to be broken during the digestive process when NSPases are included in the animal's diet. However, in order to mimic this effect in vitro, the dose of NSPase required to effect cell wall rupture is in vast excess to that used commercially. Moreover, the conditions needed during the in vitro process are quite different to those the enzyme would encounter during the digestive process. Taking this into consideration, and the fact that NSPases continue to be effective despite the fact that through cereal breeding, cereals are far less viscous than they once were, there must also be other mechanisms to explain their efficacy. More recently, the concept of NSPase acting as a pseudo or pre-prebiotic has been proposed. This centers on the value of the product of enzyme activity rather than solely the benefit of removing a substrate. For example, when arabinoxylan, a complex xylose and arabinose polysaccharide found in cereal cell walls, is broken down by the action of a xylanase, short xylo-oligosaccharides are produced. These oligosaccharides are known to exhibit many of the characteristics of the classic prebiotic, namely that they are not digestible by animal's endogenous enzymes, not directly absorbed, and most importantly, will promote the growth of considered as beneficial species of bacteria, such as bifidobacteria and lactobacilli. This clearly has benefits in terms of promotion of host gut health.

PHYTASE

Whereas most enzymes used in the feed industry have been leveraged from other industrial platform uses, be it food, textiles or industrial use, phytase stands alone in that it was developed exclusively for the animal feed industry. Phytase degrades phytic acid, or inositol hexaphosphate, to inositol monophosphate and in the process releases five phosphate moieties. In the form of phytic acid, the phosphate is largely unavailable to the monogastric as it lacks sufficient phytase activity to enable consistent release. As a result, most monogastric animal feed has to be supplemented with inorganic phosphates to meet the requirements of the animal as there is insufficient available phosphorous, P, in the diet to satisfy their needs. Two elements of this problem contributed the development of the phytase industry. The first was that this supplementation of inorganic phosphate resulted in diets containing high levels of total P simply to meet the digestible P contents of the diet. The result is that the undigested P is excreted in the manure which ultimately adds to the phosphate loading of the land on which the manure was distributed. Such P loading became critical in the Netherlands in the early 1990s. This was due to the concentration of a significant poultry and pig industry in a relatively small land mass, which resulted in accumulation of P in the soil over time with the threat of eutrophication of waterways increasing as a result. Legislation was introduced which limited the amount of P that could be spread

per hectare of land, which in effect forced the industry to consider reducing the population of animals by 25%–30% (which was economically inviable for many producers) or consider a technological solution to enhance the availability of the unavailable phosphate from phytic acid. Thus, the phytase industry was born, albeit in a limited way in that its use was restricted to geographies where the penalty for P pollution drove the economics for inclusion, which for several years restricted use to the Netherlands and some parts of northern Germany. In the mid to late 1990s the DelMarva area in the United States also started using phytase as part of a P pollution abatement program. If it were not for the penalties for excess manure P the use of phytase was not economical and thus its use was limited.

Up until the late 1990s, phytase was a relatively minor player in the feed enzyme industry but this changed with the development of the second problem noted in the previous paragraph, namely that of supply of inorganic phosphate. P is the third most expensive nutrient in monogastric feeds and the cost of the phosphates was subject to global supply and demand. The vast majority of rock phosphate is used in fertilizer manufacture with approximately 3% being used in animal feed. This source is finite and supply and demand were such that prices started to increase at the same time that prices of the phytase fell, resulting in the enzyme "costing in" to many diets and thus usage increased. At the outset there was only one supplier of phytase on the market which was derived from *Aspergillus niger*, but as it grew a second product derived from *Peniophora lycii* entered the market and competition drove prices down further. Nevertheless, the costs per tonne treated were still five to ten times what they are today. In the mid-2000s several *Escherichia coli* variants entered the market and today there are additional products derived from *Citrobacter*, *Yersinia*, *Hafnia*, *Butiauxella* and further evolved *E. coli* products. These newer phytases are more biologically effective per molecule and hence much more cost efficient, resulting in the use of higher dosages at much lower inclusion costs. Whereas phytases were used originally to extract 30%–40% of the phytate phosphorus in a typical diet, they are commonly used today to extract 50%–75%. Moreover, the recent recognition that phytate (IP6) and its lower esters (IP5,4,3) are antinutrients in their own right, has lead to a completely different use of this enzyme in monogastric nutrition, namely phytate destruction for maximum animal growth efficiency. Usage rates as a result are increasing significantly, as much as two to four times the normal commercial usage for simple Ca and P release. Moreover, more widespread use in this new application has resulted in increased consequential benefits in animal production that were not foreseen, for example antioxidant status, semen quality, and shifts in the microbiome. The latest findings suggest that we have much to learn if we are to use this enzyme class optimally in sustainable animal production.

AMYLASE

Amylase, or an amylolytic activity, was possibly the first documented enzyme [1] used in broiler feeding. Whether or not the amylolytic activity was the main

benefit of the product is impossible to tell. There are currently several amylase products on the animal feed market, originating from *Bacillus* spp. They are commonly presented as part of a combination product. That is, they are produced as individual activities and blended with others produced elsewhere. It is generally understood that the production of amylase by the pancreas of animals is an extremely efficient process and does not limit digestion of starch. However, there are circumstances where it is conceivable that this is not correct. For example, in the very young chick, while the gastrointestinal tract is still developing, it is likely that amylase production and secretion is sub-optimal. Classen [11] suggested that young turkeys and piglets may be most likely to need exogenous amylase. The fact that products are often presented as combinations means that the true benefit of an amylase, when provided singularly, is difficult to ascertain from the literature. As these products were beginning to become established, Mahagna et al. [12] showed that an amylase-containing product gave a significant performance benefit to 7-day-old chicks, but not beyond that point. Around the same time, Zanella et al. [13] demonstrated that such products, containing amylase, improved ileal starch, protein and fat digestibility in broilers. Recently, authors have demonstrated the benefit of a combination product containing three activities, over the individual activities [14]. In that case, of the single enzymes, only the xylanase had an impact on 42d FCR but the combination of xylanase, amylase, and protease was better again. This implies that amylase (and indeed protease) work better in the presence of a xylanase.

PROTEASE

As pressure in world protein supply increases, producers are looking to reduce protein in their diets. Proteases are gaining momentum as a category for this reason, as they are seen as able to support reduced protein in the diet. Protease enzymes are usually derived from, and produced using expression in, *Bacillus* spp. Like amylase, protease is often used as part of a combination product and the efficacy of the singular enzyme is often difficult to assess in the literature. A greater number of protease-specific products and literature do exist, but the evidence for their value is equivocal. Simbaya et al. [15] demonstrated that protease had an impact on broiler Feed Conversion Ratio (FCR) in a dose−responsive manner and that the effect may be dependent on the protein meal used in the diet. It has been suggested that protease is particularly effective on reduced-protein diets [16]. However, Ding et al. [17] did not find an interaction between protein content and protease, but increasing protease dose improved broiler feed conversion ratio and increased protein digestibility. Most recently, there has been more focus on investigating the varying properties of different classes of protease, which may impact performance in the animal [18]. Strategies have been suggested to include protease and synthetic amino acids, to reduce crude protein in the diet [19].

HUMAN FOOD APPLICATION

Although the use of active enzymes, for the purposes of health and nutrition, is common in animal diets, direct supplementation of enzymes in human food is not common. This is for several reasons: primarily the lack of application but also because of regulatory barriers. Enzymes are much more likely to be used during food manufacture and as processing aids and some common examples are discussed below. However, as the understanding of the antinutritional properties of phytate increases, there is the realization that it is implicated in decreasing mineral and protein nutrition. Therefore, phytases have been included in so-called micronutrient powders and have become available as a food pre-treatment, in an attempt to improve mineral nutrition [20], particularly in developing countries. In a review [21] of recent experiments, it was clearly demonstrated that phytase has beneficial effects in human Fe and Zn gastrointestinal absorption from food containing high levels of phytates.

BEVERAGE MANUFACTURE

Pectinases have been used in food production since the 1930s. However, as the understanding of the structure of fiber, has increased, so have the applications for pectinases. These enzymes are broadly hydrolytic, with varying classifications depending on the specific pectic substrate. The most significant class is acidic pectinases, usually derived from fungal sources, most commonly *Aspergillus*. These are used for clarification of clear juices, such as apple and pear, and for stabilizing cloudy citrus juices [22]. They may also help improve yield during juice extraction [23]. A second group, alkaline pectinases, often from *Bacillus*, are used for treatment of waste water from juice manufacture [22].

The wine industry, whose main raw material is grape juice, also use pectinase preparations for improving yields and clarity. Often, *Aspergillus* pectinase preparation may contain glycosidase side activities. This has been seen as an advantage by some, as these enzymes have been implicated in producing flavor compounds in the wine [24].

BAKERY APPLICATIONS

One of the most common applications for enzymes in food technology is in the bakery. Several categories of enzymes are used, under the heading of dough improvers, which results in a significant body of literature which overlaps considerably with animal food applications. This is because one of the most significant enzyme classes in the sector is xylanase, usually of microbial origin. They have been shown to improve the rising process, which then results in the desirable characteristic of increased loaf volume [25]. The precise mechanism is unclear, but it is likely to be related to the ability of xylanases to change the

physicochemical properties of arabinoxylan [26]. Different xylanases may be more or less effective depending on their specificity for water-insoluble or water-soluble arabinoxylan. Water-insoluble arabinoxylan may hold water in a dough and prevent proper and necessary gluten development. On the other hand, water-soluble arabinoxylan is viscous and may be therefore support dough structure, gas retention, and subsequent loaf volume [26]. In common with the animal feed sector, thermostability of xylanases is a key development area for the bakery sector. Although in animal feed, increased thermostability is desirable, to allow enzymes to withstand feed processing, the relationship between thermostability and bread quality is less clear in breadmaking [27].

Of similar importance to the bakery sector is amylase, commonly applied for anti-staling properties. On hydrothermal treatment, such as baking, starch gelatinizes and loses its crystalline, granular structure. Re-association of those polymers on cooling (so-called retrogradation) creates polysaccharides with changed characteristics and behaviors. Amylase may help this situation by changing the level of crystallinity in retrograded starch and also by interfering with the gluten–starch interaction [28]. Amylase may also play a part in flavor development in bread, via two mechanisms. Firstly, producing fermentable sugars which support the microbial production of flavor compounds, and secondly by producing reducing sugars available for such reactions as Maillard's [29,30].

Another key group of enzymes in the dough-improver category is lipid oxygenases. These are common in white bread manufacture as they can help in bleaching of carotenoids, but also because they improve mixing tolerance and rheological properties [31]. However, it has been suggested that lipid oxygenases may be implicated in the production of off-flavors in the final product, so they should be used with care.

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CHAPTER

Selection, engineering, and expression of microbial enzymes

1

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1.1 INTRODUCTION

Enzymes are biological molecules that catalyze the essential chemical reactions that maintain life. Enzymes are synthesized by all living organisms. Native and recombinant enzymes are tools in major sectors of the biopharmaceutical, agricultural, and chemical industries, acting in very diverse areas of medicine, diagnostics, food, feed, detergents, textiles, leather, paper, pulp, and plastics. Enzymes extracted from edible plants and animal tissues as well as those produced by microorganisms (bacteria, archaea, yeasts, and fungi) have been used in food production for centuries. For example, rennet, which is a mixture of natural enzymes obtained from calf stomach, has been used for a long time in the production of cheese, and enzymes produced by yeasts are utilized for fermentation of grape juice in wine production and of barley in that of beer.

Isolation of enzymes from living cells started in the 20th century, driving their large-scale utilization in the food industry. Nowadays, microorganisms are the main source of commercial practical enzymes. Enzyme sealers are continuously optimizing selection, recombination, expression and production of enzymes for industrial use (Fig. 1.1).

Biotechnology (genetic engineering) has the more accurate available methods for the optimization of enzyme production, starting from the transfer of the encoding genetic sequence from a plant, animal or microorganism to the expressing microbe, yeast, or fungi. It should be underlined that, in such conditions, the produced enzyme is not genetically modified but only produced by a genetically modified organism.

The global industrial enzymes market is very competitive with Novozymes being the largest player in the industry, followed by DSM and DuPont, among others. The companies mainly compete on the basis of product quality, performance, use of intellectual property rights, and the ability to innovate, among other

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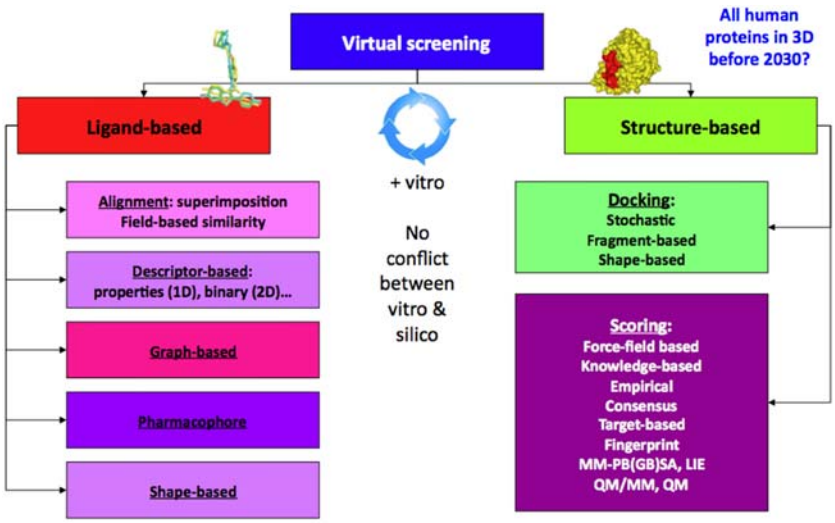

FIGURE 1.1

Screening steps for the discovery of new proteins (enzymes).

factors. North America and Europe are the largest consumers of industrial enzymes although the Asia Pacific region is undergoing a rapid increase in enzyme demand in China, Japan, and India, reflecting the size and strength of these countries' economies [1].

1.2 PRINCIPAL APPLICATIONS OF MICROBIAL INDUSTRIAL ENZYMES

The development of performant and economically affordable new enzymes is the subject of permanent research efforts. It is obvious that progress in screening, genetic engineering, and expression of recombinant enzymes is fundamental for the production of new enzyme products.

Enzyme applications in medicine are growing rapidly, being already extensively used in therapeutics in health issues associated with enzymatic deficiency and digestive disorders, as well as in diagnostic procedures such as ELISA and diabetes [2]. At present, most prominent medical uses of microbial enzymes are removal of dead skin, and burns by proteolytic enzymes, and clot busting by fibrinolytic enzymes. Several new enzymes for medicinal applications are expected to be developed in the coming years based on the processes in the screening and expression techniques.

In the food industry, enzymes are used not only to improve food production but also to improve food components, such as aroma, flavor, texture, color,

nutritive value, and appearance. For instance, baking enzymes are used for providing enhancement of organoleptic characteristics of more or less all types of bakery products. The baking enzymes industry is expected to reach $700 million by 2019 growing at a rate of more than 8% from 2013 to 2019. Enzymes (proteases, lipases, esterases, lactase, aminopeptidase, lysozyme, lactoperoxidase, transglutaminase, catalase, etc.) in dairy industries are parts of the tools required for the quality of the outcome products. This is also the case for alcoholic and nonalcoholic beverages. The deep understanding of the use of microbial enzymes in food industries has allowed the development of better markets of more safe products of higher quality.

The use of enzymes in animal diets exploded in the 1990s. Feed enzymes are gaining importance as they can increase the digestibility of nutrients, improve feed utilization by animals, and reduce the footprint of intensive husbandry [3]. The global market for feed enzymes is expected to reach almost $1.3 billion by 2020. Feed enzymes essentially used for monogastric animals (swine and poultry) are phytases, proteases, α-galactosidases, glucanases, xylanases, α-amylases, and polygalacturonases [4].

In vitro enzyme-catalyzed synthesis of polymers is an environmentally safe process that has several advantages over conventional chemical methods [5]. Biopolymers are environmentally friendly materials as these are synthesized from renewable carbon sources via biological processes, degrade biologically after use, and return to the natural environment as renewable resources, such as CO_2 and biomass [6]. Biopolymers, such as polyesters, polycarbonates, and polyphosphates are used in various biomedical applications, e.g., orthopedic devices, tissue engineering, adhesion barriers, control drug delivery, etc. [7].

With increasing awareness of sustainability issues, uses of microbial enzymes in the paper and pulp industry have grown steadily to reduce the adverse effects on ecosystems. Enzymes are also used to enhance deinking, and bleaching in the paper and pulp industry, and waste treatment by increasing biological oxygen demand and chemical oxygen demand [8].

The leather industry discharges and waste cause severe health hazards and environmental problems [9]. The biodegradable enzymes are efficient alternatives to improve the quality of leather and help to reduce waste [4].

The textile industry is responsible for enormous production of waste from production facilities, bleaching chemicals, and dye, and is one of the largest contributors to environmental pollution [10]. In such industrial activities, enzymes are used to allow the development of environmentally friendly technologies in fiber processing and strategies to improve the final product quality [11].

The applications of enzymes in cosmetics has been continuously increased. Enzymes are used as free radical scavengers in sunscreen cream, toothpaste, mouthwashes, hair waving and dyeing, etc. [12].

Detergents, which are the prime industrial application of enzymes today amounting to about 30% of the total sales of enzymes, are expected to grow fast

and have contributed significantly to the growth and development of industrial enzymes. Enzymes in detergent products are used to remove several compounds such as protein, starch, oil, and fat-based stains. Detergents are used in miscellaneous applications such as dishwashing, laundering, domestic, industrial, and institutional cleaning [13].

In the past, waste was traditionally disposed of by digging a hole and filling it with the waste material, but this mode of waste disposal is impossible to sustain. Classically, waste was and is still treated by high-temperature incineration and/or chemical decomposition (base-catalyzed dechlorination and UV oxidation). These methods are complex, uneconomical, and lack public acceptance. The associated deficiencies in these methods have focused efforts toward harnessing modern-day bioremediation processes as suitable alternatives. The accumulation of biological micropollutants and toxic chemicals can lead to environmental hazards which can be minimized through bioremediation.

Bioremediation is performed by a microorganism or an enzyme-mediated transformation or degradation of contaminants into nonhazardous or less-hazardous substances. Bioremediation can be effective only where environmental conditions permit microbial growth and activity. Its application often involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate, knowing that the process of bioremediation is very slow. Most bioremediation systems operate under aerobic conditions, but anaerobic environments may also permit microbial degradation of recalcitrant molecules. Both bacteria and fungi rely on the participation of different intracellular and extracellular enzymes, respectively, for the remediation of recalcitrant organopollutants. The use of culture-independent molecular techniques has certainly facilitated the understanding of bacterial community dynamics, and assembly, and has helped to provide understanding of the specifics of bioremediation, which has resulted in safer and more reliable techniques.

Laccases (*p*-diphenol: dioxygen oxidoreductases) constitute a family of multicopper oxidases produced by certain plants, fungi, insects, and bacteria, that catalyze the oxidation of a wide range of reduced phenolics. Laccases are known to occur in multiple isoenzyme forms, each of which is encoded by a separate gene [14] and, in some cases, the genes have been expressed differently depending upon the nature of the inducer [15]. Many microorganisms produce intra- and extracellular laccases capable of catalyzing the oxidation of ortho- and paradiphenols, aminophenols, polyphenols, polyamines, lignins, and aryl diamines, as well as some inorganic ions [16–18]. These enzymes are involved in the depolymerization of lignin, which results in a variety of small phenols. In addition, these compounds are utilized as nutrients for microorganisms or repolymerized to humic materials by laccase. Among the biological agents, laccases represent an interesting group of ubiquitous, oxidoreductase enzymes that show promise of offering great potential for biotechnological and bioremediation applications [19]. The substrate specificity and affinity of laccases can vary with changes in pH. Laccases can be inhibited by various reagents such as halides (excluding iodide),

azide, cyanide, and hydroxide. Different laccases appear to have differing tolerances toward inhibition by halides, indicating differential halide accessibility. Laccase production is sensitive to the nitrogen concentration in the culture media. Additionally, laccase activity in fungal cultures can be amplified by the addition of diverse aromatic compounds such as gallic acid, ferulic acid, xylidine, guaiacol, syringaldazine, and veratryl alcohol, which have been extensively used to encourage laccase production.

The use of enzymes for waste management is extensive and a number of enzymes are involved in the degradation of toxic pollutants. Industrial effluents as well as domestic waste contain many chemical commodities, which are hazardous or toxic to the living being and ecosystem. Microbial enzyme(s), alone or in combinations, are used for the treatment of industrial effluents containing phenols, aromatic amines, nitriles, etc., and are becoming important tools for the degradation of several toxic pollutants such as aromatic polycyclic hydrocarbures, heavy metals, pesticides, etc.

Enzyme-based processes for production of fine chemicals are rapidly gaining practical significance owing to more economical high-purity products in an ecoenvironmentally acceptable manner [20]. Enzymes are preferred in industrial chemical synthesis over conventional methods due to their high selectivity, i.e., chiral, positional, and functional group specificities [21,22].

Plant biomass is an important source of monomers and energy that is stored in the form of complex polymers, primarily hemicelluloses cellulose and lignin. The transformation of these polymers into sugars and monomers enables a large set of applications downstream [23]. The saccharification process is currently carried out by thermochemical pretreatment followed by the use of a mixture of microbial enzymes (e.g., lytic polysaccharide monooxygenases, xylanases, arabinofuranosidases, cellobiohydrolases, endoglucanases, β-glucosidases, etc.) that can work synergistically [24,25]. Plant waste sources that are used for the production of biochemical and second-generation agrofuels include agricultural by-products (e.g., cereals straw, sugarcane bagasse, etc.), wood residues and nonfood crops, such as switchgrass. Such are attractive as they do not seem to compete with food production and utilization [26,27].

Lignocellulose is the most abundant complex of polymers on the earth and it is continuously produced. It is estimated that a ton of cereals harvested is accompanied by the production of two to three tons of lignocellulose-rich residues. The cost-effective transformation of nonfood lignocellulose to starch could revolutionize agriculture and reshape the bioeconomy, while maintaining biodiversity and minimizing agriculture's environmental footprint. The transformation would not only promote the cultivation of plants chosen for rapid growth rather than those optimized for starch production, but it would also efficiently use marginal land for the production of the biomass required to meet the increasing needs for renewal chemicals and agrofuels. The cost-effective release of soluble fermentative sugars from lignocellulosic materials through enzymatic hydrolysis is essential in a new generation lignocellulosic biorefineries.

Lignin is present in all vascular plants making vegetables firm and crunchy, and supplying food and feed fiber. It serves also to regulate the transport of liquid in the living plant. Lignin is an organic substance binding the cells, fibers, and vessels in lignified elements of plants. It is a complex aromatic heteropolymer composed by radical polymerization of guaiacyl, syringyl, and *p*-hydroxyphenyl units linked by β-aryl ether linkages, biphenyl bonds, and heterocyclic linkages. It is extremely resistant to degradation [28] and has many internal H bonds. It is generally bonded in complex and various ways to carbohydrates hemicelluloses and cellulose. Lignins are actually not a single compound. All are complex, amorphous, three-dimensional polymers that have in common a phenylpropane structure; i.e., a benzene ring with a tail of three carbons. In their natural unprocessed form, they are so complex that none of them has ever been completely described, and they have molecular weights that my reach 15,000 daltons or more. Although most of them contain certain carboxylic acids, lignins are not acids and as they occur in nature (protolignins) have been grouped into several types, characteristic of hardwoods, softwoods, and grasses. Within each type there is much variation. Lignins differ from species to species, and from one tissue to the next in the same plant even within different parts of the same cell wall.

Sugarcane bagasse, corn stover, and wheat straw are among the most available resources for the production of cellulosic ethanol. Some studies have shown that lignin is able to boost the activity of the cellulose oxidizing enzyme lytic polysaccharide monooxygenase [29].

As mentioned, biomass appears to be a promising alternative and a renewable source for chemicals and agrofuels. Significant achievements have already been made in the production of ethanol from biomass, primarily starch and sugar-rich components. Lignin can account for up to 40% of the dry plant biomass weight. Most of the biorefinery schemes focus on utilizing easily convertible fractions—cellulose and hemicellulose—while lignin remains relatively underutilized. After holocellulose, it is the most abundant renewable carbon source on Earth and between 40 and 50 billion tons per year are produced worldwide, mainly as waste products. Efficient lignin utilization requires its depolymerization to low-molecular weight phenolics and aromatics that can then serve for chemical syntheses of high-value products. There are two ways of degradation of lignin—chemical and biological. An important advantage of the enzymatic approach is the lack of yield loss associated with the thermal decomposition of lignin, and the opportunity to direct lignin biodegradation utilizing selective microorganisms and/or enzymes. Furthermore, the biocatalysis takes place at lower energy input and reduces the environmental impact. Bacteria do not possess the regular peroxidases that fungi employ for lignin degradation. This may be due to intrinsic difficulties in expressing these rather complex proteins that are typically glycosylated, contain several disulfide bonds, and incorporate several calcium ions and a heme cofactor [30]. The bacterial laccases also seem to be suited to large-scale recombinant enzyme production. Some studies revealed that by optimizing expression conditions, various bacterial laccases can be produced in *Escherichia coli* [31].

Cellulose, hemicellulose, and lignin are recalcitrant molecules and it is difficult to improve their biotechnological exploitation. One of the possibilities to increase the use of cellulose and hemicellulose is to remove the lignin. The removal of lignin allows that the cellulose and hemicellulose are more easily hydrolyzed by cellulases and hemicellulases. Ligninolytic enzymes cause disruption of the lignin "seal" by oxidation of covalent bonds and increase the accessibility of hydrolytic enzymes. After this process, more sugars can be obtained for bioconversion. In this way these enzymes acting together should perform a complementary action in order to obtain an improvement in the degradation of plant cell walls.

Among cellulose-degrading microorganisms, a common feature is the predominance of endoglucanase activity. However, one of the best cellulolytic enzyme producers is the fungus *Trichoderma reesei* that produces a cellulolytic enzyme complex enriched in exocellulases (exoglucanases). In particular, the cellulolytic system of *T. reesei* is made up of two exoglucanases, Cel7A (CBHI) and Cel6A (CBHII), and at least five endoglucanases, Cel7B (EGI), Cel5A (EGII), Cel12A (EGIII), Cel61A (EG IV), and Cel45A (EGV), that cooperate synergistically. Of these, Cel7A is the most important sugar-producing enzyme comprising 60%−75% of the cellulase system in a *T. reesei* filtrate. Also, cellobiases or beta-glucosidases that hydrolyze the exocellulase product into individual monosaccharide (glucose) are present at low amounts. Because the reaction product of exoglucanases is the dimer cellobiose, which exhibits strong product inhibition, cellobiase is a key enzyme because hydrolysis of cellobiose into glucose decreases the inhibition [32].

Hemicellulases are also important enzymes such as α-L-arabinofuronosidase, β-mannanases, α-glucoronidases, β-xylosidases, and xylanases. Hydroxycinnamic acids, e.g., ferulic acid, are esterified to hemicellulose, making covalent bonds between hemicellulose and lignin which constitute a "seal" surrounding cellulosic fibrils. This composite structure of lignocellulosic cell walls constitutes an additional limitation to biodegradation, thus feruloyl esterase is another key enzyme involved in the delignification process. Most of the feruloyl esterases have been shown to act synergistically with cellulases, xylanases, and pectinases to break down complex plant cell wall carbohydrates [31].

The biopolymer lignin (hydrophobic phenylpropanoid compound) is oxidized and degraded by an extracellular ligninolytic system mainly composed of lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), laccase, and auxiliary H_2O_2-generating oxidases (e.g., aryl alcohol oxidase and glyoxal oxidase). These enzymes have shown enormous biotechnological potential as they can be used in a wide variety of lignin-degradation processes. It is well known that basidiomycetes of ecological white-rot group (white-rot fungi) are the most efficient lignin degraders. They also have great potential for bioremediation of phenolic-rich or aromatic xenobiotic-containing wastewaters, e.g., agroindustrial effluents such as olive oil mill wastewater, winery effluents, and azo-dyes containing textile effluents. Bioprocesses carried-out by basidiomycetes producing ligninolytic enzymes

are also used in solid-state incubation to improve animal feed by catalyzing the removal of natural toxic compounds from agroindustrial residues such as *Jatropha* seed cake and olive leaves. The experimental results shown great potential since the removal of antinutritional compounds to nontoxic levels was observed [19].

Lignocellulosic crop residues such as cereal straws (e.g., wheat straw) are annually produced in huge amounts. The ability of ligninolytic enzymes from white-rot fungi to selectively degrade the lignin biomass fraction has great potential for applications in industries processing lignocellulosic materials to produce cellulose, biofuels, or cellulose-enriched feed for ruminants in a biorefinery concept. In fact, one of the main challenge aspects of biotechnology improvement of biomass use is the enzymatic pretreatment in order to break down and remove the lignin exposing unprotected holocellulose, thus providing available sugars for subsequent use (e.g., animal feed or bioconversion into renewable biofuels). White-rot fungi exhibiting higher ligninolytic activities (laccase, MnP, and LiP) associated with a lower activity of cellulolytic enzymes (especially exocellulases) are the most promising enzyme-producing fungi for the delignification process. Using this approach, after this enzymatic pretreatment, the increase of holocellulose accessibility can be evaluated by incubations in the presence of cellulolytic enzymes and measuring the liberated reducing sugars. Experimental results revealed a degree of delignification of about 50% and a three- to four-times increase in the cellulose accessibility [33].

The production of sugars and agroethanol from lignocellulosic materials is still a costly process, and requires, for positive environmental effects, the production of strong and low-cost enzymes. Today, cellulosic ethanol is twice as expensive as gasoline and the proportion is similar for the production of bioactives. There are more than a few generations of ethanol: first generation—ethanol achieved from the broth feedstock; second generation—ethanol obtained from the by-products of agroindustry; and third generation—microalgae as feedstock for the production of ethanol. In relation to the bioethanol of the second generation, the feedstock can be sugarcane bagasse or straw, wheat bran, dry corn, sawdust, and other coproducts that are considered to be of low cost but high energy value.

It is expected that the production cost of cellulases will be reduce from 5 to 1 US cent per liter from now to 2020. The world leaders in the domain (Novozymes, Genencor, and Iogen) would like to reduce the price of cellulases within few years. Several microorganisms can be used as providers of cellulases. *T. reesei* is today a paradigm for the commercial-scale production of different plant cell wall degrading enzymes mainly cellulases and hemicellulases. These enzymes have a long history of safe use in industry and well-established applications are found within several processing industries. However, these enzymes are to be used for the saccharification of cellulosic plant biomass to simple sugars which can be further used as biorefinery products, and thus compete with chemicals produced from fossil sources or from agricultural activities. Additional efforts

are needed to reduce costs and maximize yield and efficiency of the produced enzyme mixtures. Among other approaches to this end is the use of genetic engineering to manipulate the biochemical and regulatory pathways that operate during enzyme production and control enzyme yield [31].

The Earth's available stocks of lignocellulosic biomass are tremendous and are distributed all around the world. Thus, the sustainability of the valorization of cellulose by cellulases seems clear. More economical feeding can most probably be obtained by improving pretreatment and enzymatic hydrolysis of cellulose. Furthermore, an application into human nutrition may be also possible. It should also be mentioned that lignocellulolytic enzymes and particularly laccases will have a fundamental role in the valorization of the nonedible plant biomass for several applications.

1.3 INCREASED UTILIZATION OF RECOMBINANT ENZYMES

In the majority of countries, enzymes used in the food and feed industries are legally considered as nutritional additives and under the authority of specific organizations such as the Food and Drug Administration (FDA) in the United States, Santé Canada (Canada), and the European Food Safety Authority (EFSA) in the European Union.

Although plants, fungi, bacteria, and yeasts produce the most enzymes, microbial sources produce enzymes that are more valuable than their equivalents from animal or vegetable sources. The benefits include minor production costs, the option of large-scale production in industrial fermenters, extensive variety of physical and chemical characteristics, prospect of genetic manipulation, absence of effects brought about by seasonality, quick culture growth and the use of non-burdensome methods.

Recombinant enzymes have been expressed in bacteria (e.g., *E. coli*, *Bacillus*, and lactic acid bacteria), filamentous fungi (e.g., *Aspergillus*), and yeasts (e.g., *Hansenula polymorpha*). The favorable and very advantageous characteristics of these species have resulted in an increasing number of biotechnological applications. Bacterial hosts such as *E. coli* can be used to quickly and easily overexpress recombinant enzymes. However, bacterial systems cannot express very large proteins and proteins that require posttranslational modifications. The main bacterial expression hosts, with the exception of lactic acid bacteria, can produce several toxins which are not compatible with the expression of recombinant enzymes in food and drugs.

The safety of the source organism is the fundamental element to be taken into account in the evaluation of an enzymatic product. A microorganism utilized in enzyme production should be well characterized and not produce any pathogen, toxin, or antibiotic. Often, the production of enzymes is based on soil microorganisms, normally in direct contact with humans and animals, and having a recognized

secure history and are Generally Recognized as Safe (GRAS status). Accordingly, the genome of the producing microorganism must be fully characterized allowing full examination of safety.

Early developments on enzymes and biocatalytic processes started to take form during the 17th and 18th centuries. It was after the 1960s that enzymatic biocatalysis became a viable industrial option with the mass production of microbial proteases and lipases for use in washing powders. Industrial enzymes have since evolved into a multibillion-dollar global market [31]. This market will probably continue to expand in the future as a consequence of developments in the biotechnology industry, the sustained need for cost-efficient industrial process, and calls for greener technologies.

1.4 BIOMINING FOR NEW AND/OR IMPROVED ENZYMES

Enzymes are one of the most important biomolecules which have a wide range of applications in industrial, nutritional, as well as biomedical fields. Today, enzymes are some of the most important molecules which have been widely used since ancient human civilizations. With growing populations and increasing needs, enzymes seem to be some of the most vital molecules that have a great impact in several sectors [31].

The harmful effects of using chemical catalysts to speed up the process have come to the fore for mankind. Many chemical transformation processes used in various industries have inherent drawbacks from commercial and environmental points of view. Nonspecific reactions may result in poor product yields. High temperatures and/or high pressures needed to drive reactions lead to high energy costs and may require large volumes of cooling water downstream. Harsh and hazardous processes involving high temperatures, pressures, acidity, or alkalinity need high capital investment and specially designed equipment and control systems. Unwanted by-products may prove difficult or very costly to produce. High chemicals and energy consumption as well as harmful by-products have a strong negative impact on the environment. An urgent need for environmentally friendly processes/biocatalysts came to light which again created a great search for innovation. In several cases, some or all of the drawbacks can be virtually eliminated using enzymes. Interestingly enzyme reactions may very often be carried out under mild conditions. They are highly specific and involve high reaction rates. Industrial enzymes originate from biological systems which can effectively contribute to sustainable development through being isolated from microorganisms which are fermented using primarily renewable resources. In addition, only small amounts of enzymes are needed in order to carry out chemical reactions even on an industrial scale. Enzymes also reduce the impact of manufacturing on the environment by reducing the consumption of chemicals, water, and energy, and the subsequent generation of waste.

Enzymes are nowadays indispensable tools in an extreme large area of activities. Some have volume and economic value preponderance. Thus, detergent enzymes as well as those aimed at waste bioremediation acquired a relevant position. Enzymes aimed at "green" biocatalysis or "white chemistry" are becoming an important emerging field for new hydrolases. Enzymes for food and feed technology belong to the bulk of industrial enzymes. The better valorization of plant biomass as well as that of nonedible ones will retain a need for screening of either more performant enzymes and/or for the application of new enzymes such as the lignolytic catalysts. On the other hand, probiotics and digestive enzymes are essential for normal digestive function. Together they play a vital role in the breakdown, delivery, and utilization of the nutrients we obtain from our diet. The use of probiotics is a very promising strategy, not only for prevention of human and animal pathologies, but also for improvement of animal productivity and food quality and safety. For example, a NisZ-producing *Lactococcus lactis* strain was isolated for the first time from an aquatic environment in 2015, and this strain may be considered as a potential probiotic for rainbow trout farming for improving the quality and safety of fish and fish products [34].

Traditionally, new biocatalysts were discovered by a cumbersome screening of a wide variety of organisms for the desired activity. A modern variant is the metagenomics approach, which involves the extraction of genomic DNA from environmental samples, its cloning into suitable expression vectors, and subsequent screening of the constructed libraries [35]. This approach has been successfully applied to isolate new biocatalysts, including carboxylic ester hydrolases from hyperthermophiles [36], and can potentially result in unique enzymes (no sequence similarity), but obviously depends on functional expression. At present, with many complete genome sequences available, bioinformatics has become an important tool in the discovery of new biocatalysts. This is a high-throughput approach for the identification, and in silico functional analysis, of more or less related sequences encoding potential biocatalysts (Fig. 1.2).

The bioinformatics approach, such as Basic Local Alignment Search Tool (BLAST), Motif Search, and InterPro, has been successfully applied in identifying new carboxylic ester hydrolase sequences in the completely sequenced genomes of several selected hyperthermophiles. Several sequences in the existing databases have previously been marked as putative esterase or lipase. However, even more carboxylic ester hydrolases can be identified when BLAST and Motif searches, in combination with pair-wise comparison with sequences of known carboxylic ester hydrolases, are used. The improvement of this methodology paralleled to outdated activity screening is the direct identification of new and varied carboxylic ester hydrolases, which would otherwise have not been detected due to a low level of expression. In order to have as many candidates as possible, sequences that were assigned a different function, but did have the characteristics of carboxylic ester hydrolases, were also included, such as acylpeptide hydrolases. The advantage of this approach compared to traditional activity screening is the direct identification

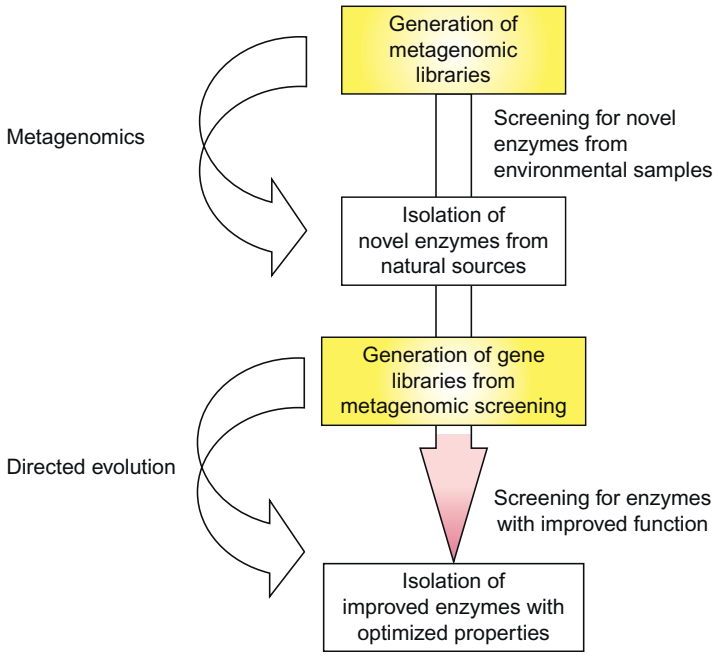

FIGURE 1.2

Summarized steps for the discovery of new enzymes.

of new and diverse carboxylic ester hydrolases, which would otherwise have not been detected due to low levels of expression [37].

In detergent, the main utilized enzymes are lipolytics (lipases and phospholipases) and proteases. Incorporated in detergents, such enzymes are able to remove natural stains from laundry at temperatures of 60°C and below. The various enzymes form complexes with the substrates (stains) and hydrolyze fixed proteins and polysaccharides as well as fats. Surfactants rinse the resulting fragments into the wash liquor. The genes for the synthesis of these enzymes, which predominantly originate from *Bacillus* strains, are optimized for the desired performance of the enzymes. They work nonspecifically and are resistant to the detergent ingredients in the liquor. During fermentation, in the industrial production of the enzymes, microorganisms secrete the enzyme into the medium to cleave proteins with high molecular weight, which swim in the broth. The fragments penetrate into the cell and serve as energy sources. After the end of fermentation, the enzymes are separated from the microorganisms, concentrated, and converted into the final product. Granulated products, containing the enzymes in the matrix, require a coating to ensure product safety. Product design is determined on the one hand by protein engineering and on the other hand by design of the final product.

Numerous categorizations of esterases and lipases into different families have been made. Horchani et al. [38] classified 53 bacterial esterases and lipases into eight families based on their sequence similarities and some of their fundamental biological properties. Several new esterases and lipases have since then been identified, including some, such as EstD from *Thermotoga maritima*, which could not be congregated into one of these eight families. Consequently, new families for these enzymes have been projected [39]. Nevertheless, this initial arrangement has provided a good basis for more sophisticated classification of the esterases and lipases. Many studies are based on sequence and structural similarity, and are available at online databases as The Lipase Engineering Database (LED), the Microbial Esterase and Lipase Database (MELDB), the Carbohydrate Active Enzyme (CAZy) database, and the ESTHER database [40].

Lipases (triacylglycerolacylhydrolase, E.C.3.1.1.3.) are ubiquitous enzymes with the industrial potential of detergents in cleaning and as emulsifiers in nutrition and cosmetics [41]. Detergent enzymes constitute about 32% of the total worldwide industrial enzyme production [42]. Thermal stability is a major requirement for a commercial enzyme such as lipase which would allow enzymatic reactions to be performed at a higher temperatures and would be helpful to increase conversion rates and substrate solubility. The detergent industries are relying on recombinant lipases such as Lipex and Lipolase (Novozymes) for formulation of biodetergents due to their stability in the presence of harsher detergent formulation ingredients such as surfactants and oxidizing agents [43]. Researchers are continuously searching for lipases from indigenous extremophilic regions for better application in the laundry detergent industry. Among the different sources (fungal, yeast, and bacterial), bacterial lipases received much attention for their ability to function in extreme environments of temperature, pH, surfactant, and oxidizing agents [44]. Moreover, bacterial lipases added to household detergents reduce or replace synthetic detergents, which have been considered as environmental pollutants [45]. It has been shown that the alkaline lipase from *Staphyloccocus arlettae* improves the oil removal from oiled cotton fabric by 21% as an additive to commercial detergent suggesting that *S. arlettae* is an interesting option for formulating an environmentally friendly detergent formulation [37].

Alkaline lipases in a detergent should have high activity and stability over a broad range of temperature and pH, and should also be compatible with different components in a detergent including metal ions, surfactants, and oxidants [46]. In past years, intense efforts have been focused on the engineering of lipases with altered properties or better performance for practical applications. Therefore, screening of new microorganisms with lipolytic activities could facilitate the discovery of novel lipases.

It is believed that the ability of phospholipases to cleave lecithin or phosphorylated lipids results in water-soluble lysophospholipids, which are more easily removed in the washing process. Attention should be focused on amino acids sensitive to oxidation in the case of bleach stability and on surface charges for the surfactant compatibility. The isoelectric point of such enzymes may be modified

by the substitution of some charged amino acids, e.g., an increase in isoelectric point may help to improve compatibility with anionic surfactants. The stability of the enzymes may be further enhanced by the creation of e.g., additional salt bridges and enforcing calcium binding sites to increase chelant stability [47].

Over the past 20 years, the development of subtilisins as typical detergent proteases has employed all the tools of enzyme technology, resulting in a constant flow of new and improved enzymes. The number of molecules identified and characterized, however, is in clear opposition to the number of molecules that are entering the market. The question that is arising is whether the next-generation detergent proteases should be based on new backbones different from subtilisins, or will the use of all available technologies (rational design, directed evolution, and exploitation of natural diversity) yield improved subtilisins, ending the current era dominated by high-alkaline subtilisins. These questions will have to be answered not only by the performance of the molecules themselves, but also by their yield in fermentation and their compatibility with existing production technologies [48].

The alkaline proteases as well as other detergent-compatible enzymes such as lipases and amylases serve now as the key components in detergent formulations. The search for detergent-compatible proteases with better properties is a continuous exercise. The current trend is to use detergent-compatible proteases that are stable over a wide temperature range. Although the proteases showing stability at elevated pH have the capacity to be used in detergent formulations, their usage can be significant if they are also stable and compatible with detergent and detergent ingredients, and they are also able to remove protein stains. Until now, there is no specification for the use of alkaline proteases as detergent additives [49].

Advances in protease engineering for laundry detergents should comprise simultaneous improvement of thermal resistance and activity at low temperatures, a rational strategy to modulate pH profiles, and a general hypothesis for how to increase promiscuous activity toward the production of peroxycarboxylic acids as mild bleaching agents [49].

In the past, wastes were traditionally disposed of by digging a hole and filling it with waste material. This mode of waste disposal was difficult to sustain owing to a lack of new places to dump the waste. New technologies for waste disposal that use high-temperature incineration and chemical decomposition (e.g., base-catalyzed dechlorination, UV oxidation) have evolved. Although they can be very effective at reducing a wide a range of contaminants, they have several drawbacks. These methods are complex, uneconomical, and lack public acceptance.

Bioremediation is the result of a microorganism or enzyme-mediated transformation or degradation of contaminants into nonhazardous or less-hazardous substances. The employability of various organisms such as bacteria, fungi, algae, and plants for efficient bioremediation of pollutants has been reported [44,45].

The process of bioremediation mainly depends on microorganisms in which enzymes attack the pollutants and convert them to innocuous products. As bioremediation can be effective only where environmental conditions permit microbial

growth and activity, its application often involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate. The process of microbial bioremediation is a very slow process. Only certain species of bacteria and fungi have proven their ability as potent pollutant degraders. Many strains are known to be effective as bioremediation agents but only under laboratory conditions. The limitation of bacterial growth is under the influence of pH, temperature, oxygen, soil structure, moisture and appropriate level of nutrients, poor bioavailability of contaminants, and the presence of other toxic compounds. Although microorganisms can exist in extreme environments, most of them prefer optimal conditions—a situation that is difficult to achieve outside the laboratory [50–52]. Both bacteria and fungi rely on the participation of different intracellular and extracellular enzymes, respectively, for the remediation of recalcitrant and lignin and organic pollutants [50,53]. Some of the inconvenience can be bypassed by the use of adequate enzyme or most often a cocktail of enzymes [54].

1.5 POTENTIAL ROLE FOR ENZYMES FROM ARCHAEA (EXTREMOZYMES)

A large number of articles on extremophiles have been published recently [50]. Due to their capability of surviving in environments under extreme conditions, both physical, such as temperature (−2 to 12°C, 60–110°C), pressure, or radiation, and geochemical, such as salinity (2–5 NaCl) and pH (<2, >9), extremophiles are a very interesting source of enzymes as well as biochemicals with extreme stability under conditions regarded as incompatible with biological materials. Recent studies show that the diversity of organisms in these extreme environments could be even greater than was initially thought [55,56]. However, because the majority of these microorganisms have not yet been isolated in pure culture, useful characterization of their enzymes still remains quite difficult. Thermophilic proteases, lipases, as well as cellulases and amylases, are being used in many different industrial applications [57].

Extreme thermophiles (growing at 60–80°C) are widely distributed among several bacterial genera such as *Clostridium*, *Thermus*, *Thermotoga*, and *Bacillus*, whereas most hyperthermophiles belong to *Archaea* such as *Pyrococcus*, *Thermococcus* or *Methanopyrus*, among others. The Taq DNA polymerase, isolated from the thermophilic *Thermus aquaticus*, had sales of several hundred million dollars. Also, enzymes from psychrophiles have become quite interesting for many industrial applications, partly because of the ongoing efforts to reduce energy consumption. Therefore, enzymes such as proteases, amylases, or lipases have great commercial potential for development of detergents to reduce wear and tear of textile fibers. Polymer-degrading activities (e.g., laccases, cellulases, xylanases) are quite interesting for the pulp and paper industry, as well as for

saccharification of pretreated lignocellulosic biomass for the production of second-generation agrofuels and nutritional applications. Cold-active enzymes also have potential for many other applications, such as extraction and clarification of fruit juices, improvement of bakery products, polishing and stone-washing of textiles, bioremediation of waters contaminated with oils or hydrocarbons and aquaculture additives. Enzymes from halophites have to cope with very high concentrations of salts (e.g., sodium or potassium chloride). Such proteins have adapted to these environments by acquiring a large number of negatively charged amino acid residues of their surfaces to prevent precipitation. Such a property has been taken advantage of by use of nonaqueous media. Xylanases, amylases, proteases, and lipases from *Halobacterium*, *Halobacillus*, and *Halothermothrix* have been produced and their potential has been reviewed. Microorganisms that can survive under extreme pH values could be good sources of thermoalkaliphilic and thermoacidophilic enzymes, such as proteases and lipases, particularly useful for applications as additives in laundry and dishwashing detergents.

Recent enzyme discoveries and developments in genetics and protein engineering have increased the reach of enzymes in industry. The vast majority of current industrial processes are performed under harsh conditions, including extremely high or low temperatures, acidic or basic pHs, and elevated salinity.

Extremophiles are mainly microorganisms which belong to the domains Archaea and Bacteria. They thrive in environmental conditions considered by human standards to be extreme. Extremophiles grow and reproduce under high temperatures in hot springs or thermal vents, low temperatures in glaciers or the deep sea, acidic and basic pH, high concentration of salts in salt lakes, etc. [58].

Microorganisms possess some membrane-specialized structures and the proteins that enable them to proliferate in extreme environments. Such microorganisms, possessing unusual cell make-up, are now proving to be assets to our industries, enabling industrial processes to work beyond the normal range. In recent years, industries have also shown an interest in extremozymes. The increasing usage of cold-active proteases, particularly in the detergent industry, is because of their catalysis at low temperature, resulting in additional benefits such as cost reduction due to minimization of energy and retention of fabric quality, which was missing during the use of phosphate. The thermolability of psychrophilic enzymes strengthens the process by virtue of curtailment of unwanted side reactions. These atypical properties place the cold-active enzymes on top in industrial demand list, as the market competes for cost effectiveness and quality product. According to recent reports, the carbohydrases account for the largest market share followed by proteases among all enzymes. The escalating demand for biocatalyst has put up challenges to modern biotechnology. These challenges can be met in either of two ways: improving the catalytic activity of the already existing enzymes or finding the novel enzymes [59].

Success with DNA polymerase allowed an increase in biotechnological utilization of enzymes from extremophiles. To fulfill the great potential, methods should be developed to overcome current bottlenecks like the current lack of

ability to produce most extremozymes on industrial large scales. Some recombinant extremozymes can be produced by mesophilic organisms such as *E. coli*, but not all of them. Taking this into account, new expression systems should be developed with extremophilic organisms as the host to achieve high expression of soluble proteins.

In many cases, enzymes from extremophiles have adapted to withstand extreme conditions on their own. These adaptations correspond to key changes in the amino acid sequences, which are translated into variations in the structure, flexibility, charge, and/or hydrophobicity of the enzymes. These changes do not follow a pattern or a specific trend. Extremophilic proteins display substantial variability in adaptations for similar extreme physical or chemical conditions. These diverse adaptations allow extremozymes to expand the ranges of optimal enzyme performance enabling enzymatic biocatalysis under the enzymatically unfavorable conditions found in most of industrial processes.

Hyperthermophiles show a broad physiological diversity, ranging from aerobic to methanogens and saccharolytic heterotrophs [60]. As they are a potential source of new biocatalysts, the genomes of several hyperthermophiles have been completely sequenced. All completely sequenced hyperthermophiles have a reverse gyrase catalyzing a positive supercoiling of their DNA and this can be seen as a marker for growth at high temperatures [61,62]. The stability of hyperthermophilic enzymes is dependent on changes in amino acid composition, such as a decrease in asparagine and cysteine residues, increased hydrophobic interactions, an increased number of ion pairs and salt bridge networks, reduction in the size of surface loops and of solvent-exposed surface, and increased intersubunit interactions and oligomeric state [63]. Besides these structural adaptations, proteins can also be stabilized by intracellular solutes, metabolites, and sugars.

Through actively bioprospecting extreme environments and /or using genetic engineering it is possible now to discover and develop extremozymes that can accurately respond to nowadays industrial processes or products.

The unique features of enzymes led to their applications in competitive bioprocesses as one of the foremost areas of biotechnology research. Much of the biosphere is under extreme conditions such as in cold oceans and dry, salty deserts). The discovery of thermostable DNA polymerases and their impact on research, medicine, and industry has underscored the potential benefits of extremozymes [50]. A great deal of current research effort is aimed at screening for new sources of novel enzymes capable of functioning in extreme conditions [23,64]. A basic understanding of the stability and function of extremozymes under extreme conditions is important for innovations in biotechnology [65]. Emerging knowledge on protein−solvent interactions are likely to aid in development of new catalysts for use in novel synthetic applications, including enzymes operating in low water activity and organic solvents, and in the development of efficient catalytic systems active in organic solvents.

It is expected that the number of archaea enzymes used industrially will increase due to their inherent stability and novel specificities. The development of

new rapid-screening technologies and improved bioinformatic approaches in combination with a new generation of sequencing methodologies of archaeal genomes and metagenomes will provide new enzymes for industrial biotechnology [66]. The enzymes can be cloned and overexpressed in easily grown hosts such as *E. coli*, allowing access to sufficient quantities of the purified enzymes for detailed biochemical and structural characterization scale-up of the enzyme production required for commercial applications can be carried out by using a fungal host system which allows export of the proteins into the growth medium for easy downstream processing. The cost of the enzyme biocatalyst is often the most expensive component of the industrial biotransformation and must be matched to the value of the end product. The stability of the biocatalyst is also an important issue. Immobilization of the enzyme can often increase its stability and allow it to be easily recovered for reuse. Thermostable enzymes are usually more robust under industrial conditions and can be used for repetitive biocatalytic conversions. The increased use of enzymes from the extremophilic archaea offers the opportunity to access biocatalysts that are naturally stable to a variety of different conditions of temperature, pH, salinity, and pressure making them better suited to different industrial processes [67].

The use of enzymes in "white biotechnology" is expected to grow with biobased materials and chemicals produced from emerging technologies predicted to rise globally to over 8 million metric tons in 2018. The initial process of enzyme discovery and optimization is still a limiting factor in the adoption of new biobased industrial processes. There is, therefore, an increasing opportunity to promote newly discovered archaeal enzymes for sustainable manufacturing to contribute to the new circular economy.

1.6 GENETIC ENGINEERING OF ENZYMES

The ability to manipulate in vitro DNA encoding for enzymes as well as for other proteins depends entirely on the availability of purified enzymes that can cleave, modify, and join the DNA molecule in specific ways. Until now, no chemical method can achieve the ability to manipulate the DNA in vitro in a predictable way. Only enzymes are able to carry out the function of manipulating the DNA. Each enzyme has a vital role to play in the process of genetic engineering. The various enzymes used in genetic engineering are as follows: nucleases, restriction enzymes, DNA ligase, kinase, phosphatase, reverse transcriptase, terminal deoxynucleotide transferase, and RnaseP.

The general strategies for obtaining recombinant enzymes follows a route from identifying a suitable DNA template to choosing the correct host expression system. Nevertheless, there is no standard operating procedure for cloning a recombinant enzyme and accordingly the best strategies should be based on the accuracy of the methods applied (Fig. 1.3).

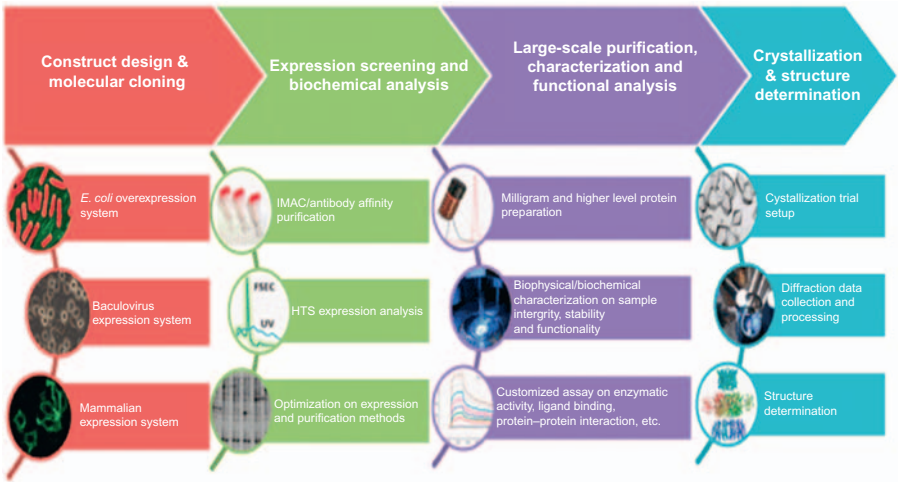

FIGURE 1.3

Screening steps for the discovery of new proteins (enzymes).

There are general and basic steps in DNA cloning. First, the desired DNA of interest is cut from the source organism using restriction enzymes. Then, the DNA is pasted into a vector and the ends of the DNA are ligated with the vector. The vector is then introduced into a host cell, which is either a fungus, a bacterium, or a yeast, and the cells are grown by a fermentation process. There are two main cloning methods that can be used according to the aim of the process—cDNA cloning and genome cloning. Since the enzymes must be in active form so that they can be expressed by the new host, cDNA cloning appears as the more suitable.

Polymerase chain reaction (PCR) has revolutionized cDNA cloning and, in some circumstances, it is not necessary to construct a library for the isolation of a particular cDNA clone. If some sequence information is available, then it is possible to clone the cDNA by PCR. It is worth trying because this approach is much faster and simpler than building and screening a cDNA library.

The major activity of cloning will involve the selection of the suitable vector and expression host as detailed [68].

1.7 SCREENING FOR MICROBIAL ENZYMES

Microbial enzymes are present in the immense natural biodiversity. Main available tools to expand the search for new enzymes are metagenome screening, genome mining in more than 2000 sequenced microbial genomes, and exploring the diversity of extremophiles.

Metagenomics- and metatranscriptomics-based approaches have been increasingly used to study lignocellulolytic microbial consortia. Recent work has successfully enriched microbial consortia using plant waste as the sole carbon source. Such microbial consortia were shown to have large capacities to degrade plant biomass, being promising for lignocellulose saccharification. A new methodology is thought to enhance the prevalence of microbes acting on the most recalcitrant part of the lignocellulose (e.g., complex hemicellulose structures, crystalline cellulose, and lignin). Clearly, a better picture of the consortial behavior will be obtained by the evaluation of the time points along the microbial growth and incubation [69].

1.8 MICROBIAL GENOMES

The recent success of genome-sequencing programs has resulted in an explosion of information available from sequence databases, thus creating an opportunity to explore the possibility of finding new natural products (including enzymes) by database mining. The next-generation sequencing platforms (454 from Roche, Solexa from Illumina, or SoLiD from ABI), hold promise to reduce the time and cost of genome sequencing. Using these platforms, as well as cutting-edge approaches such as resequencing, allows the completion of multiple whole genomes in less than 2 weeks. So far, more than 2000 genome sequences and draft assemblies are available in the NCBI database. Two approaches are being followed to discover new enzymes. On the one hand, genome hunting is based on searching for open reading frames in the genome of a certain microorganism. Sequences that are annotated as putative enzymes are subjected to subsequent cloning, overexpression, and activity screening. Another approach, called data mining, is based on homology alignment among all sequences deposited in databases. Using different bioinformatics tools (e.g., BLAST), a search for conserved regions between sequences yields homologous protein sequences that are identified as possible candidates for further characterization. After the best hits to genes from an isolated genome are identified, all subsequent analysis is performed using the genes of the isolate genomes, not the genes of the metagenome at hand. The synergy between multiple next-generation screening platforms and the increased accuracy and data processing speed of downstream bioinformatics, biostatistical and machine-learning pipelines continue to push the boundaries of genomic and metagenomics data analyses [34].

1.9 METAGENOMIC SCREENING AND FUNCTIONAL SCREENING OF (META)GENOMIC LIBRARIES

It is known that from the numerous microbes inhabiting the biosphere, less than 1% can be cultivated through standard microbiological techniques. This fact is

very important in that we do not know the physiological role of the tremendous majority of the microbiota. Metagenomics has appeared as an alternative strategy to conventional microbe screening by preparing a genomic library from environmental DNA and systematically screening such a library for the open reading frames potentially encoding putative novel enzymes. Such methodologies allow a much faster and elaborative genomic/genetic profile generation of an environmental sample at a very acceptable cost. Full shotgun metagenomics has the capacity to fully sequence the majority of available genomes within an environmental sample (or community) [69]. Metagenomic screening is mostly based on either function or sequence approaches. On the one hand, function-based screening is a straightforward way to isolate genes that show the desired function by direct phenotypical detection, heterologous complementation, and induced gene expression. On the other hand, sequence-based screening is performed using either PCR or hybridization procedures. Usually, the common procedure is to use a set of degenerated primers that have been designed based on consensus amino acid sequences. Studies from different habitats such as volcanic vents, arctic tundra, cow rumen, marine environments and termite guts have yielded microbial enzymes with potential for biocatalytic applications.

Function-based screening of metagenomic libraries is somehow problematic mainly due to insufficient, biased expression of foreign genes in *E. coli* as this bacterium is usually used as a surrogate host. To overcome such hurdles, alternative bacterial host and expression systems are currently being examined including *Streptomyces lividans*, *Pseudomonas putida*, and *Rhizobium leguminosarum*, among others. Hit rate (probability of identifying a certain gene) also depends on other factors such as size of the target gene and the assay method. Enzyme activities are usually assayed on agar plates supplemented with different substrates Sensitivity of agar plate-based screening can be improved by using cell lysates, screening for genes giving resistance to toxic compounds, or linking the target activity to the expression of a reporter gene such as green fluorescent protein or β-galactosidase. Also, development of flow cytometry based screens such as SIGEX are leading the way as they enable more rapid screening of large metagenomic libraries. Genes obtained through sequence-based screening are limited to those having homology to the sequences used as probes.

Practically immense types of environments remain available for checking for new enzymes. Even among the environments that have been studied by metagenomics over the last 20 years, it seems that only 11% have been studied with this goal in mind [65]. Functional analysis of samples taken from as-yet unexplored habitats is bound to yield original and exceptional microbial biocatalysts.

The quality of extracted gDNA or the directly extracted eDNA (culture-independent approach) is a fundamental aspect and they should not be degraded and be as pure as possible. DNA could be purified and size-selected on agarose gel or by ethanol or PEG/NaCl precipitation [66]. When the quality of the extracted DNA has been checked, the DNA is digested with restriction enzymes to the desired insert-size and to obtain compatible ends for further cloning. The

restriction enzyme is chosen according to the type of ligation envisaged (blunt or sticky ends), whether and where the extracted DNA is methylated (some restriction enzymes are sensitive to *dam*), ligation for introduction into host cells [70]. When the cloning vector is to be used in different hosts, it is recommended to use a shuttle vector or a broad-host-range vector containing more than one replication origin, suitable for expression in various hosts [70].

Heterologous expression is a big challenge in functional screening of (meta) genomic libraries. The transformed host cell must be able to express the foreign DNA and ensure proper folding of the resulting protein(s). Expression can be predicted by bioinformatics [69]. An example of a hurdle is that some codons are unrecognized by the host cell and can lead to ineffective translation, production of truncated polypeptides, or formation of inclusion bodies after translation resulting in inactive proteins (Figs. 1.4 and 1.5).

Host cells for constructing functional metagenomics DNA libraries must respect several requirements: they must be transformable, genetically accessible and modifiable, easily detected (sensitivity of bacteria to some specific antibiotic is generally used), and show no activity on the screening medium. Ideally, they should show as few enzymatic activities as possible for functional screening.

Metagenomics studies are commonly applied to investigate the specific genomes that are present within an environmental community under study. Moreover, when performing full shotgun metagenomics, the complete sequences of protein coding genes (previously characterized or novel) as well as full operons in the sequenced genomes can offer invaluable functional knowledge about the community [69].

E. coli is the more frequently used bacterial host for expression of recombinant proteins. Other bacterial species have been used in (meta)genomic library screens such as *P. putida* as well as its variant *Pseudomonas antartica*, *Thermus thermophilus, Bacillus subtilis*, and *S. lividans* [71]. A close phylogenetic relationship between the expression host and the organism from which the foreign DNA derives should favor heterologous expression, and the efficiency of multihost screenings in the identification of enzymes or molecules is clear.

Yeasts and fungi can also be used as screening hosts. Yeasts such as *Saccharomyces cerevisiae* (whose genetics is well known and for which many genetic tools are available), *Pichia pastoris* (with which excellent protein production yields are achieved), and *H. polymorpha* (often used for the expression of phytases) are widely used for their numerous advantages in high-level heterologous expression of genes encoding for enzymes [72]. These organisms combine the advantages of unicellular cells since they are easy to grow and manipulate genetically with those of eukaryotic cells, such as better protein processing than in prokaryotes, allowing posttranslational modifications and glycosylation [73]. The biggest limitations of using yeasts in functional screening could be poor recognition of heterologous promoters (especially if they are bacterial), low transformation yields (the libraries are then constructed in *E. coli* and screened in yeast),

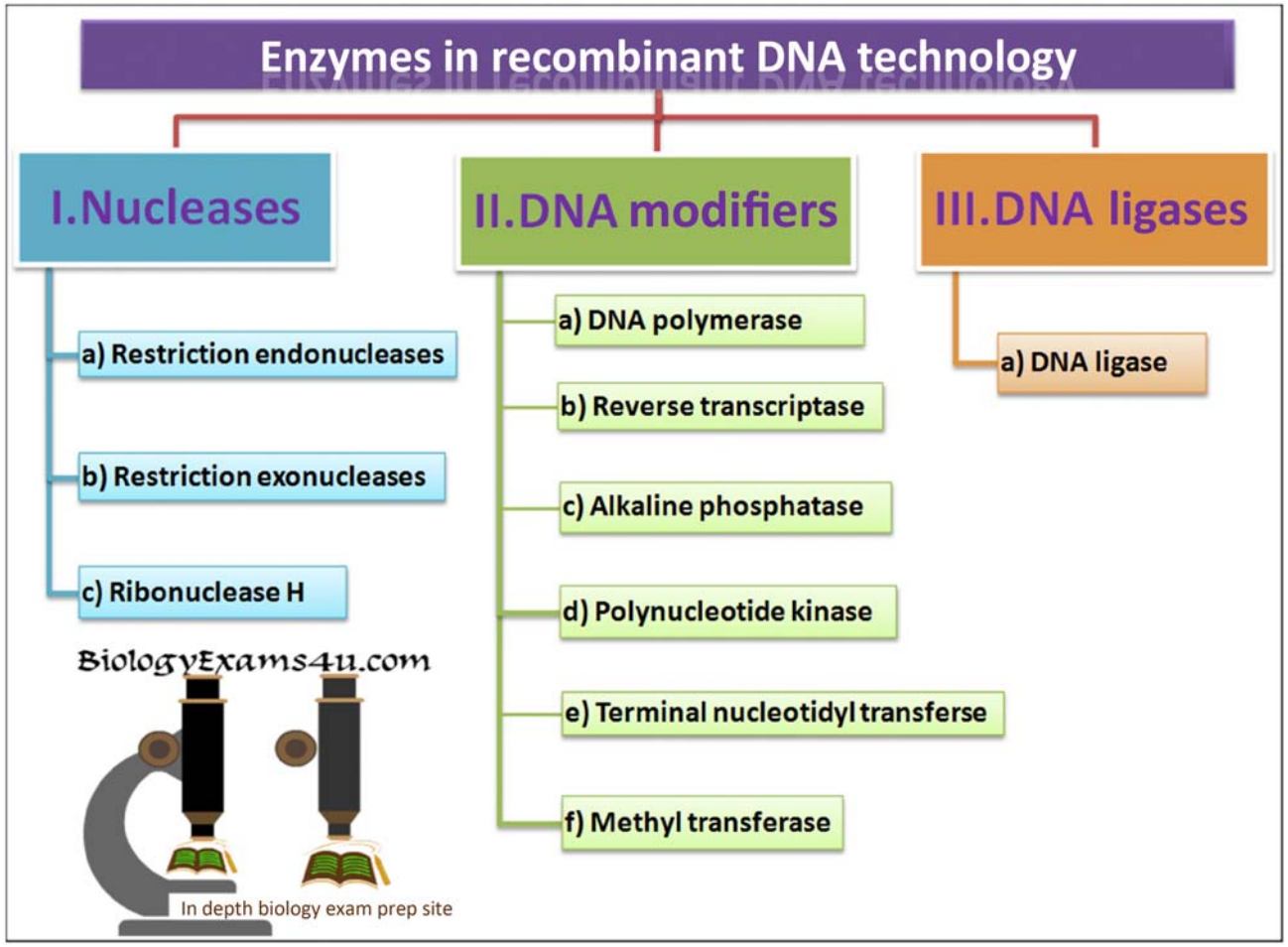

FIGURE 1.4

Schema of enzymes in recombinant technology.

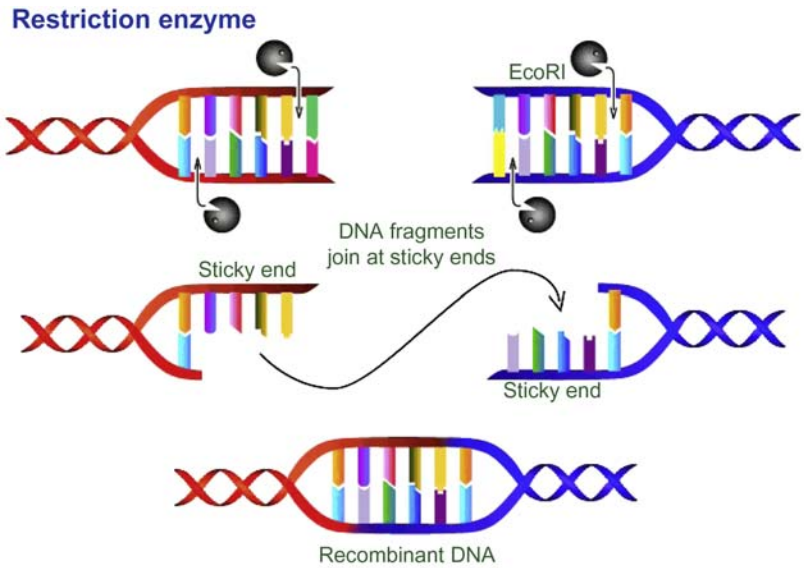

FIGURE 1.5

Role of restriction enzymes in DNA recombination.

and the multiple enzymatic activities displayed by yeasts (the host should be mutated in all genes encoding enzymes of interest).

More than 7000 enzyme types are currently listed in the BRENDA database. They are classified into six classes: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. Varying the screening conditions, even if time consuming, can be useful for providing knowledge for the selection of clones with particular properties and/or for further characterization of the enzymes responsible for detected activities.

There is an urgent need to develop function-based screening methods for genes and enzymes that belong to enzyme classes other than hydrolases and that are of relevance to biotechnology [74]. Many biotechnologically relevant enzymes including hydrolases and oxidoreductases have already been discovered by metagenomic screening. It is not surprising that the majority of metagenome-derived enzymes that have been characterized biochemically mainly originated from function-based screenings [69]. Functional screening can also be performed with a mutant host cell impaired in a vital enzymatic activity. The advantage of heterologous complementation is that the host cell is equipped to produce a protein having the same function, and that only clones producing the enzyme sought are viable [74]. This method is very sensitive, but it is applied mostly to the identification of metabolites, as few vital enzymes are sought.

To obtain a wide diversity of novel enzymes, different strategies should be combined, such as working with cultivable and noncultivable organisms, using

sequence- and function-based approaches, performing multihost screenings, and constructing libraries in both plasmids and fosmids.

Another technical option for obtaining "tailor-made" enzymes is to try to construct synthetic genes able to encode for the suitable characteristics of the enzyme. To our best knowledge, the first enzyme (phytase) obtained from a full synthetic gene expressed in *H. polymorpha*, named "Consensus" was developed in 1997 [75]. The enzyme is resistant to temperatures up to 90°C, has the capacity of refolding after denaturation, and has an activity equivalent to that of *Aspergillus niger* [76–78].

1.10 CONCLUSIONS AND PERSPECTIVES

Microbes have been utilized since ancient human civilizations, as early as early as 6000 BC in Mesopotamia. They are huge sources for industrial enzymes due to the easy availability and fast growth rate. Genetic changes using recombinant DNA technology can easily be performed on microbial cells for elevated enzyme production and scientific development [79]. Microbial enzymes have gained global recognition for their widespread uses in various sectors of industries, e.g., food, agriculture, chemicals, medicine, and energy. Enzyme-mediated processes are rapidly gaining interest because of reduced process time, low energy input, cost-effective, nontoxic, and ecofriendly characteristics [72]. Moreover, with the advent of recombinant DNA technology and protein engineering, a microbe can be manipulated and cultured in large quantities to meet increased demand [11]. Associated driving factors that motivate the use of microbial enzymes in industrial applications are increasing demand of consumer goods, the need for cost reduction, natural resources depletion, and environmental safety [72]. Isolation and purification of enzymes is very costly and their recovery for further utilization is difficult [80].

The main application areas for new or improved microbial enzymes are detergents, pollutants, waste treatment, and valorization of nonedible plant biomass.

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CHAPTER

Intellectual property on selection, expression, and production of enzymes

2

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2.1 INTRODUCTION

Intellectual property (IP) is related to creations of the intellect for which a monopoly is assigned to designated owners by law. IP rights (IPRs) are granted to the creators of IP, and include patents, trademarks, industrial design rights, fabrication secrets, and in some countries trade secrets. Besides industrial and commercial goods, artistic works including music and literature, as well as discoveries, inventions, words, phrases, symbols, and designs can all be protected as IP.

The development of industrial goods such as food and feed additives, including enzymes is time consuming and costly. For enzymes the development processes imply selection, genetic engineering, expression, upscale production, formulation, registration, and promotion. It is not surprising that enzyme production companies spend continuous actions to protect their inventions. In such processes the fabrication secrets are most probably the simplest way to get protection. However, in the case of fraud by competitors there are almost no means of returning the knowledge back to the owner and this only other means of IP can be really protective.

The IP laws have evolved over centuries. However, it was not until the 19th century that the term "intellectual property" began to be used, and it was not until the late 20th century that it became commonplace in the majority of the world.

IP contributes enormously to national, state, and individual economies. Several industries across economy rely on the adequate enforcement of their patents, trademarks, and copyrights, while consumers use IP to ensure they are purchasing safe, guaranteed products. IP rights are worth protecting, both domestically and abroad. For many businesses, IP protects more than just an idea or a concept. It protects genuine business assets that may be integral to the core services of the business and overall long-term viability, it keep ideas, protects business growth, and as a whole it is less complicated than expected. It is also generally assumed that IP creates and supports high-paying jobs, drives economic growth and competitiveness; strong and enforced IPRs protect consumers and families, help to generate breakthrough solutions to global challenges, and

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encourage innovation and reward entrepreneurs. Bringing all of these important and diverse points together is the fact that protecting IP is a nonpartisan issue that is shared by a broad coalition of interests. Such rights are embraced by all sectors of industry—small, medium, and large companies alike—and by labor organizations, consumer groups, and other trade associations.

IP protection is critical to fostering innovation. Without protection of ideas, businesses and individuals would not reap the full benefits of their inventions and would focus less on research and development. Similarly, writers and artists would not be fully compensated for their creations and cultural vitality would suffer as a result.

IPRs are often confusing and sometimes the topic of heated debates. There are those who question the worth of creative products and projects, claiming design and art are things that anyone can do, regardless of training, experience, or any inherent ability. As creatives, a deterioration of IPR is a dangerous possibility. By going public with our work, we have no recourse to prevent others from using our discoveries, designs, books, or any other artwork without paying us or even offering proper credit.

And yet, creatives are just as often guilty of violating these rights as those who are not in a creative profession, and sometimes they are even more likely to violate these rights. It is fundamental to have more respect for creators and their work, regardless of the perceptions of what is thought about their work and their processes. If something is created by someone else, one needs to respect that and abide by their wishes when it comes to use, credit, and compensation.

There are hundreds of excellent books dealing with IP (e.g., [1,2]). The biggest edition companies, such as, e.g., Springer and Elsevier, participate in the Access to Research for Development and Innovation (ARDI) program which is coordinated by the World Intellectual Property Organization (WIPO) as part of the Research for Life (RFL) program. RFL is a public−private partnership that includes the United Nations agencies WIPO, the World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO), and the United Nations Environment Program (UNEP), the International Association of Scientific, Technical and Medical Publishers (STM), leading academic libraries, and technology partner Microsoft [3].

In developing countries, local innovation is important to the success of national economies. Young scientists and their research institutions need to access the latest developments in technology found in scientific and technical literature for rapid patenting and commercialization of their inventions. However, without sufficient access to journals, their ability to learn from scientific information is limited and efforts are slow and arduous. Through ARDI, developing countries are given direct access to high-quality published subjects from several publishers allowing select academic and research institutions to develop and innovate more effectively, and patent offices to protect and reward innovation more efficiently.

WIPO is the leading global forum for the international promotion of IP as a force for innovation and creativity allowing a positive change to be achieved. As

a specialized agency of the United Nations, WIPO assists its 185 member states in developing a balanced international IP legal framework to meet society's evolving needs. It provides business services for obtaining IP rights in multiple countries and resolving disputes. It delivers capacity-building programs to help developing countries benefit from using IP and it provides free access to unique knowledge banks of IP information.

2.2 ORIGINS AND HISTORY OF INTELLECTUAL PROPERTY

The history of IP is an old and surprising one. It begins 500 years BC when the Greek state Sybaris made it possible for citizens to obtain a 1-year patent for "any new refinement in luxury." Patent, trademark, and copyright laws have become more complicated in the ensuing centuries, but the intent remains the same. Countries establish IP laws to foster creativity and to make it possible for their inventors to reap the benefits of their or transmitted ingenuity. Mentions of copyrights, patents, and other matters of IP law are sparse in early history. It is not until medieval Europe that some major and well-known legislation was passed; the first was the Statute of Monopolies, British law, established in 1623 [4,5]. At the time, all major industries were controlled by guilds. Each guild held considerable power, with the government endowing them with the ability to dictate what products and raw materials could be imported as well as how those items would be produced and sold. Moreover, the guilds were responsible for bringing all new innovations to the marketplace, essentially giving them ownership and control over inventions even if they had nothing to do with their creation [6]. IP law dates at least as far back as medieval Europe. In those times, "guilds," or associations of artisans in a particular industry, were granted authority by the governments to control the regulation and conduct of the various industries. These guilds exercised control over what items could be imported, marketed, and produced, and the manner in which new inventions, devices, and procedures could be introduced to the stream of commerce. Because the authorities for these guilds were given by the governments, and because they concentrated the power to regulate an industry in a select few, and were not earned by innovation, skill, or creativity, these guilds did far more to stifle creativity and invention than to encourage it. IP law at that time was driven not by an interest in creation and innovation, but rather by political and religious motivations. For example, the 1556 establishment of the Stationers' Company's monopoly in England was largely intended to help limit the Protestant Reformation movement's power. By putting the entire printing industry in the control of this company, the government and church could prevent the dissemination of ideas [7,8]. Although the Stationer's Company monopoly expired in 1694, it was not until the early 18th century that the guild system was definitively broken in England. From that point forward, more specific statutes and British common law continued to develop and

refine IP law. Most notably, the Statute of Anne was enacted in 1709–10 [9], granting an initial 14-year protection period and a possible 14-year renewal.

IP designation seems to have originated as an alternative to literary property as a name for copyright—particularly in Romance language jurisdictions. In France, "droit d'auteur" was much more frequently related to literary property, but the name IP was also widely used. In Italy and Spanish-speaking jurisdictions, IP seems to have had significantly more traction as an official name for copyright and related rights. The German equivalent was used with the founding of the North German Confederation whose constitution granted legislative power over the protection of intellectual property ("Schutz des geistigen Eigentums") to the confederation. Through this lens, something becomes apparent that a number of us overlooked: the occurrences of IP in 19th century and the early 20th century English language discussions may sometimes have meant the umbrella concept and others meant just copyright. At the same time, there were 19th century commentators in English and French who clearly used IP with its current, umbrella concept meaning: those uses provided the backdrop—and justification—for the clever, quiet, mid-20th century rebranding of WIPO's predecessor, The United International Bureaux for the Protection of Intellectual Property (BIRPI), in which the final "I" drifted from referring to industrial property toward standing for IP. This is now dominant and ubiquitous as the umbrella name and concept for patents, trademarks, copyrights, neighboring rights, and a variety of other legal tools that protect intangible values. There was a proposal in England in 1870 to subsume copyright, patents, and trademarks under a unified law of "Mental Property," enforced by its own "Court of Mental Property," and that was surely better than the proposal that came a decade later that all these subjects should be called "Brain Property." It seems interesting to pursue such debates—particularly in a discourse that feels obliged to footnote all but the most obvious propositions—it is also important to consider all that came before. Details of IP history and growth are available in Ref. [10]. Until recently, the purpose of IP law was to give as little protection as possible in order to encourage innovation. Historically, therefore, they were granted only when they were necessary to encourage invention, limited in time and scope.

2.3 CURRENT PERSPECTIVES AND PROSPECTIVES OF INTELLECTUAL PROTECTION AND RIGHTS

In last few decades, with the advancement of globalization, trans-border transactions have increased. Many companies are now multinational firms. The rights associated with them intellectually are country specific. IPRs are specific to the owners and provide the rights to owners such that no one other than a person having consent from the owner can use it, or defines conditions under which it can be used [11]. IPRs are characterized by risk in innovation, global competition, high investment of

both capital and time, as well as the most important human resources needed for the introduction into the market. Due to the increase in global trade, a reduction in the barriers to the marketing of products has occurred. In the trade in goods and services, the IPRs have become the most common targets of infringement. However, the developers are taking care to ensure that at least the cost of product development can be recovered from the market.

As already mentioned, IP refers to any original creation of the human intellect such as artistic, literary, technical, or scientific creation. IPRs—from the French "droits intellectuels"—refers to the legal rights given to the inventor or creator to protect their inventions or creations for a certain period of time. To enjoy the IPRs there are certain protection tools designed such as patent, copyright, trademark, industrial rights design, etc. Such protection is known as Intellectual Property Protection. The IPRs are the legal rights granted by the government or governing council which allows the owner of the IP to completely benefit from the work commercially for a certain period of time. But the owner has to disclose all the information related to the work in lieu [12].

IPRs are generally territorial with the exception of copyright which is applicable to all members of the Berne convention. The copyright provides rights to the owners such that no else can use the IP commercially without asking for or having the consent of the owner. Most geographical indications are not liable to get renew. These rights are just sold, traded, and bought in the same was as the general property business. Regarding patents, a patent can be owned in other countries as well as a patent owned in the home country through the Patent Cooperation Treaty. However, for owing IPRs, the work must be original and novel also. Contributory work which is obvious or already known to the public cannot be given IPRs. However, modifications of the existing products could be granted a patent [13].

2.4 INTELLECTUAL PROPERTY LAW IN THE UNITED STATES

When the American colonies secured independence from England, each colony (except Delaware) passed its own copyright law. This, of course, led to a severe degradation in the value of patents and copyrights. In order to rectify intercolonies unenforceable problems, to reward innovation, and to greatly facilitate commerce among the states, the drafters of the Constitution felt that IP law should be within the province of the federal (national) government, not the state governments. This constitutional power has had, for the most part, the effect of ensuring that federal law is the overwhelming authority for all law regarding IPRs. The Supreme Court has even gone so far as to rule that Congress, with its complex and detailed system of IP laws, has developed a scheme which was pervasive. In addition to federal law being the primary source of IP law in the United States, federal law gives courts "exclusive" jurisdiction in most IP cases.

In these types of cases, which are often known as "idea submission" cases, courts will generally use contract law to protect the IP interest, assuming three conditions are met: the idea at issue must be "novel and concrete," the parties must have had an express or implied agreement under which the idea was turned over, and the recipient must have actually used the idea. In addition to these three, all of the general requirements for contracts (mutual assent, consideration, legal subject matter, competent parties, etc.) must also be present for a court to enforce IPRs under a contract law theory.

Shortly after the United States broke away from Great Britain, most of the 13 colonies had established their own systems for IP protection. The one exception to this was Delaware. However, it was soon apparent that having each state operate its own system of IP protection was problematic, leading to the establishment of federal laws that had precedence over any state laws.

2.5 OWNERSHIP RIGHTS

The Statute of Monopolies changed by allowing the author or inventor to retain their ownership rights. Monopolies, in the form of government-sanctioned guilds, were no longer granted. The law also guaranteed the inventor a 14-year period during which he had the exclusive right to govern how his invention was used.

Other significant legislation came in 1710 with the Statute of Anne. This law similarly provided a 14-year term of protection. It also gave the inventor the option of seeking a 14-year renewal term. Aimed largely at copyrights, this law granted authors rights in the recreation and distribution of their work.

2.6 GLOBAL INTELLECTUAL PROPERTY

In 1883, the Paris Convention came into being. It was an international agreement through which inventors could protect their innovations even if they were being used in other countries. Writers came together in 1886 for the Berne Convention, which led to protection on an international level for all forms of written expression as well as songs, drawings, operas, sculptures, paintings, and more. Trademarks began to gain wider protection in 1891 with the Madrid Agreement while the offices created by the Paris and Berne Conventions eventually combined to become the United International Bureau for the Protection of Intellectual Property, the precursor of today's WIPO, which is an office of the United Nations [6–8].

Through centuries of development and innovation, inventors and creators now have dozens of options when it comes to IP.

2.7 PATENTS

A patent is a form of right granted by the government to an inventor, giving the owner the right to exclude others from making, using, selling, offering to sell, and importing an invention for a limited period of time in exchange for the public disclosure of the invention. The protection is granted for a limited period, generally 20 years from the filing date of the application. More directly, it is a shortened version of the term letters patent, which was a royal decree granting exclusive rights to a person, predating the modern patent system. Similar grants included land patents, which were land grants by early state governments in the United States, and printing patents, a precursor of modern copyright. An invention is a solution to a specific technological problem, which may be a product or a process and generally has to fulfill three main requirements: it has to be new, not obvious and there needs to be an industrial applicability [14].

In principle, the patent owner has the exclusive right to prevent or stop others from commercially exploiting the patented invention. In other words, patent protection means that the invention cannot be commercially made, used, distributed, imported or sold by others without the patent owner's consent.

Patents are territorial rights. In general, the exclusive rights are only applicable in the country or region in which a patent has been filed and granted, in accordance with the law of that country or region. In modern usage, the term "patent" usually refers to the right granted to anyone who invents any new, useful, and nonobvious process, machine, article of manufacture, or composition of matter. Some other types of IPRs are also called "patents" in some jurisdictions: industrial design rights are called "design patents" in the United States, "plant breeders' rights" are sometimes called "plant patents," and "utility models" and "Gebrauchsmuster" are sometimes called "petty patents" or "innovation patents." The additional qualification "utility patent" is sometimes used (primarily in the United States) to distinguish the primary meaning from these other types of patents. Particular species of patents for inventions include biological patents, business method patents, chemical patents, and software patents.

2.7.1 PROTECTION BY A PATENT

In principle, the patent owner has the exclusive right to prevent or stop others from commercially exploiting the patented invention. In general, the exclusive rights are only applicable in the country or region in which a patent has been filed and granted, in accordance with the law of that country or region. As mentioned the protection is granted for a limited period, generally 20 years from the filing date of the application. A patent is a set of exclusive rights granted by a sovereign state to an inventor or assignees for a limited period of time in exchange for detailed public disclosure of an invention. An invention is a solution to a specific technological problem and is a product or a process.

The procedure for granting patents, requirements placed on the patent, and the extent of the exclusive rights vary widely between countries according to national laws and international agreements. Typically, however, a granted patent application must include one or more claims that define the invention. A patent may include many claims, each of which defines a specific IPR. These claims must meet relevant patent ability requirements, such as novelty, usefulness, and nonobviousness [15]. Under the World Trade Organization (WTO) TRIPS Agreement, patents should be available in WTO member states for any invention, in all fields of technology, provided they are new, involve an inventive step, and are capable of industrial application. Nevertheless, there are variations on what is patentable subject matter from country to country, even among WTO member states. TRIPS also provides that the term of protection available should be a minimum of 20 years [16,17].

2.8 SPECIFIC ASPECTS OF INTELLECTUAL PROPERTY ON ENZYMES

The world's principal enzymes producers (AB Vista, BASF, Novozymes, DSM, VTR Bio-Tech, DuPont, Youtell Biochemical, Smistyle, SunHY, and Adisseo) have as a global objective the selection and development of microbial catalysts efficacious for a number of different applications such as detergents, starch, agrofuels, ethanol, textiles, human food, and animal feed. In recent years, the global industrial enzyme market size increased year by year, registering an annual growth of 5%, approximating US $4.2178 billion in 2014. At present, the global industrial enzyme market is basically an oligopoly. In 2014, Novozymes, accounted for a 44% market share while that for Dupont and DSM was 20% and 6%, respectively. Regionally, Europe and North America have the largest demand for industrial enzymes, occupying nearly 80% in sharp contrast to some 9.4% in China. Stimulated by the growing demand for enzyme preparations and favorable policies, China's industrial enzyme preparation output reached 1.1657 million tons in 2014. For years to come, industrial enzyme output will keep 10% growth rate, from 1.5487 million tons today. Emerging application fields for enzymes are chemical catalysis and bioremediation. Enzymes in biotechnology can make a significant contribution to global environment improvement, but this effort comes with heavy IP protection. Enzymes can strongly reduce or even remove the need for inorganic P in animal feed, and replace artificial coloring, flavors, and preservatives. They are found, e.g., in cheese, beer, and wine, which could not be manufactured without them. Enzyme technology is considered as a "green technology" and contributes to saving resources, to increasing the utilization of alternative natural resources, and to reducing water waste and pollution, It can be foreseen that a better valorization of plant biomass by adequate new enzymes will increase in the years to come. Screening for new enzymes and their practical development is

quite expensive so that the IP generated must be protected against copying by competitors. This is usually done by patenting the enzyme or its production method or, most usefully, the process in which it is to be used. Patenting will be initiated as soon as there is evidence that an innovative discovery has been made.

It is an evidence that intellectual "capital" has an enormous role in the creation and marketing of industrial products. Nevertheless, a large number of small and medium sized enterprises do not devote enough attention to the protection of their IP. In today's commercial environment, the concurrent advantage for any company is based on innovation and on the expression of the original developments, correctly protected by the system of IP.

The biggest enzyme developers and producers have globally a general aggressive strategy of patenting. Novozymes is often cited as an example on how to deal efficaciously with IP protection in the global domain of enzymes. The company had, in early 2017, more than 7000 patents or patent applications in the field and has a dynamic strategy in the concession of licenses allowing profits from the royalties. Others, such as AB Vista, e.g., aimed exclusively at enzymes for animal nutrition, has over 550 patents and its owned IP is growing.

Commercial secrets can cover several fields of IP, including informatics programs. However, the strength of such protection is relatively weak. Correct management of the IP assets in industry and marketing contribute to an increase in the enterprises value as seen by investors and financial institutions.

Despite the inherent advantages of extremozymes, the actual number of available extremophilic biocatalytic tools is very limited. The large technical gap between producing an enzyme under laboratory conditions and obtaining a final commercial product is still a problem for the development of novel biocatalysts [18]. Recent molecular biology techniques have allowed scientists to tailor a specific microorganism more and more, to produce not only the enzyme with desired special characteristics such as thermostability, stability in acidic or alkaline environment, and retaining the enzyme activity under severe reaction conditions such as in the presence of other metals and compounds, but also with high yields of enzymes [19]. Extremophiles and their enzymes have an extensive foothold in the market that is expected to keep growing. To fulfill the great potential, innovative methods will have to be developed to overcome current roadblocks. New expression systems will have to be developed with extremophilic organisms as the host to achieve high expression of soluble proteins [20]. The potential for IP of extremozymes is thus very important.

Ideally enzymes should not need excessive formulation which is a supplementary step in the production and has relatively high cost. However, near all enzymatic products available nowadays are handicapped by weakness in one or more points such as dust production, low mixability capacity, weak resistance to the harsh conditions of composed feed preparation—pressure, moisture, and temperature, poor resistance to acidic denaturation and to proteolytic hydrolysis in the stomach [21]. Formulation is often considered as an art and generally the precise details of the methods used to stabilize enzyme preparations are kept secret or protected by strong patents.

Formulation cost is a fundamental determinant factor in the use of enzymes in any technological process. Hundreds of enzymes are available at very different prices. As enzymes are catalytic molecules, they are not directly used up by the processes in which they are used. Their high initial cost, therefore, should only be incidental to their use. However, due to denaturation, they do lose activity with storage time. They should be stabilized against denaturation to be utilized in an efficient manner [22].

For the moment, crops used in swine and poultry feeding are in direct competition with humans. Regarding aquafeed production, the limit for fish capture needed for fish meal production is recognized to have been exceeded, with dramatic effects on the environmental equilibrium of the oceans. Also, 70% of the total extracted quantities by the industrial fisheries is supplied for meal production and such fish is edible for humans.

All the alternative feedstuffs require exogenous efficacious enzymes. The usefulness of such enzymes will increase and facilitate by appropriate enzymes or enzyme cocktails with some reinforced properties: strong natural activity, improved heat resistance, low production cost, long storage life, resistance to denaturation and proteolysis, broad pH range activity, etc. The potential for producing biochemicals, agrofuel, and nutritional compounds will require strong research activity into their formulation. The screening and development of extremozymes appear as a research field allowing, when successful, on the one hand the valorization of plant biomass and on the other hand a huge reduction in formulation costs [23]. The hydrolysis of lignocellulose will have a very important economic role in the future and the polymer will be one of the more useful raw materials. That is already the case in the conversion of some lignocellulosic mass into agroethanol [24,25]. Reduced fossil energy supplies, pollution concerns, and economic reasons have stimulated the interest in lignocellulose as a source of fuel, nutrients, and biotechnological tools in the paper and food industry. Many extremophile catalysts can be identified in genome sequences and their characterization will allow new opportunities of practical utilization [26,27].

Two groups of the principal lignolytic enzymes—cellulases and laccases—are foreseen to have a fundamental role to play in the valorization of plant biomass [28]. Today, cellulosic ethanol is twice as expensive than gasoline, and the proportion is similar for the production of bioactives. It is expected that the production cost of cellulases will be reduced from 5 to 1 US cents per liter from now to 2020. The world leaders in the domain (Novozymes, Genencor, and Iogen) would like to reduce the price of cellulases within a few years. All together they have a large number of patented cellulases. The National Renewable Energy Laboratory (NREL) has also a set of strong patents on these enzymes [29]. The Earth's available stocks of lignocellulosic biomass are tremendous and distributed all around the world. Thus, the sustainability of the valorization of cellulose by cellulases seems clear. More economical feeding can most probably be obtained by improving pretreatment and enzymatic hydrolysis of cellulose. Furthermore, an application into human nutrition may also be possible. Genetic engineering will be

intensively used to manipulate the biochemical and regulatory pathways that operate during cellulases production and control enzyme yield [30]. Up until now, more than 150 laccases have been isolated and characterized; the majority of them were isolated from wood-destroying white rot fungi, whereas plant laccases are much less studied. The ability of laccases to attack and degrade lignin in conjunction with laccase mediators is currently viewed as one of the fundamental potential applications for greater lignin utilization [31]. Several fungi produced lignin degrading enzymes such as ascomycetes and basidiomycetes. Some anaerobic microbial species can hydrolyze lignin in the gastrointestinal tract of ruminants, using a complex mixture of enzymes. Currently the catalytic properties of laccases are being exploited for a range of technological applications such as the bioremediation of soils and water, and for the development of environmentally friendly processes in biorefinery [32]. Furthermore, the laccases' hydrolytic activity on plant biomass aimed at animal as well as at human nutrition has a tremendous potential. In the food industry, laccases are used for the elimination of undesirable phenolic compounds in baking, juice and beer processing, wine stabilization, and bioremediation of waste water. Laccases have demonstrated potential and sometimes are already being used for the degradation of antimicrobials (antibiotics and sulfamides) and other drugs (biogenic amines, toxic compounds present in fermented food and beverages) [33]. Details on decontamination by laccases can be obtained in Ref. [23]. There are numerous patents on laccases and an extensive review on these enzymes has been published in Ref. [34].

In ruminant nutrition, white rot fungi selective lignin degraders can be potential alternatives to current methods that utilize potentially toxic chemicals and expensive equipment. In this respect, future research should focus on optimization of culture conditions and gene expression allowing the development of superior fungal strains to degrade lignin in biomass [35].

2.9 ECONOMIC AND ETHICAL ISSUES OF INTELLECTUAL PROPERTY, DEBATES, AND TRENDS

IP protection is widely thought to be even more important to the rapidly growing biotechnology industry, where the development of new techniques of genetic engineering of new life-forms employing such techniques can be extremely expensive. Biotechnology firms argue that, if they were unable to prevent rivals from imitating their creations, they would not be able to recoup their costs and thus would have no incentive to invest in the research and development necessary for scientific breakthroughs. Companies selling goods and services over the Internet have made similar claims concerning the importance of their domain names [36].

The growth and increasing importance of IPRs have stimulated a vigorous debate concerning the justification for and the appropriate contours of this body

of law. The debate has largely centered around four theories. The first and most prominent of these is an outgrowth of utilitarianism. Specifically, utilitarians urge lawmakers to craft IP regulations carefully in order to strike an optimal balance between the socially desirable tendency of such laws to stimulate the creation of inventions and works of art and their partially offsetting tendency to curtail the widespread public enjoyment of these products. A second theory was inspired by the writings of the 17th-century English philosopher, John Locke, and specifically by his account of the origin of IPRs. Proponents of this theory argue that a person who labors on unowned resources has a natural right to the fruits of his efforts and that the state has a duty to respect and enforce that natural right. A third theory grew more loosely out of the writings of the 18th- and 19th-century German philosophers Immanuel Kant and Georg Wilhelm Friedrich Hegel and out of a sentiment, common in western Europe, that artists and authors should enjoy certain "moral rights." This approach is premised on the notion that private-property rights are crucial to the satisfaction of fundamental human needs, among which is the need for creative expression. IPRs are thus justified either because they protect artifacts through which authors, artists, and inventors have expressed their "wills" or because they create social and economic conditions conducive to creativity. A fourth, less-well-defined theory contends that IPRs can and should be shaped so as to help foster the achievement of a just and aesthetically sophisticated culture. Advocates of this approach emphasize the capacity of copyright, patent, and trademark systems—if properly crafted and limited—to promote a vibrant democracy and a participatory and pluralist civil society.

Together, the proponents and critics of the four perspectives have generated a cacophonous debate in journals of law, economics, and philosophy. On occasion, lawmakers have been moved by this debate. In the 1990s, scholars of all four stripes denounced the growth in the United States of the right of publicity. Another example of scholarly influence involves the proliferation of patents on methods of doing business. Patents of this sort were rarely granted in any jurisdiction before 1998, when an influential USA court decision led to a surge in applications for and grants of business-method patents. Scholars have been almost unanimous in denouncing this development, and in part this opposition led the Patent and Trademark Office to revise its procedures to limit the availability of such patents. Despite the strengthening of IP, the growing economic and cultural importance of IPRs, and a widespread view that such rights are socially desirable, the future of IP remains in some doubt. IPRs are threatened principally by the proliferation of technologies that facilitate the violation of copyright and patent rules. At the beginning of the 21st century, the sector most affected by these technologies was the music industry as the combination of compression technologies and copying systems led to widespread unauthorized copying and distribution of digital music. In other fields the situation is much less critical [37,38].

2.10 COPYRIGHT

A copyright gives the creator of an original work exclusive rights to it, usually for a limited time. Copyright may apply to a wide range of creative, intellectual, or artistic forms, or "works." Copyright does not cover ideas and information themselves, only the form or manner in which they are expressed. The 1886 Berne Convention first established recognition of copyrights among sovereign nations, rather than merely bilaterally. Under the Berne Convention, copyrights for creative works do not have to be asserted or declared, as they are automatically in force at creation: an author need not "register" or "apply for" a copyright in countries adhering to the Berne Convention, which also results in foreign authors being treated equivalently to domestic authors, in any country signed onto the Convention [39]. As soon as a work is "fixed," i.e., written or recorded on some physical medium, its author is automatically entitled to all copyrights in the work, and to any derivative works unless and until the author explicitly disclaims them, or until the copyright expires. In the United States the Copyright Law includes all amendments enacted by Congress through June 30, 2016. It includes the Copyright Act of 1976 and all subsequent amendments to copyright law; the Semiconductor Chip Protection Act of 1984, as amended; and the Vessel Hull Design Protection Act, as amended. The Copyright Office is responsible for registering IP claims under all three [40]. Copyrights are generally enforced by the holder in a civil law court, but there are also criminal infringement statutes in some jurisdictions. Copyright subsists for a variety of lengths in different jurisdictions. The length of the term can depend on several factors, including the type of work (e.g., musical composition, novel), whether the work has been published, and whether the work was created by an individual or a corporation. In most of the world, the default length of copyright is the life of the author plus either 50 or 70 years. In the United States, the term for most existing works is a fixed number of years after the date of creation or publication [41]. In 1998, the length of a copyright in the United States was increased by 20 years under the Copyright Term Extension Act. This legislation was strongly promoted by corporations which had valuable copyrights which otherwise would have expired, and has been the subject of substantial criticism on this point [42].

Traditionally, the author of an article was required to transfer the copyright to the journal publisher. The biggest scientific publishers had edited their own rules to deal with copyright laws and issues (e.g., as for Elsevier [43]). Most of the publishers claimed this was necessary in order to protect author's rights, and to coordinate permissions for reprints or other use. However, many authors, especially those active in the open access movement, found this unsatisfactory, and have used their influence to effect a gradual move toward a license to publish instead. Under such a system, the publisher has permission to edit, print, and distribute the article commercially, but the author(s) retain the other rights themselves. Even if they retain the copyright to an article, most journals allow

certain rights to their authors. These rights usually include the ability to reuse parts of the paper in the author's future work, and allow the author to distribute a limited number of copies. In the print format, such copies are called reprints; in the electronic format, they are called post-prints. Some publishers, e.g., the American Physical Society, also grant the author the right to post and update the article on the author's or employer's website and on free e-print servers, to grant permission to others to use or reuse figures, and even to reprint the article as long as no fee is charged. The rise of open access journals, in which the author retains the copyright but sometimes needs to pay a publication charge, such as the Public Library of Science family of journals, is another recent response to copyright concerns. According to Gadd et al. [44,45], academic authors are generally and primarily interested in preserving their moral rights, and that the protection offered to research papers by copyright law is way in excess of that required by most academics. The protection offered to research papers by copyright law exceeds the protection required by most academic authors. It also raises concerns about the level of protection enforced by e-journal license agreements. In UK law, there are two main moral rights: (1) the right to be identified as author; and (2) the right to object to derogatory treatment of a work. The interest in limiting modification and aggregation may also have its roots in such moral rights considerations. It seems that the protection offered by the rights metadata promises to provide adequate protection for academic authors, whilst granting end-users the "open access" to the research literature that they desire.

2.11 OTHER SUBJECTS ON INTELLECTUAL PROPERTY

Several other topics on IP should be briefly mentioned. Details are available in many publications (e.g., Refs. [46,47]).

2.11.1 INDUSTRIAL DESIGN RIGHTS

An industrial design consists of the creation of a shape, configuration, or composition of pattern or color, or combination of pattern and color in three-dimensional form, containing aesthetic value. An industrial design can be a two- or three-dimensional pattern used to produce a product, industrial commodity, or handicraft.

2.11.2 PLANT VARIETIES

A new variety of a plant must, amongst other factors, be novel and distinct, and for registration the evaluation of propagating material of the variety is examined.

2.11.3 TRADEMARKS

Trademarks are recognized signs, designs, or expressions distinguishing products or services of a particular trader from the similar products or services of others.

2.11.4 TRADE SECRETS

There is no formal government protection granted for trade secrets. Each business takes measures to guard its own trade secrets. By exchanging limited exclusive rights for disclosure of inventions and creative works, society and the patent/copyright owner mutually benefit, and an incentive is created for inventors and authors to create and disclose their work. Some commentators have noted that the objective of IP legislators and those who support its implementation appears to be "absolute protection." In recent years there has been much debate, which remains open, on the desirability of using IPRs to protect cultural heritage, including those that are intangible, as well as on risks in turn to commodities derived from this possibility.

2.12 MORAL ISSUES

The relationship between IP and human rights is a complex one. Nevertheless, there are moral arguments for IP. Arguments that justify IP fall into three major categories. Personality theorists believe IP is an extension of an individual, utilitarians believe that IP stimulates social progress and pushes people to further innovation, and lockeans argue that IP is justified based on deservedness and hard work. Violation of IPR, called "infringement" when related to patents, copyright, and trademarks, and "misappropriation" with respect to trade secrets, may be a transgression of civil law or criminal law, depending on the type of IP involved, jurisdiction, and the nature of the action.

2.13 INFRINGEMENTS—PATENTS, COPYRIGHT, TRADEMARK, ETC.

Patent infringement is provoked by the use or sale of a patented invention without permission from the patent holder. The scope of the patented invention or the extent of protection is defined in the claims of the granted patent. There is a possibility in many jurisdictions to use a patented invention for research. Such possibility does not exist in the United States unless the research is done for purely philosophical purposes, or in order to gather data in order to prepare an application for regulatory approval of a drug. Depending on the jurisdictions, patent infringement cases are handled under civil law, but several jurisdictions

incorporate infringement in criminal law also. Enforcement of copyright is generally the responsibility of the copyright holder.

The Anti-Counterfeiting Trade Agreement (ACTA) signed in 2011 and 2012 by many countries including the United States, Japan, Switzerland, EU, etc., and which has not entered into force, requires that its parties add criminal penalties, including incarceration and fines, for copyright and trademark infringement, and obligated the parties to actively police for infringement. In the EU, the agreement signature resulted in intense public and Parliamentary debates.

2.14 CONCLUSIONS

IP is related to creations for which a monopoly is assigned to designated owners by law. IPRs are granted to the creators of IP, and include trademarks, patents, industrial design rights, fabrication secrets, and in some countries trade secrets.

The development of industrial microbial enzymes imply selection, genetic engineering, expression, upscale production, formulation, registration, and promotion. Fabrication secrets are most probably the simplest way to get protection, but in case of frauds by competitors there are almost no means to return the knowledge back to the owner. IP contributes enormously to national and state economies. IPRs are often confusing and sometimes the topics of heated debates. IPRs are characterized by risk in innovation, competition globally, high investment of both finance and time, and most importantly human resources needed for the introduction into the market. Due to global trade increases, the IPRs have become the most common targets of infringement. Patents are territorial rights tools for protection of IP, giving to the owner the right to exclude others from making, using, selling, offering to sell, and importing an invention for a limited period of time (generally 20 years), in exchange for the public disclosure of the invention. A patent may include many claims, each of which defines a specific IPR.

The world principal enzyme producers have as a global objective the selection and development of catalysts efficacious for a number of different applications such as detergents, starch, agrofuels, ethanol, textiles, food, and animal feed. Enzymes in biotechnology can make a significant contribution to global environment improvement, but this effort comes with heavy IP protection. The biggest enzyme developers and producers have a generally aggressive strategy of patenting. Despite the inherent advantages of extremozymes, the actual number of available extremophilic biocatalytic tools is very limited. The large technical gap between producing an enzyme under laboratory conditions and obtaining a final commercial product is still a problem for the development of novel biocatalysts. Ideally enzymes should not need important formulation which is a supplementary step in the production and has relative high cost. However, near all enzymatic products available nowadays are handicapped by weakness in one or more points such as dust production, low mixability capacity, week resistance to the harsh

conditions of composed feed preparation—pressure, moisture, and temperature, poor resistance to acidic denaturation and proteolytic hydrolysis in the stomach. Formulation is often considered as an art and generally the precise details of the methods used to stabilize enzyme preparations are kept either secret or preferably protected by strong patents.

The scope of the patented invention or the extent of protection is defined in the claims of the granted patent. Patent infringement is provoked by the use or sale of a patented invention without permission from the patent holder. There is a possibility in many jurisdictions to use a patented invention for research. Such possibility does not exist in the United States unless the research is done for purely philosophical purposes, or in order to gather data in order to prepare an application for regulatory approval of a drug. Depending on the jurisdictions, patent infringement cases are handled under civil law, but several jurisdictions incorporate infringement in criminal law also.

The ACTA signed in 2011 and 2012 by many countries including United States, Japan, Switzerland, EU, etc., and which has not entered into force, requires that its parties add criminal penalties, including incarceration and fines, for copyright and trademark infringement, and obligated the parties to actively police for infringement. In the EU, the agreement signature resulted in intense public and Parliamentary debates.

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PART

I

Phytases

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CHAPTER

General aspects of phytases

3

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3.1 INTRODUCTION

Phytate is one of the prime antinutrient factors in the plant-based diet because monogastric animals and human digestive systems are incapable of metabolizing phytate. Phytate serves as the primary storage form of phosphate in plant seed and is stored in protein storage vacuoles in the aleurone cell layer or the embryo of the seed. It accumulates during seed development until the seeds reach maturity and accounts for about 60%−70% of total phosphorus (P) content in cereals, legumes, nuts, and oil seeds. However, this stored P remained unutilized either by humans, poultry, swine, or agastric animals because they lack the intestinal phytate degrading enzyme called phytase [1]. Dietary presence of phytate can also form insoluble complexes mainly with cations such as calcium (Ca), magnesium (Mg), zinc (Zn), copper (Cu), and iron (Fe), thereby limiting the bioavailability of these minerals. Owing to the strong chelation of mineral ions, phytate can intensely restrict the absorption and digestion of dietary minerals by humans as well as animals. Consequently, low bioavailability of the minerals can lead to deficiencies in human populations, particularly where staples like rice, wheat, and maize are the major source of nutrition. Besides, there are also reports documenting that phytate can form complexes with proteins, ultimately resulting in decreased protein solubility, enzymatic activity, and proteolytic digestibility. Traces of phytate in basal diet can also interact with various digestive enzymes such as amylase, pepsin, trypsin, and acid phosphorylase to reduce their, activity which eventually can diminish nutrient utilization [2,3]. Ca salt of phytate can blend with lipid to form metallic soaps in gut lumen, depriving the energy utilization from lipid sources [4]. When the diet of young chicks was supplemented with fat and phytate, the majority of the fat was excreted as soap fatty acids [5]. Reports have also confirmed that phytate can negatively affect vitamin utilization, as a result animal feeds with high phytate contents often lead to off-feeding, emaciation, retarded growth, and reproduction failure [6,7]. It is also well anticipated

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that in the case of livestock production systems, the excretion of un-utilized phytate can result in the accumulation of P in soil and water. A high P burden will lead to the eutrophication of streams, lakes, or ponds.

Removal of phytate (i.e., dephytinization) increases the bioavailability of P, cations, and also enhances the nutritional value of the meal. Various strategies for dephytinization have been proposed including milling of cereals, cooking, fermentation, moistening, autolysis, etc. These processing methods could reduce phytate to a certain extent, but also account for the loss of minerals and other nutrients. Taking into account the constraints of these methods and to maximize the nutritive value of a plant-based diet, it is requisite to catalyze the hydrolysis of undigestable phytate. In this venture, the use of exogenous phytate-degrading enzyme, phytase, appears to be an ultimate solution for degrading phytate. Furthermore, it is also well documented that during the course of food processing or preparation, phytate is not fully hydrolyzed by endogenous phytase enzyme. In order to increase the mineral bioavailability to a greater extent, phytate must be reduced to very low levels. For these issues, addition of exogenous phytase is preferred. Phytases are phosphohydrolytic enzymes that initiate the stepwise removal of phosphates. Phytase supplementation in animal feedstuffs has become an efficient strategy to improve bioavailability of P. This also minimizes the amount of excreted phytate-bound P by animals into the environment. The application of phytase in animal feed has also been documented to improve protein utilization of plant-sourced feed ingredients and also tends to reduce dependence on fishmeal diet [2,8–12]. Therefore, application of exogenous phytase has a vast potential in animal farming and aquaculture. To date, commercial phytase products have been mainly explored as animal feed additives typically for swine and poultry diets. In the last few years, more emphasis has been given to the supplementation of phytase for aquaculture species with the aim to maximize nutrient utilization and growth, as well as in human nutrition. Many researchers have reported a convincing improvement of food products by adding microbial-based phytase during food processing for bread making [13], plant protein isolates [14], corn wet milling [15], and the fractionation of cereal bran [16]. Unfortunately, in spite of the immense potential of phytase in processing and manufacturing of food for human consumption, no phytase product for human food application is commercially available.

3.2 PHYTASES

3.2.1 BACKGROUND

Phytases are phosphohydrolytic enzymes which are chemically known as myo-inositol(1,2,3,4,5,6)-hexakisphosphate phosphohydrolase. Phytases initiate the stepwise removal of phosphate groups from myo-inositol hexakis phosphate. The enzyme sequesters orthophosphate groups from the inositol ring of phytic acid to

produce free inorganic P along with a chain of lower phosphoric esters (inositol pentaphosphate to inositol monophosphate) as intermediates (Fig. 3.1). Thus, by releasing bound P in food/feed ingredients of plant origin, phytase makes more P available to fuel the demand for biological process such as bone formations and membrane stability. As mentioned earlier, phytase not only frees the P from plant-based diets but also increases the bioavailability of calcium and magnesium, as well as protein and lipid.

Lei and Porres [17] described four classes of phosphatase activity which can degrade phytic acid and display different catalytic efficiencies, structure, mechanism of action, and biochemical properties. Among these, histidine acid phosphatases are the most widely used phytases in animal feeds. The other three phytase groups include β-propeller phytases, cysteine phosphatases, and purple acid phosphatases. Most of these phytate-degrading enzyme phosphatases are widely present in the germinating grain and soybeans seedlings [18].

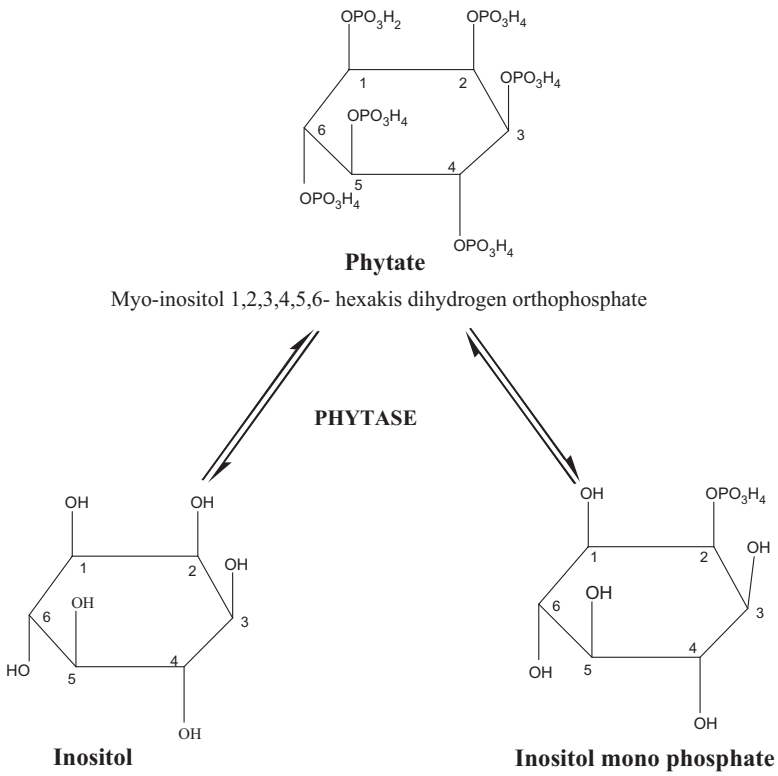

FIGURE 3.1

Schema of phytase action.

3.2.2 UNIT OF PHYTASE ACTIVITY

Activity of phytase is expressed as FYT, FTU, PU, and U. All these units have the same denotation; one unit of phytase is defined as the quantity of enzyme that liberates 1 μmol of inorganic-P per minute from 0.0015 mol/L sodium phytate at pH 5.5, and 37°C [19]. This definition offers a useful measure of phytase activity and represents a simple benchmark measurement under well-defined assay conditions such as pH, temperature, duration, mineral content, agitation, etc. As per estimation, inorganic P/phytase equivalence in animal diets suggests that 300−600 FTU/kg of diets can release 0.8 g of digestible phosphorus and replace either 1.0 or 1.3 g of P from mono- and dicalcium phosphate, respectively.

3.2.3 HISTORY OF PHYTASES

Phytase activity was first documented in rice bran [20] followed by the blood of calves [21]. Afterward its presence was also documented in diverse groups including bacteria, yeast, fungi, and plants. Human and most monogastric animals lack phytase enzyme in their digestive track, therefore, for phytate hydrolysis they depend on the small intestinal mucosal or bacterial enzymes associated with large intestine or on nonenzymatic hydrolysis by gastrointestinal acidity. In general, endogenous phytase activity of humans and animals is insignificant in contrast to plant and microbial phytase.

The phytase, commercially named Natuphos was the first introduced into the market in 1991 [22] and was obtained from *Aspergillus niger*. Subsequently, it began gaining popularity worldwide as an animal feed supplement. Moreover, its prospective for human nutrition [23] and in animal nutrition [24] is also being broadly investigated.

In 1999 it was demonstrated that some phytases of bacterial origin such as that of *Escherichia coli* were more active than those produced by fungi [25−27]. The first phytase obtained from a full synthetic gene expressed in *Hansenula polymorpha*, named "Consensus" was developed in 1997 [28]. The obtained enzyme is resistant to temperatures up to 90°C and has an activity equivalent to that of *A. niger* phytase [29−31]. A large number of phytases have been described over several years and their properties have been described by various authors [32−36].

3.3 CLASSIFICATION OF PHYTASES

Phytases have been classified based on (1) the pH of activity and (2) the site initiating the dephosphorylation of phytate molecule at different positions on the inositol ring, generating different isomers of the lower inositol phosphates.

3.3.1 PH OF ACTIVITY

Phytases can be grouped as acidic, neutral, or alkaline phosphatases depending on the optimum pH of activity. The optimal activities for these groups are documented at pH 5.0, 7.0, and 8.0, respectively [8]. Most of the microbial phytate-degrading enzymes belong to the acidic type (typically histidine acid phosphatases) [37]. Likewise, most of the plant phytases tend to have a pH optimum at 5. Bioefficacy of plant as well as microbial phytases of different sources can differ in their pH, as shown in Table 3.1. Furthermore, due to the applicability in human food and broader substrate specificity, the acidic phytases are explored to a greater extent than those of alkaline phytases.

3.3.2 SITE OF HYDROLYSIS

Based on the initiation of phytate dephosphorylation at different positions on the inositol ring, three classes of phytase enzymes have been proposed by IUPAC-IUBMB (the International Union of Pure and Applied Chemistry and the International Union of Biochemistry and Molecular Biology). These are 3-phytase (EC 3.1.3.8 or myo-inositol hexakisphosphate 3-phosphohydrolase), 5-phytase (EC 3.1.3.72 or myo-inositol hexakisphosphate 5-phosphohydrolase) and 6-phytase (EC 3.1.3.26 or myo-inositol hexakisphosphate 6-phosphohydrolase). The former liberates the P moiety at position C3, whereas the latter initiates release, respectively, at positions C5 and C6 of the myo-inositol hexaphosphate ring. This distinctive hydrolysis feature can generate different isomers of the lower myo-inositol phosphates esters (IP6-IP5-IP4-IP3-IP2-IP1), via a progression of step-wise dephosphorylation reactions, and ultimately leads to the production of free myo-inositol. It is also acknowledged that each class of phytase differs in their structural property as well mechanistic action to hydrolyze phosphate from phytate. Interestingly, Lei and Porres [40] documented that the majority of phytases have pronounced stereo specificity and prefer equatorial phosphate groups over axial groups.

3.3.2.1 3-Phytases (EC 3.1.3.8)

3-Phytases are found mainly in fungi and bacteria, and to date these are the largest group of phytases reported throughout the living world. They are structurally homologous to β-propeller phosphatase or histidine acid phosphatases. β-Propeller phosphatases are tightly bound to three Ca ions and need two adjacent phosphate groups to bind to the "cleavage site" and to the "affinity site" before hydrolysis can proceed. The end product is believed to be inositol-triphosphate either Ins (1,3,5)P3 or Ins(2,4,6)P3 [41,42]. Recent evidence propose that Ins(2,4,6)P3 is the sole end product [43]. A well explored 3-phytases is isolated from baker's yeast, *Saccharomyces cerevisiae*. The sequential dephosphorylation of phyate by an *S. cerevisiae* phytase is illustrated in Fig. 3.2. Oh et al. [44] found that the

Table 3.1 Properties of Phytases From Different Sources

Source	pH	Temperature (°C)	Specific Activity (U/mg) at 37°C
Plant Source			
Buttercup squash	4.8	48	
Canola seed	4.5–5	50	
Faba beans	5	50	636
Hazel seed	5		
Legume seeds	8		
Lily pollen	8	55	0.2
Lupin seeds	5	50	539, 607, 498
Mung beans	7.5	57	2.4
Navy beans	5.3	50	
Peanut	5	55	
Rapeseed	5.2	50	
Scallion leaves	5.5	51	500
Soybean seeds	4.5–4.8, 4.5–5	55,58	2.4
Sunflower	5.2	55	
Tomato roots	4.3	45	205
Typha latifolia pollen	8		
Barley	5; 6	45, 55	117, 43
Maize seedling	5	55	2.3
Maize root	5–5.1	35–40	5.7
Oat	5	38	307
Rice	4, 4, 4.6	40	
Rye	6	45	517
Spelt	6	45	262
Whole meal wheat	5.15	55	
Wheat bran	5		
Wheat bran	5.6, 7.2		
Wheat bran	6, 5.5	45, 50	127, 242
Crude extract wheat	6	45	
Microbial Sources			
Aspergillus caespitosus	5.5	80	–
Aspergillus fumigatus	5.0–6.0	60	23–28
Aspergillus niger	5.0–5.5	55–58	50–103
Aspergillus oryzae	5.5	50	11
Aspergillus terreus	5.0–5.5	70	142–196
Penicillium simplicissimum	4	55	3
Emericella Nidulans	6.5		29–33

(*Continued*)

Table 3.1 Properties of Phytases From Different Sources *Continued*

Source	pH	Temperature (°C)	Specific Activity (U/mg) at 37°C
Myceliophthora thermophila	5.5		42
Spilosoma castellii	4.4	77	418
Cladosporium	3.5	40	909
Klebsiella pneumoniae	5.5, 5.5	50, 60	224, 297
Klebsiella aerogenes	4.5, 5.2	68	
Peniophora lycii	5.5	58	1080
Thermomyces lanuginosus	6	65	110
Bacillus amyloliquefaciens	7.0–8.0	70	20
Bacillus subtilis	6.5–7.5	55–60	9.0–15
Citrobacter braakii	4	50	3457
Escherichia coli	4.5	55–60	811–1800
Klebsiella terrigena	5	58	205
Lactobacillus sanfranciscensis	4	50	–
Pantoea agglomerans	4.5	60	23
Pseudomonas syringae	5.5	40	769
Candida krusei	4.6	40	1210
Pichia anomala	4	60	–

Sources: *Greiner and Konietzny [38], Bohn et al. [39].*

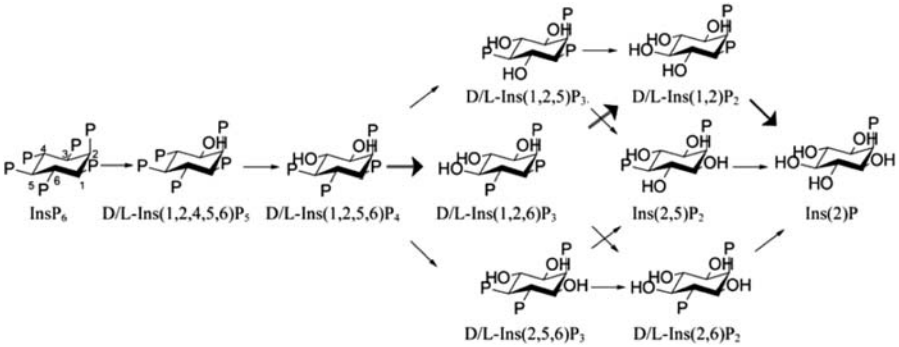

FIGURE 3.2

Dephosphorylation of phytic acid by the 3-phytase from *Saccharomyces cerevisiae*. Hydrolysis of phytic acid initiates on C3 of the inositol ring and the adjacent phosphates are removed consecutively. Thick arrows indicate major pathway of dephosphorylation; thin arrows indicate possible pathways.

Adapted from Greiner et al. [45].

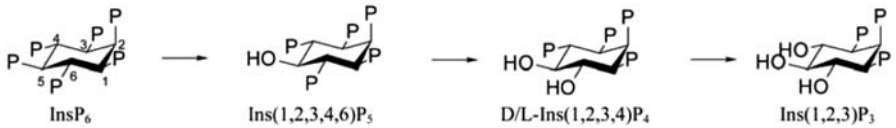

FIGURE 3.3

Dephosphorylation of phytic acid by the 5-phytase isolated from lily pollen. Hydrolysis initiates at site C5 of the inositol ring and the adjacent phosphates are removed consecutively.

Adapted from Barrientos et al. [47].

expression of *S. cerevisiae* can be stimulated when grown in a medium containing InsP6. The yeast-based 3-phytase has been intensively explored because of its potential role during baking.

3.3.2.2 5-Phytase (EC 3.1.3.72)

Interestingly, to date only a single 5-phytase has been reported. It has been detected in lily pollen, and is the only phytase type that initiates hydrolysis with an attack of a phosphate group in the plane of symmetry (Fig. 3.3). The highest activity of this pollen phytase is recorded at pH 8.0 and temperature 55°C [46]. Its amino acid structural conformation is comparable to that of histidine acid phosphatases phytase and much higher homology is found higher toward multiple inositol polyphosphate phosphatase from humans or rats.

3.3.2.3 6-Phytases (EC 3.1.3.26)

6-Phytases (EC 3.1.3.26) act on the carbon atom next to C5 of the inositol ring. However, the official name should be 4-phytase, but traditionally it has been called a 6-phytase. In general, the activity is higher in weak acidic environments (pH $4 \sim 6$) with an optimum temperature between 40°C and 60°C. Molecular weight data suggest that they are usually $50 \sim 70$ kDa and have Michaelis–Menten constants (*K*m) in the range of $10^{-5} \sim 10^{-4}$ mol/L phytate. High molecular weight 6-phytases from tobacco roots (164 kDa) [48] and the highly active phytase from wheat (*K*m 0.5 μmol/L phytate) [49] have also been documented. In this group, numerous structurally different phytases are found including purple acid phosphatase, ADP phosphoglycerate phosphatase, and acid phosphatase.

3.4 SOURCES OF PHYTASES

Phytases are extensively distributed among various life forms. Microorganisms are the greatest potential source of phytase followed by plants. Broadly, there are four possible sources of phytase—plant phytase, microbial phytase, phytase generated by the small intestinal mucosa, and gut-associated microfloral phytases.

3.4.1 PLANT PHYTASES

Phytase enzymes have been isolated and characterized from a number of plant sources—wheat, rice, rape seed, soybean, maize, and rye (Table 3.1). Most of these plant phytases initiate the hydrolysis of phyate at position C6 of the myo-inositol hexaphosphate ring, therefore considered as type 6 phytases. However, in soybean, major InsP5 is DL-Ins(1,2,4,5,6)P5 and thus soybean phytase is a 3-phytase [50]. Activity of wheat phytases (EC 3.1.3.26) were first reported in 1929 by Posternak and Posternak [51]. Two enzymes, Phy1 and Phy2, have previously been purified from wheat bran [52] and two isozymes with the N-terminal amino acid have also been identified [49]. Increased levels and desired properties of phytase in the plants can be achieved by inserting the cloned microbial genes (typically from *A. niger, Bacillus subtilis, Aspergillus fumigatus, E. coli, and Schwanniomyces occidentalis*) encoding for the phytases (Table 3.2). So far, only one transgenic plant expressing a phytase has been developed in order to deal with the issue of dietary phytate in human nutrition. Transgenic plant expressing phytase from *A. fumigatus* gene has been developed to improve white or polished rice as a source of iron [53,54]. Although some feed ingredients contain native phytase activity, steam-pelleting during the manufacture of many commercial feeds results in substantial losses of this intrinsic phytase activity.

3.4.2 MICROBIAL PHYTASES

Bacteria and fungi comprise the most important sources of phytase. Microbial sources of phytases are listed in Table 3.1. Among yeasts, *A. niger*, *Aspergillus ficuum*, *A. fumigatus*, and *S. cerevisiae* are commonly used strains for commercial production of phytases. Hydrolysis of dietary phytate by extrinsic microbial phytase was investigated first by Nelson et al. [55]. They detected improved phosphorus utilization in chicks reared with maize−soybean meal diets containing preparations of *Aspergillus*. For bread production, phytase derived from *S. cerevisiae* is of particular importance. Likewise, cell-bound phytase from *Pichia anomala* and *Candida krusei* have potential applications in food processing because it remains stable even at high temperature and acidity [56,57]. At present several commercial microbial phytases are available on the market with different trade names (Table 3.3). In addition, a group of marine yeasts producing phytase are capable of producing alkaline phytase. Thus, their applicability in the bioremediation of marine phosphorus pollution can be well anticipated. Different bacteria such as *E. coli*, *B. subtilis*, *Klebsiella terringa*, *Lactobacillus* sp., *Pseudomonas* spp. can degrade phytate during growth through production of extracellular phytases [58].

3.4.3 MUCOSAL PHYTASE DERIVED FROM SMALL INTESTINE

In pigs, jejunum is the main site for intestinal mucosal phytase activity. Phytase activity is also reported in small intestine of humans but has very limited ability

Table 3.2 Phytase Expression in Genetically Engineered Plants

Phytase Source	Host Plant	Tissue
Aspergillus niger	Tobacco	Leaf
A. niger	Tobacco	Leaf
A. niger	Tobacco	Seed
A. niger	Tobacco	Root
Bacillus subtilis	Tobacco	Root
B. subtilis	Tobacco	Leaf
A. niger	Alfalfa	Leaf
A. niger	Arabidopsis	Root
B. subtilis	Arabidopsis	Root
Escherichia coli	Arabidopsis	Seed
A. niger	Clover	Root
A. niger	Sesame	Root
A. niger	Soybean	Callus
A. niger	Canola	Seed
A. niger	Potato	Leaf
Consensus	Potato	Root
Aspergillus fumigatus	Rice	Seed
Schwanniomyces occidentalis	Rice	Leaf
E. coli	Rice	Seed
A. niger	Wheat	Seed
E. coli	Sugarcane	Callus

Source: *Greiner and Konietzny [38].*

Table 3.3 Commercially Available Microbial Phytases

Company	Country	Phytase Source	Production Strain	Trademark
AB Enzymes	Germany	*Aspergillus awamori*	*Trichoderma reesei*	Finase
Alko Biotechnology	Finland	*Aspergillus oryzae*	*A. oryzae*	SP, TP, SF
Alltech	USA	*Aspergillus niger*	*A. niger*	Allzyme phytase
BASF	Germany	*A. niger*	*A. niger*	Natuphos
BioZyme	USA	*A. oryzae*	*A. oryzae*	AMAFERM
DSM	USA	*Peniophora lycii*	*A. oryzae*	Bio-Feed Phytase
Fermic	Mexico	*A. oryzae*	*A. oryzae*	Phyzyme
Finnfeeds International	Finland	*A. awamori*	*T. reesei*	Avizyme
Genencor International	USA	*Penicillium simplicissimum*	*Penicillium funiculosum*	ROVABIO
Roal	Finland	*A. awamori*	*T. reesei*	Finase
Novozymes	Denmark	*A. oryzae*	*A. oryzae*	Ronozyme Roxazyme

Source: *Cao et al. [59].*

for phytate degradation [60]. Interestingly, it was shown that when offered with phosphorus-inadequate diets, many animals including humans can elevate intestinal phytase and phosphatase activities [61]. Phytase activity in the small intestine has been widely documented in the piscine group. In Tilapia *Oreochromis niloticus* × *Oreochromis aureus* phytase activity is localized in the small intestinal brush border membrane (BBM) [62]. Likewise, intestinal BBM phytase activity is also documented in hybrid striped bass *Morone saxatilis* × *Morone chrysops* and common carp *Cyprinus carpio* [63].

3.4.4 GUT MICROFLORAL PHYTASES

Gut microfloral phytases are primarily evident in the large intestine of pigs. The phytase-producing bacterial flora in the foregut and hindgut regions of the gastrointestinal tracts of many freshwater fish have been isolated [64]. Recently, a novel phytase gene, *appA*, was isolated by degenerate polymerase chain reaction and genomic library screening from *Buttiauxella* sp. *GC21*, a bacterium isolated from grass carp (*Ctenopharyngodon idella*) intestine. The phytase gene could be successfully expressed in *E. coli* with high expression levels [65]. However, bacteria colonies present in the colon of humans have limited ability to degrade phytate. The significance of phytase produced by resident bacteria in nonruminants has not been demonstrated and is probably negligible. To date, the supplementation of gut microfloral derived phytase as food additive is very limited.

3.4.4.1 Suitability of genetically modified phytases

In order to evaluate the health concerns of genetically modified plant and microbial phytases, pigs and poultry were offered up to 20-fold higher doses than the recommended levels (500 U/kg). General necropsy and histological examination of liver, kidney, and tibial tissues revealed no adverse effects [66]. It was also evident that the performance, phosphorus availability and bone mineralization values also increased.

3.5 APPLICATION OF PHYTASE

3.5.1 PHYTASES AS FOOD ADDITIVES

Supplementations of phytate-hydrolyzing enzymes in food have multiple benefits. Phytase-mediated degradation of phyate, and the release of phosphorous and minerals is of immense importance to human and animal nutritionists as well as to ecologists. The nutritive value of bread is enhanced by supplementation of phytase which reduces the phytate content and stimulates the endogenous α-amylase activity by making more calcium available. Along with lowering phytate content in doughs and fresh breads, fermentation time was curtailed by phytase addition without affecting the dough pH. An upsurge in bread volume and an improvement

in crumb texture were also recorded following phytase addition [13]. Other texture parameters such as gumminess and chewiness were also decreased. These enhancements in bread quality are believed to be linked to triggered α-amylase activity incited by the phytase enzyme. In humans, the absorption of nonheme iron from wheat bread rolls is almost doubled when these food items are treated with fungal phytase [67]. Likewise, research on humans confirms that iron absorption from porridges based on flours from rice, wheat, maize, oat, sorghum, and wheat-soy increased many fold by adding phytase to the porridge [68]. The positive feature of phytase enrichment during tofu preparation is also demonstrated on human health [69]. Soymilk is generally not preferred owing to the high level of phytate (0.56%), supplementation of phytase can significantly reduce the phyate level in soyabean milk [70]. Tarhana, a traditional Turkish fermented and dried cereal food, is a good source of minerals (Ca, Mg, and K). The fermentation medium with baker's yeast as a phytase source resulted in a momentous decrease in phytic acid as well as augmenting total amounts of minerals and proteins [71]. Chapathi, commonly called "roti," is a staple food in parts of India and neighboring countries. It is prepared from whole-wheat flour that contains considerable amount of phytic acid. Application of yeast *Candida versatilis* as a source of phytase during the preparation of chapatti dough can reduce the level of phytate by 40% [72].

3.5.2 PHYTASES AS FEED ADDITIVES

Research on pigs shows that enrichment of maize, wheat, and triticale with microbial phytase increased phosphorus availability by 38%, 12%, and 15%, respectively [73]. Addition of microbial phytase in maize and soybean diet is also documented to increase the zinc bioavailability for pigs [74]. The increased bioavailability of phytate bound phosphorous in response to phytase reduces the phosphorous excretion by animals, and thus limits the influx of phosphate load into the natural/(aqua) culture water bodies. This ultimately prevents the incidence of eutrophication or algal bloom. Phytase supplementation in pigs basal feed can cut down phosphorus content in fecal matter by up to 60% [75]. In chickens, supplementing the feed with exogenous phytase can remarkably improve the absorption of amino acids, Ca, Na, and phytate-P [75,76]. Positive effects of phytase inclusion on weight gain was reported for both broilers as well as pigs [77].

3.5.3 PRODUCTION OF PLANT PROTEIN ISOLATES AND CONCENTRATES

The application of plant protein isolates and concentrates in food production is increasing owing to their good nutritional value. However, high content of phytate present in plant products (seeds and grains) and its interaction with endogenous

proteins reduces the solubility of the proteins. Therefore, it is apparent that phytate can adversely affects the yield and quality of the protein isolates in the processed plant concentrates. Supplementing exogenous phytase during the production process has been demonstrated to significantly increase the protein yields and almost comprehensively remove of myo-inositolhexakis-, pentakis-, tetrakis-, and trisphosphates from the final plant protein isolate [14,78]. Phytate-reduced plant protein isolates are also marked by an improved mineral bioavailability, amino acid profile, as well as their protein digestibility. Consequently, these treated protein isolates are recommended as suitable protein sources for infant formulae. Furthermore, corn steep liquor is used in the fermentation industry for the production of compounds such as enzymes, yeast, antibiotics, amino acids, as well as a high-energy liquid animal feed ingredient. However, phytate present in the maize can get into the corn steep liquor and specify an undesirable component. By supplementing phytase together with plant cell wall degrading enzymes to the steep liquor, phytate-free corn steep liquor can be obtained [15,79].

3.5.4 SOURCE OF MYO-INOSITOL PHOSPHATES

The cellular transmembrane signaling and the mobilization of calcium from the intracellular reserves are regulated by the inositol phosphates and phospholipids. The inositol phosphates also act as enzyme stabilizers, enzyme substrates for various metabolism, as well as potential drugs blockers [80]. Phytase plays a crucial role in various inositol phosphate preparations [81,82].

3.6 HEALTH BENEFITS OF PHYTASES AND POTENTIAL CONCERNS

We have already mentioned that supplementing the basal diet with phytase can significantly increase the body's ability to absorb and assimilate important minerals such as Ca, Mg, Zn, and Fe (Fig. 3.4) Thereby, it is reasonable to speculate that it might be a promising approach to reduce mineral deficiency in susceptible groups especially childbearing women, vegetarians, and people primarily dependent on plant-based foods, typically in the developing world. In addition, phytase can break down phytate, so we have less possibility of building up excess insoluble complexes in the digestive tract. Phytase also breaks down bound forms of P/phosphate in the body; this is one more way by which it facilitates the absorption of this mineral. P is an essential element for the growth and protection of bone density, therefore it is obvious that phytase-mediated augmentations in P assimilation will help to prevent bone loss and reduce osteoporosis. Phytate can also bind with proteins or amino acids at low and neutral pH [3,83]. Complex formation of protein or amino acids with phytate may occur in foodstuffs in the

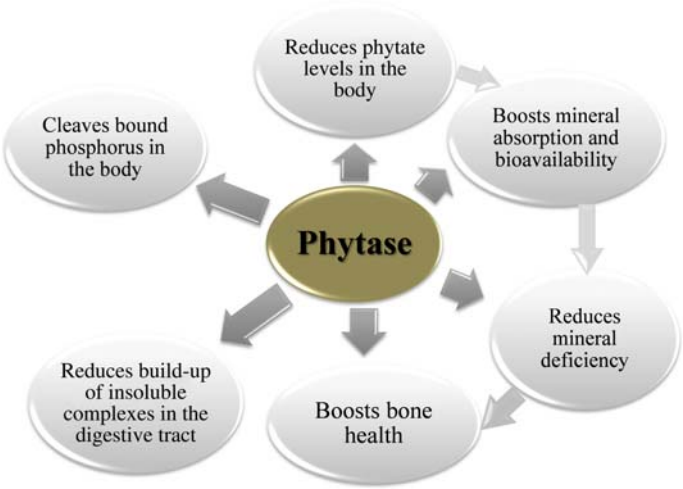

FIGURE 3.4

The health benefits of supplementing phytase in diet.

native state, and may be formed in the upper gastrointestinal tract. Phytate can also form complexes with proteolytic enzymes which may occur in the upper gastrointestinal tract. These potential phytate−protein/enzyme complexes can reduce the utilization of proteins as well as functionality of proteolytic enzymes. Most of the researches with phytase were focused on measuring the effects of phytase on P and mineral utilization, amino acid digestibility was rarely evaluated. In broilers, Ravindran and Bryden [84] reported that half of the amount of amino acid bound to phytate was liberated after the addition of phytase. A noticeable amino acid digestibility was improved in broilers when phytase was added to a 23% crude protein maize−soybean diet [66]. Apparent and true ileal digestibilities of amino acid in Large White turkey female poults fed a maize−soybean meal diet improved when 750 U phytase/kg diet was added [85]. In broilers, supplemental phytase (1200 U/kg feed) enhanced ileal digestibilities of protein of three cereals (maize, sorghum, and wheat), four oilseed meals (soybean meal, canola meal, cottonseed meal, and sunflower meal) and two cereal by-products (wheat middlings and rice polishings) [84]. Similar findings have been reported in weaner and growing-fattening pigs [86] where microbial phytase improved the apparent ileal and total tract digestibility of crude protein by 2.3%−12.8% units [87,88]. In cannulated pigs, Mroz et al. [12,89,90] reported that microbial phytase enhanced the apparent ileal digestibility of many amino acids, including essential ones. The increments in phytase-mediated P and protein digestibility were reflected by a parallel reduction in excretion of these nutrients.

A question arising is to what extent should dietary phytates be hydrolyzed by added phytases. On the one hand, it has been clearly demonstrated that adequate dosages of phytase can degrade all the phytates present in food- and feedstuffs

[91]. On the other hand, several authors attributed to inositol-6-phostate (IP-6) a protective role against dysplasia with increasing demonstration in animal models in recent years [92–94]. These facts are of more importance in human nutrition than in animal nutrition since the lifespan of farmed animal is generally not enough long to develop neoplasias.

3.7 CONCLUSION AND PERSPECTIVES

Until the last few years, the application of exogenous phytase enzymes was mainly confined to animal feed, but recently scientists and entrepreneurs started to realize their great potential as food supplementation for the human health. Removal of phytate, or at least part of it, by adding exogenous phytase can result in significant improvement of the bioavailability of essential minerals. Therefore, phytase seems to be a potential tool to reduce the risk of mineral deficiency among populations depending on unrefined cereals or pulses as basal diet as well as for nutritionally vulnerable groups such as pregnant women, strict vegetarians, and dwellers of underdeveloped countries. Furthermore, use of phytases in processing and manufacturing of foods such as bread, plant protein isolates, corn steep liquor, etc., can positively influence the purity and yield of the products. However, one should bear in mind that the use of phytase for human consumption is still in the experimental phase and also no ultimate phytase for all food applications exists. Therefore more research is needed to screen the diverse sources of phytases, to optimize their catalytic activities, and to enrich them with preferable features for intensifying their propriety for food applications, as well as to establish the level of phytate hydrolysis respecting the potential protective role of IP6.

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CHAPTER

Phytase in animal feed

4

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4.1 INTRODUCTION

In many plant-based ingredients, phytic acid is a common compound that functions as a way to store phosphorus (P) in a stable form and is particularly high in most cereal grains and oilseeds. This can range from 0.08 to 1.13 [1] (Fig. 4.1A), but some reports show this can be upward of 8.36% in wheat bran [2]. Although phytase is also correspondingly high (Fig. 4.1B), phytase from plants is considered to be highly heat labile, and is often destroyed during pellet manufacture. Therefore, in nonruminant animals that lack or have limited endogenous phytase, phytic-P is largely indigestible and may lead to deficiencies in P. Such deficiencies can decrease bone mineralization, causing difficulties in animal locomotion, and in terms of postharvest quality, can increase incidences of broken bones and bloody breast meat [3].

Additional dietary P can be supplemented to ensure this essential nutrient does not becoming limiting, however the underutilized phytic-P is subsequently excreted to the environment [4,5]. This is a particular concern due to the well-known effects of excessive P discharge promoting eutrophication in waterways [6]. Moreover, phytic acid can decrease the utilization of various nutrients in different ways. Firstly, phytic acid binds with cation groups on various nutrients that include minerals, proteins, amino acids, and starches in largely inaccessible forms (Fig. 4.2). Therefore, in feeds that contain high amounts of plant-based ingredients, phytic acid may decrease the absorption of many essential nutrients. Secondly, phytic acid can directly alter the physiological status of animals. Some of these effects include phytic acid inhibiting various digestive enzymes [7], particularly trypsin activity [8,9] as well as potentially irritating the gastrointestinal system of the animals [10,11]. Thirdly, diets high in phytic acid were shown to decrease the expression of some genes responsible for appetite and glucose/sodium absorption in fish and piglets, respectively [7,12]. These multifaceted consequences can potentially reduce animal growth and health; therefore, methods to reduce phytic acid in terrestrial and aquatic animal diets are generally viewed as advantageous.

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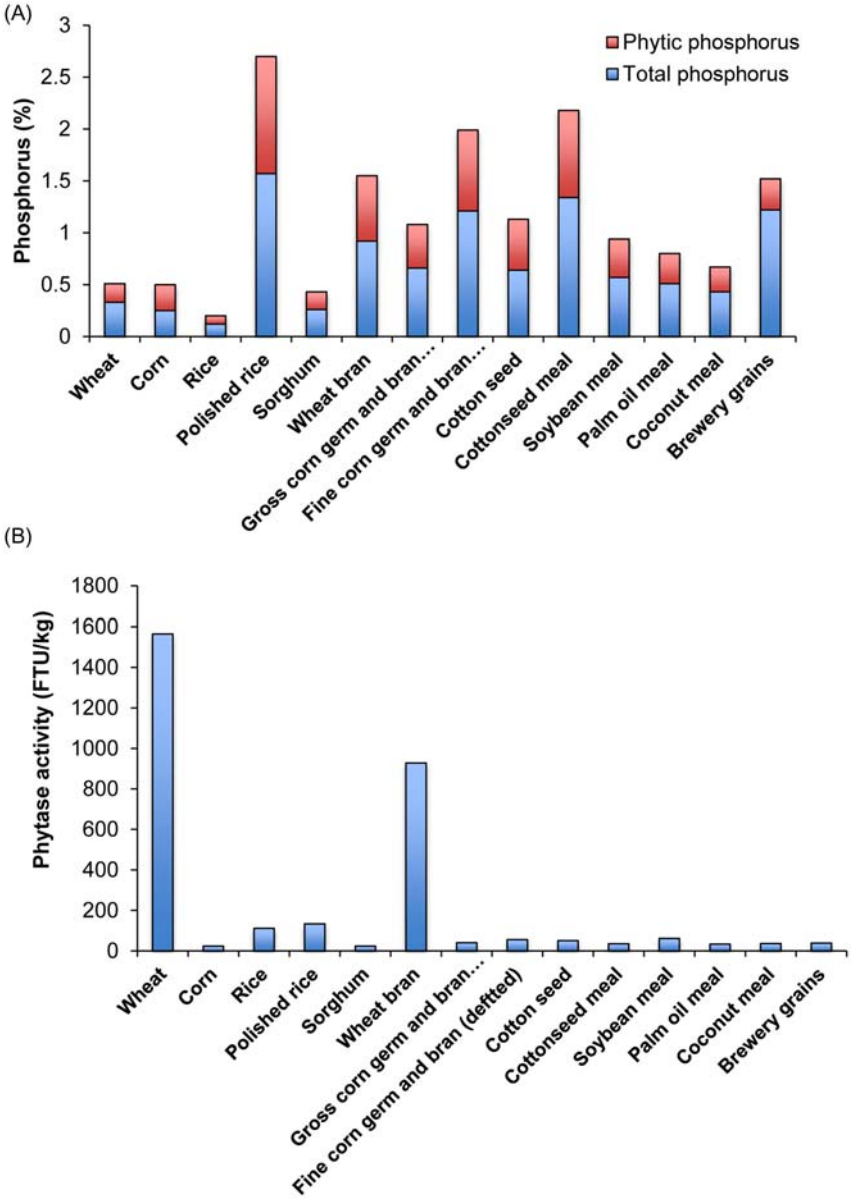

FIGURE 4.1

The amount of phytase-phosphate (%) (A) and inherent phytase activity (B) in various feeds commonly used in the diets of terrestrial and aquatic animals.

Data compiled from Godoy S, Chicco C, Meschy F, Requena F. Phytic phosphorus and phytase activity of animal feed ingredients. Interciencia 2005;30:24–7.

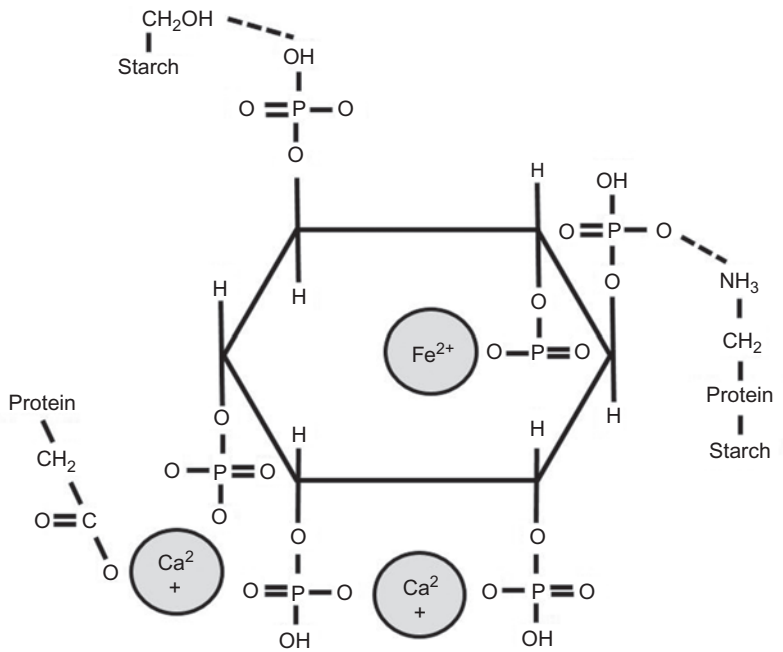

FIGURE 4.2

Phytic acid binding to minerals, proteins, and starches.

One effective way to accomplish this includes the use of phytase. Phytases are a class of enzymes that catalyze the removal of phosphate from either phytic acid or phytate (salt form) and can be classified as histidine acid phosphatases (HAPs), β-propeller phytase (or alkaline phytase), purple acid phytase, or protein tyrosine phosphatase. These are largely considered safe additives in animals feeds [13,14], but the most commonly used in commercial feeds are microbial HAPs from the fungi *Aspergillus niger* or bacteria such as *Escherichia coli* and *Buttiauxella* spp. [2]. The type of microbial phytase is sometimes not reported in the scientific literature, but rather only the activity. Providing such information is important to potentially explain differences among studies and in future investigations this should be encouraged. In this chapter, such information will be provided when possible.

4.2 FACTORS INFLUENCING PHYTASE EFFICACY

The efficacy of phytase hydrolyzing phytic acid within various plant-based ingredients in vitro depends on various factors including pH, duration/temperature of incubation, presence of P and calcium (Ca), and interactions with other enzymes [2,15,16]. For example, crude phytase (at 10,000, 100,000, and 340,000 FTU/kg;

Natuphos) was more effective at hydrolyzing phytic acid in canola meal compared to purified (by gel filtration) phytase [15]. Newkirk and Classen [15] suggested that the presence of nonphytase enzymes in crude phytase, such as proteases and/or cellulolytic enzymes, increased the number of available sites for phytase thus leading to improved efficacy. This suggestion has been supported in later in vitro studies [17] as well as during live trials on broiler chickens [18,19] and fish [20].

The activity of phytase is also highly dependent on pH conditions and generally the optimal pH range for phytase activity produced from *A. niger, E. coli,* and *Saccharomyces cerevisiae* is between 2.0 and 5.8 [2]. This is because lower pH conditions promote the disassociation of phytate to phytic acid, which therefore increases solubility as well as reducing inhibitory interactions of the associated minerals to phytase [21,22]. The most potent inhibitors to phytase activity are divalent cations that include zinc, followed by iron, manganese, calcium, and finally magnesium [22]. For example, the inclusion of zinc at 2500 mg/kg in phytase-supplemented diets decreased P digestibility in pigs, but this was not observed in the other tested zinc level of 100 mg/kg [23]. Understanding this relationship is important because metals, and in particular zinc, are often included in the diets of broilers and pigs to promote growth [24] as well as to minimize diarrhea in postweaning pigs [23].

It is important to note that the stomachs of various terrestrial and aquatic animals are within the optimal range for phytase activity. Therefore, when phytase is supplemented, the stomach is the predominant site for most phytic acid hydrolysis [25,26]. On the other hand, without phytase supplementations, some phytate degradation can occur, but this mainly occurs in the cecum [27] (Fig. 4.3). This may be due to the presence of intestinal bacteria that are capable of degrading phytic acid as observed in broilers [28] as well as fish [29].

When including phytase in feeds, heat is of particular concern for nutritionists and feed manufacturers since high temperatures that are often used during pellet manufacturing can reduce phytase activity. For example, in an early study it was shown that when temperatures exceeded 80°C during steam pelleting, P and Ca utilization was reduced in pigs, which was believed to be from phytase naturally within the ingredients being degraded [30]. Similarly, when microbial phytases were added in an ingredient mix and then steam pelleted (67−70°C), this led to an approximate loss of half the activity [31]. The optimal temperature for most microbial phytases is around 60°C, and when temperatures exceed 80°C, phytase activity substantially reduces [2]. This has prompted increasing investigations to design more heat-stable phytases [32], which would be of particular relevance for fish feeds that are often made by extrusion with temperatures generally exceeding 100°C [33]. One of the ways to bypass this issue is by pretreating ingredients known to be high in phytic acid. However, it is certainly worth noting that when comparing increasing phytase inclusion levels (0, 200, 400, 600, 800, and 1000 FTU/kg) in extruded diets, the optimal phytase dose was 400 FTU/kg for mineral digestibility in rainbow trout [34].

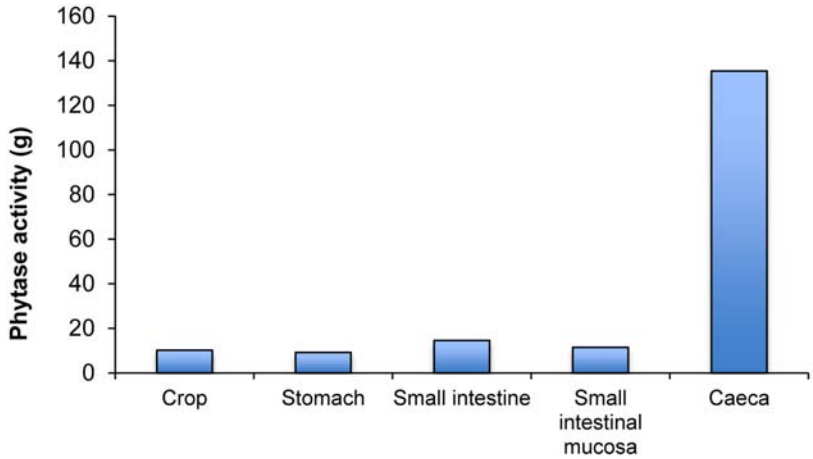

FIGURE 4.3

Phytase activity (μmol phytic acid/h) in the digestive tract of laying hens fed diets without phytase supplementations.

Data compiled from Marounek M, Dušková D, Skřivanová V. Hydrolysis of phytic acid and its availability in rabbits. Br J Nutr 2003;89:287–94.

4.3 EFFICACY OF DIETARY PHYTASE TO GROWTH AND NUTRIENT UTILIZATION IN ANIMALS

The inclusion of dietary phytase in terrestrial and aquatic animals commonly leads to an improvement in mineral absorption, particularly for P and Ca (e.g., Refs. [35–37]). It is generally recommended that phytase be added at an FTU/kg range of 500–2000 to effectively replace P supplementations [2]. Such inclusions are environmentally friendly in P-deficient feeds, but this may not always be reflected in enhanced growth performance, feeding efficiencies, or amino acid digestibility. Indeed, in some cases, phytase was an ineffective growth promoter in broiler chickens [38–41], turkeys [42], pigs [35], and some fish species [43–45]. Contributing factors may include differences in the host species, gender, phytase type/level, inclusion method, levels of dietary P and Ca, as well as different ingredients that have varying levels of endogenous phytase and enzyme resistant compounds.

Nevertheless, phytase supplementations are generally beneficial to the host animal and some recent studies indicate that levels exceeding the normal dose improve the gastrointestinal health of broilers [11,28]. In a recent example, the inclusion of a "super-dose" of phytase at 6000 FTU/kg enhanced the growth performance and feeding efficiencies of early chicks, but not at their later grower or finishing stages [11]. Overall, it was concluded that rather than improving P availability, phytase may have reduced phytic acid induced irritation on the

intestine [11]. Similarly, there were indications that high phytase levels (5000 FTU; *E. coli* 6-phytase) in P-deficient diets improved the gastrointestinal health of broilers based on increased ileal short chain fatty acid (SCFA) levels, which was likely due to enhancing lactic acid bacteria (LAB) that included *Lactobacillus* sp. and *Enterococcus* sp. [28]. Research in this area appears to be promising since LAB and SCFAs may act as growth promoters and effective prophylactics to disease in both terrestrial and aquatic animals (e.g., Refs. [45–48]). Moreover, SCFAs are slightly acidic compounds and this may further improve phytase activity by reducing intestinal pH [49].

Other methods to increase SCFA levels, and potentially phytase activity, may also include the use of prebiotics such as fructooligosaccharides (FOSs), which are the most commonly used prebiotic in broiler diets [49]. The inclusion of FOSs has been shown to increase intestinal SCFAs in both terrestrial [49] and aquatic animals [50], but research on any potential interaction between FOSs and phytase is limited. In one study, however, two different dietary levels of FOS (0.5% and 1.0%) without or with phytase (500 FTU/kg) were compared [51]. It was shown that the best combination for growth and P digestibility was 0.5% and 1.0% FOS with phytase, respectively, but these were not significantly different compared to the sole use of phytase [51]. Since prebiotics are well known to act as immunostimulants, more research in this area should conducted.

Although P is an essential nutrient for animals, it has been shown that additional P supplementations reduce intestinal SCFAs in animals by inhibiting bacterial fermentation [28] as well as phytase activity [18,41,52]. In terms of the latter, a similar observation was made with Ca since high dietary levels of P and/or Ca reduced phytate-P digestibility in various animals including rats [53], turkeys [54], chickens [52,55], and pigs [56]. Optimal C:P ratios of 1:1 to 1.4:1 have been suggested for optimal phytase activity [54,57]. This interaction is likely due to Ca binding to phytic acid, thus reducing their efficiency [58] as well as phytase not being as necessary when additional P is added.

In addition to phytase being more effective in P-deficient diets, nonphytase enzymes may also be effective. For example, Selle et al. [18] compared diets with low P and phytase (500 FTU/kg), without or with xylanase (2000 U/kg), to a control diet that had adequate amounts of P (and no phytase) in broilers. While it was found that phytase significantly improved growth, feed intake, ileal nitrogen, amino acid, energy, sodium, Ca, and P digestibility in broilers, a combination of phytase and xyalanse had a synergistic activity on these parameters. It was suggested that the xylanase degraded the cell structures in the plant-based ingredients, thus allowing more sites for phytase [18]. Similar findings were later observed in broilers and tilapia when phytase was combined with a cocktail of carbohydrate-degrading enzymes [19,20,59].

Although proteins and amino acids can bind with phytic acid, the available literature suggests that phytase supplementations are more effective at improving nitrogen and amino acid digestibility in broilers [24] rather than pigs (e.g., Refs. [37,60,61]) or fish [34]. Interestingly, the inclusion of phytase actually led to a slight reduction

in nitrogen absorption and retention in pigs [61] and a significant reduction in some amino acids in rainbow trout *Oncorhynchus mykiss* [34]. The exact cause(s) for these findings still remains elusive, particularly since phytase has a clear benefit in improving mineral digestibility in both pigs (e.g., Ref. [61]) and fish [34].

Indeed, similar to in terrestrial animals, phytase supplementations generally improve mineral digestibility in fish [34]. The implications of such supplementations to growth or digestibility of other nutrients appear to depend on the species, feed formulations, as well as the method of delivery. Positive growth effects of dietary phytase were found in channel catfish *Ictalurus punctatus* [62,63], pangus *Pangasius pangasius* [64], rohu *Labeo rohita* [65], African catfish *Clarias gariepinus* [66] and the freshwater prawn *Macrobrachium rosenbergii* [67]. On the other hand, dietary phytase had no effect on the growth or feeding efficiencies in sea bass *Dicentrarchus labrax* [43], Japanese flounder [68], Atlantic salmon *Salmo salar* [69], Korean rockfish *Sebastes schlegelii* [44], Japanese sea bass, *Lateolabrax japonicus* [4], or yellow catfish *Pelteobagrus fulvidraco* [45].

Understanding the exact reasons for these differences is complicated. This is particularly valid considering there are large differences in the efficacy of phytase among the same species. For example, when using plant-based diets, phytase at 3000 or 2000 FTU/kg (microbial 6-phytase; *Aspergillus oryzae*) improved the growth, feeding efficiencies, and digestibility of various ingredients in *O. mykiss* [70,71]. In contrast, lower doses of 1000 or 1400 FTU/kg in the same species had no effect on the growth or feeding efficiencies when using a plant-based diet, but did improve P digestibility [72,73]. These discrepant findings may be related to differences in the phytase levels, but this does not appear to explain contradictory findings in tilapia. For example, it was recently shown that phytase (1000 FTU/kg; *A. niger*) improved P utilization and the gut microbial community in tilapia, but on the other hand, caused intestinal inflammation and upregulated the expression of stress-related genes [74]. This combination of beneficial and harmful effects in tilapia was suggested as the cause for no growth improvement and it was suggested that a different phytase type be explored. Furthermore, two separate studies on tilapia showed that phytase additions in soybean-meal-based diets, actually reduced lysine and alanine digestibility [75] as well as protein utilization [76]. In contrast, Liebert and Portz [77] found that a similar phytate type and level in a soybean-meal-based diet improved tilapia growth and nutrient utilization. It may be possible that these discrepant findings were due to differences in the delivery of phytase. This is because in the study by Liebert and Portz [77], phytase was mixed with the dietary ingredients, whereas in the studies by Riche and Garling [75] and Riche et al. [76], soybean meal was pretreated with phytase by incubation (and activated by citrate). These latter authors [75,76] suggested that the degradation of phytic acid might have made lysine and methionine more susceptible to being destroyed by high heat conditions during pellet manufacture. Another possibility included phytic acid reducing the harmful effects of other antinutritional factors and/or reducing the leaching of soluble nutrients by forming complexes [75,76].

This certainly seems plausible since phytic acid at 1.0% or 1.5% improved shrimp *Penaeus japonicus* growth [78] and Zn bioavailability to blue tilapia *Oreochromis aureus* [79], respectively. On the other hand, incubating soy concentrate with phytase to remove all phytic acid improved feeding efficiencies, protein, Zn, Ca, and magnesium digestibility in *S. salar* [80]. Interestingly, a later study on the same salmonid species showed that a combination of phytase (2000 FTU/kg) and phytic acid (at 1%) significantly improved both growth and feeding efficiencies in *S. salar* [69]. These results indicate that the phytase delivery method and interactions with other compounds in fish are likely species-specific and continued research in this area should help to optimize the efficacy of phytase supplementations. In particular, there is increasing interest in the use of dietary acidifiers, such as some organic acids.

4.4 USE OF PHYTASE WITH ORGANIC ACIDS

One of the potential strategies to improve the efficacy of phytase in the growth and nutrient utilization in both terrestrial and aquatic animals is by adding organic acids [81]. Organic acids are slightly acidic compounds that are "generally regarded as safe," with one or more carboxyl groups in their structure. These have been used for many decades in the livestock feeds due to their potential improvements to nutrient digestibility and resistance to disease [82]. The most commonly used include propionic, formic, lactic, and citric acids and their salts, and among these, citric acid has been the most studied regarding potential interactions with phytase [81].

Rather than citric acid having any direct ability to degrade phytic acid [83,84], it is believed that a synergistic effect can occur between citric acid and phytase in two different ways. Firstly, citric acid is a competitive chelator and by removing minerals that would otherwise bind to phytic acid, this would reduce their inhibitory actions to phytase [22]. Secondly, among organic acids, citric acid has the strongest acidifying properties and a reduction in pH within the gastrointestinal tract of the animal is well known to enhance phytase activity. Similarly with phytase, combinations with citric acid appears to be particularly effective when animals are fed P-deficient diets [39].

Generally, a combination of citric acid with phytase is reportedly not often recommended in commercial applications, but a recent literature review on broilers indicates this can be effective at improving both growth and mineral utilization [81]. For example, the inclusion of citric acid/sodium citrate (at a 1/1 ratio) at 6% and in combination with phytase (1450 FTU/kg) significantly increased the early growth of chicks as well as feeding efficiencies and tibia ash compared to the sole use of citric acid/sodium citrate [85]. In a later study on chicks, a similar finding was also observed using citric acid (3% or 4%) and phytase (300 FTU/kg) [86]. In rohu, *L. rohita*, a combination of citric acid (at 3%) and phytase

(produced from *A. niger*; 500 FTU/kg) synergistically improved mineral bioavailability [65]. In contrast, the growth-promoting effects of phytase (600 FTU/kg) in the diets of chicks were neutralized by citric acid (at 2%) [87]. Similarly, the combined use of citric acid and phytase had no synergistic effect on the growth and/or nutrient utilization in broilers [36,41], pigs [88], or catfish *P. fulvidraco* [45]. In fact, Centeno et al. [41] found that citric acid (at 2% or 5%) decreased broiler growth, but in contrast, the sole use of citric acid at 0.2% was a growth promoter in *P. fulvidraco* [45]. Further investigations regarding potential interactions of citric acid and phytase with other ingredients such as Ca and P as well as the age of the animals may help to explain these differences.

Information on the combined use of other organic acids with phytase is limited but generally is successful [89–92]. For example, a combination of phytase (410 FTU/kg; *A. niger*) with formic (0.8%–1.6%) or lactic acid (1.6%–3.2%) significantly improved growth as well as dry matter, ash, Ca, and P digestibility compared to the sole use of phytase in pigs [89]. However, Jongbloed et al. [89] found that the optimal combination appeared to be formic acid with phytase as opposed to lactic acid in pigs. Similarly, in broiler chickens, a proprietary organic acid blend (lactic, formic and citric acids) with phytase (1000 FTU/kg; Natuphos) had a beneficial and interactive effect on growth and feeding efficiencies [90]. Emami et al. [92] also found that an organic acid blend (formic and propionic acid) with phytase (500 FTU/kg) significantly improved the growth performance, feeding efficiencies and digestibility of protein and P in broilers compared to either a positive (adequate P level) or negative control diet (deficient P level). Interestingly, another benefit appeared to be enhanced immunity based on higher total Ig, IgM, IgG, as well as a better intestinal histomorpholoy [92]. Such combinations may have significant implications in disease management and, indeed, the prophylactic properties of organic acids are well known. Therefore, further research into combinations of organic acids with phytase in the diets of both terrestrial and aquatic animals appears promising.

4.5 CONCLUSIONS

Various factors can influence the efficacy of phytase to growth performance, feeding efficiencies, and nutrient digestibility in both terrestrial and aquatic animals. Overall, however, the inclusion of phytase can be recommended as an effective and safe way to improve mineral utilization. For optimal performance, phytase-supplemented diets should not receive any additional P or Ca since these minerals bind with phytase leading to reduced activity. Moreover, this practice will help minimize excessive P discharge to the environment. An improvement to mineral utilization, however, may not necessarily lead to better growth or feeding efficiencies and is likely due to various interactions between dietary formulations, the phytase type, and inclusion levels among studies. There are some recent

researches that indicate that phytase levels exceeding the normal recommended dose appear to show additional success at not only improving growth but also the gastrointestinal health of animals. Such high levels may not necessarily be cost-effective in all situations, but one strategy to improve phytase activity is by the inclusion of some organic acids (citric, lactic or formic acid) as well as nonphytase enzymes (proteases and xylanases).

This area appears to be particularly promising since there are indications that such combinations may lead to synergistic or added benefits to the productivity and health of animals, which could have significant implications for disease management. This area of research is still young and should receive increasing attention, such as potential interactions with probiotics, prebiotics, organic acids, or other natural products known for their health-promoting characteristics. Such research may further improve productivity in both terrestrial and aquatic animals in a more environmentally friendly and cost-effective way.

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CHAPTER

Perspectives of phytases in nutrition, biocatalysis, and soil stabilization

5

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5.1 INTRODUCTION

The first phytase to be introduced into the market, in 1991, was obtained from *Aspergillus niger*. In 1999 it was demonstrated that some phytases from bacterial origin such as *Escherichia coli* were more active than those produced by fungi [1,2].

The first phytase obtained from a full synthetic gene expressed in *Hansenula polymorpha*, named "Consensus" was developed in 1997 [3]. The enzyme is resistant to temperatures up to 90°C and has an activity equivalent to that of *A. niger* [4–6].

Besides the recognized positive effects of phytases on the digestive utilization of phosphors (P) from phytates, beneficial effects are attributed to phytases on the bioavailability of energy and amino acids [7]. Effects on energy are controversial since in the better cases what is observed is a very marginal positive tendency and questions arise on the proposed E "matrices" for phytase. On the contrary, the improvements in the amino acids biodisponibility are systematic and clear in swine while they can be variable in poultry [8].

In humans, Fe and Zn deficiencies are serious problems of health in several regions of the world, particularly in children and pregnant women, even if both minerals are present in enough quantity in the plant foodstuffs consumed in less-developed countries. It is known that the biodisponibility of Fe and Zn is inhibited by the phytates. It has been demonstrated that phytates also inhibit Ca, Mg, and Mn [9]. The hydrolysis of phytates increases the availability of several cations and accordingly improves the nutritive value of food [10].

Several methods have been proposed to improve the nutritive value of plants such as genetic engineering, fermentation, immersion, germination, and treatment with phytases that can be used in pretreatment or as food additives. The significant reduction of phytates can be obtained in cereals and leguminous seeds and improve the Fe absorption [11]. In a synthesis of 12 essays performed in several countries [12] it was clearly evidenced that phytase has beneficial effects on the Fe and Zn absorption present in foodstuffs with high levels of phytates. With the

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aim of achieving a more coherent application of phytases in human and animal nutrition including the cost, the development of new phytases or formulations is needed. In human nutrition the determination of the recommended dosages must be performed.

Besides the food and feed applications of phytases, two potential applications of the enzyme should be mentioned. On the one hand, the addition of phytase to sandy soils originate the formation of cements starting from crystals of Ca phosphate and can be a form of stabilization of sandy areas [13]. On the other hand, the evaluation of the possible utilization of phytases in industrial biocatalysis should be pursued [14].

5.2 NUTRITION

5.2.1 THE PHYTASE STAKE IN ANIMAL NUTRITION

Phytates [myo-inositol-(1,2,3,4,5,6)-hexakisphosphate] are a large group of organic P compounds present in nature in the form of salts of phytic acid, or inositol phosphates. They are most likely found in eukaryotic organisms, especially plants, where they stand for the most significant source of P storage in seeds. Cereal grains have abundant inositol phosphate as well, and are commonly used as animal feed in agribusiness.

The wide presence of phytate in the natural environment and its essential function of P storage suggests growing interest in this topic by ecological and environmental scientists. Nonetheless, this group of substances has been particularly significant in the animal nutrition field [15–17].

This is due to the fact that monogastric animals (such as pigs and poultry) naturally do not have in their digestive system the corresponding enzyme to degrade phytate: phytase [18,19]; this is also the case in human systems [15].

Since phytate cannot be hydrolyzed by this enzyme, P remains fixed and chemically incorporated in the phytate molecular structure. As a result, there is no liberation of available P and its absorption by intestinal tissues is considerably restricted. Hence, supplemental phosphate is required in these animals' diets in order to compensate for this deficiency and to maintain high productivity [20,21].

Owing to the additional phosphate, nonruminant animals excrete substantial P concentrated in manure, which is detrimental in several ways. Financially, it represents an investment loss for the producer. Environmentally, as reported by Correll [22], it can initiate a serious problem in aquatic ecosystems, called eutrophication.

The long-term fertilization of agriculture lands with manure leads to an accumulation of fixed P in the soil, inducing the emergence of P pools. This overload of phosphate compounds infiltrates soil and reaches lakes, rivers, and other aquatic environments. The bacteria present in theses ecosystems consume all the oxygen in the body of water, in order to decompose the oversupply of nutrients. The consequences are critical: the death of aquatic fauna due to the hypoxia (lack

of oxygen) and a considerable reduction in water quality. Such diffuse pollution is now recognized as a serious agriculture risk and several examples of regional-scale deterioration in areas of intensive livestock operations have been taking place (see Fig. 5.2 for more details). To manage this matter, numerous strategies in nutritional manipulation have been tested to improve the ability of these beings to digest phytate; Bohn et al. [23] discussed some of the strategies. For instance, the selection of "low-phytate" concentration mutant grains as a feed and also the development of transgenic monogastric animals that are able to produce phytase.

However, these attempts have not shown themselves to be extremely relevant. It is exactly the present context where phytases insert themselves as a key component and promising solution. The most successful answer found to this issue until now has been the supplementation of animal diets with microbial originated phytase. It became a standard practice in most large-scale animal feeding operations and is even mandated by law in some states of the United States [20]. It has proven to be extremely effective in reducing the excessive P excretion in manure. Moreover, this technique has the added benefit of improving mineral nutrition because the phytase hydrolyzes the metal-complexed-phytate in a dephosphorylation process, releasing those metals in cationic form and making the organic P available for absorption.

Despite the advances in the range and disponibility of information on the use of phytases in animal nutrition, the environmental impacts of manure-derived inositol phosphates and associated dietary manipulations are not fully well understood. Since inositol phosphates can no longer be considered ecologically or environmentally benign, there is an important demand for more study and investigation in areas with the greatest future potential solutions for P soil stabilization: the phytases.

5.2.2 THE USE OF PHYTASE IN ANIMAL'S DIET—A PROMISING ALTERNATIVE

Many recent researches were able to prove that the implementation of the phytase in diets is actually beneficial and may present some satisfying results.

For instance, Rachmawati and Samidjan [24] analyzed the effect of phytase activity in the artificial feed of a kind of shrimp, the giant tiger prawn (*Penaeus monodon*). The enzymatic dose of 1000 FTU/kg of feed significantly affected raw protein and total protein digestibility, nutrient efficiency utilization and relative growth rate. Once the antinutritional effect was inhibited by the degradation of phytic acid by phytase activity, an increase in both diet digestibility and nutrient efficiency growth was observed.

In the same field of study, Hassaan et al. [25] also provided evidence that microbial phytase had significant effects on some nutritional and biological parameters in Nile tilapia (*Oreochromis niloticus*) fish. Both growth performance and weight gain were significantly improved on plant-protein-based diets using 1000 FTU/kg of phytase, which was suitable for blocking the effect of phytic acid

and releasing the chelated protein and minerals. One unit of phytase activity (FTU) is defined as the amount which liberates one micromole of inorganic phosphorus per minute from an excess of sodium phytate at 37°C and pH 5.5 [26].

Furthermore, Menezes-Blackburn et al. [27] demonstrated that inorganic P availability in cattle manure could be increased by the inoculation of a phytase from *Bacillus* sp. MQH-19. In fact, the authors proved that bacteria-inoculated cattle presented higher release of Pi compared with the uninoculated ones; the addition of fresh phytate also increased the Pi release ratio in samples. Also, even after a stabilization of the used manure, there was still a notable amount of organic P available for further enzymatic degradation. In addition, it was stated that not only phytase-producing microorganisms were present in the manure before inoculation, but also the phytase from the used inoculant was more plentiful and/or more efficient in hydrolyzing the freshly added phytate. For this reason, the authors believe that an appearance of small amounts of Pi in manure could naturally be provoked by the activity of native bacteria stimulated by phytate enrichment. Further research is required to improve the efficiency and survival of the inoculated bacterium, and to identify the native microorganisms incited by phytate enrichment.

Calabi-Floody et al. [28] also assessed the effects of phytase in cattle manure, but in this case the authors implemented an immobilization mechanism of an acid phosphatase—a type of phytase—using natural clays and nanoclays as support materials. It was evidently demonstrated that the use of phytase is a valuable and attractive biotechnological alternative for the management of nutrition quality and should be further explored.

5.2.3 IMPLICATIONS FOR HUMAN NUTRITION

With the fast-food culture, the current generation and several others have been following an unhealthy eating lifestyle, which includes a poor diet that may lead to inadequate nutrient intakes. According to the WHO [29] it is estimated that more than 2 billion people (over 30% of world's population) suffer from anemia, which is mostly caused by iron (Fe) deficiency. The same context is valid for zinc (Zn), where a similar contingent of people might be affected. These deficiencies may be a reflection of: (1) a low concentration of the two minerals in commonly consumed foods; (2) a lack of vegetables and meat in the diet; and (3) the presence of inhibitor substances in the iron and zinc uptake process. Being known as the most important antinutritional factor for mineral availability, phytic acid has a strong tendency to readily form complexes with Zn^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, and Fe^{2+} (in decreasing order of stability), seizing and making them unavailable for absorption by the body. Phytases, then again, have the important role of catalyzing the hydrolysis of the former substance and consequently breaking down these mineral complexes, releasing thereby active ligand sites and likewise mineral cations for absorption.

However, in relation to mineral availability in humans, the subjects and consequences of the dietary supplementation of phytate have caused some controversy. On the one hand, some researchers claim that there are risks of Zn and Ca

deficiencies from high-phytate diets, while on the other hand some of them state that these assumptions are exaggerated [30]. Indeed, recent discoveries suggest that free phytate (i.e., phytic acid) might have some benefits in human nutrition. In particular, in the oncology field in medicine, it has been shown that phytate has protective effects against colonic cancer, since it seems to only affect the malignant cells, and not the healthy ones. According to Midorikawa et al. [31], phytate is one of the most promising cancer chemopreventive agents because it does not cause any damage to DNA, and that it acts as an antioxidant and anticancer agent by chelating metals. This factor may partially explain the epidemiological association of high-fiber diets containing elevated phytic acid concentrations, with lower incidences of certain cancers [32].

Besides, there are several other benefits of the use of phytase as an inhibitor of the antinutritional effects of phytate in human nutrition. First, it evidently causes a reduction of phytic acid in the organism, avoiding the formation of insoluble complexes with minerals. Then, it boosts the body's capacity to absorb minerals, increasing thus their bioavailability, especially the vital minerals such as Ca, Mg and Fe. Consequently, there is a significant reduction in mineral deficiency diseases, such as anemia, many of which are due to Fe deficiency. Furthermore, a study concluded that a phytase commonly used for animal nutrition had a promising performance and several applications for human digestion, particularly for human intestinal alkaline phosphatase. This enzyme is extracted from the fungus, *Aspergillus*, and it was proved to have a broad pH range with at least 80% of the maximal activity at pH values, and optimal activity for phytate hydrolysis [30]. The results are important because they offer possibilities to reduce mineral deficiency in vulnerable groups such as child-bearing women, vegans, vegetarians, and people in developing countries.

Added to that, with the use of phytase in human nutrition, there is a remarkable decrease in the chances of antinutritional-substances accumulation in the digestive tract, such as phytic acid and its salts. Phytase releases the available P from the mineral phytate complexes, which as is known to be an essential element for the growth and protection of bone tissues. Therefore, this enzyme activity may also avoid bone loss, improve overall bone strength, and prevent osteoporosis, seeing as it increases the P availability.

5.2.4 PERSPECTIVES

Phytate restricts the path of mineral bioavailability and protein digestibility in human and animal nutrition. However, scientists from all over the world have proved that phytases are an efficient approach to solving this issue. The inclusion of this enzyme in food and the consequent reduction of phytate levels in plant-based feed via genetic engineering have been seen as promising areas. Application of phytases in the food business seems to be a promising strategy, both nutritionally and economically. Phytases are recognized for their ability to reduce the need for supplemental phosphorus in diets, which minimizes the P levels in manure and the

risk of environmental damage. Yet, it is not so simple to find the ideal type of phytase, which can fulfill all requirements and also predict any side effects. Hence, there are still many tests to be conducted to approve the effectiveness of phytase in each particular kind of situation. For the development of science, researchers should invest in some still not completely understood subjects, such as isolating novel and best phytate-hydrolyzing enzyme microorganisms and optimizing their catalytic features, thermal tolerance, and specific activity via genetic engineering to generate an ideal phytase for food application [29].

Section 5.3 (Biocatalysis) presents the action of phytases, classification, sources, and recent applications.

5.3 BIOCATALYSIS

Phytases are enzymes responsible for catalyzing the hydrolysis of phytic acid in different positions of the inositol ring and releasing phosphorus, zinc, and other minerals in inorganic form, resulting in loss of ability of that acid to chelate metal ions to form complexes, which in turn increases the absorption of minerals by living organisms [33–35]. For further details, see Fig. 5.1.

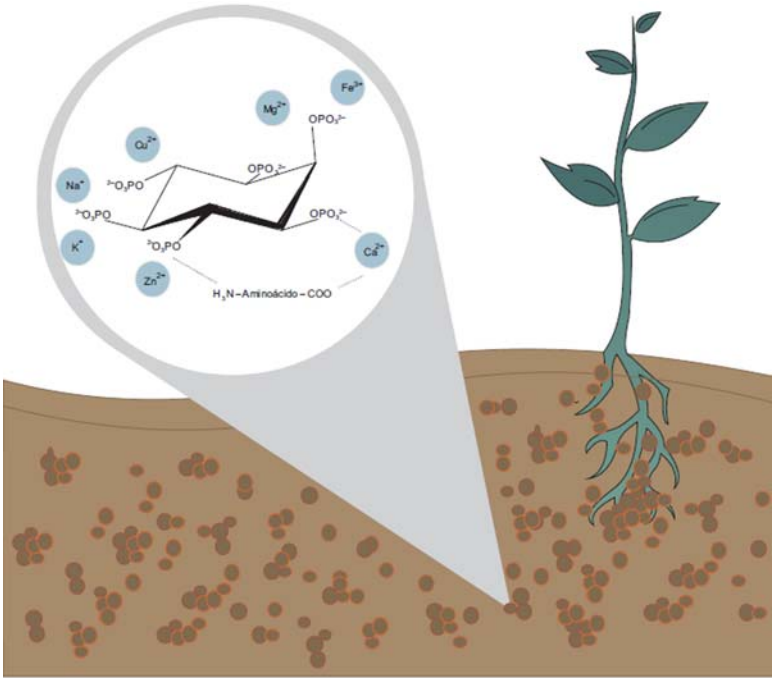

FIGURE 5.1

Phytase and soil biofertilization/stabilization.

The International Union of Biochemistry and Molecular Biology (IUBMB) [36], classifies the phytases according to how they start dephosphorylation of phytic acid at different positions of the inositol ring. The IUBMB currently list three distinct classes of phytases: 3-phytase (EC 3.1.3.8), which hydrolyzes the ester link myo-inositol hexakisphosphate in the third position, releasing 1D-myo-inositol 1,2,4,5,6-pentakisphosphate and phosphate; 4-phytase (EC 3.1.3.26), which in turn hydrolyzes the fourth position of this acid, releasing 1D-myo-inositol 1,2,3,5,6-pentakisphosphate and phosphate; and 5-phytase (EC 3.1.3.72), hydrolyzing the fifth position, releasing 1L-*myo*-inositol 1,2,3,4,6-pentakisphosphate and phosphate.

Several sources of phytase has been studied in recent years, including: bacteria [37], yeast [38], fungi [39,40], grain [41], animals [42], and plants [43,44], among others.

Phytases have been studied as a way to increase the power of several living beings to reduce the need for later nutritional implementation due to unavailability of minerals already present in the food. Searches related to the supplemental diets of many living beings with this enzyme have been made, e.g., humans [37,45], ruminants [33], pigs [46,47], chickens [33,48,49], turkeys [19], fishes [50,51], salmons [52], shrimps [24] and beans [53].

Studies have been performed to develop new application forms for phytases in bread formulation for higher digestibility of minerals [54–56], in animal feed, to aid in growing species, to reduce phosphorus levels in the soil, and to reduce environmental pollutions [27,37]. Studies on the metal ion content changes in barley malt production with the addition of phytase [57], treatment with phytase in the raw material of beer and agroethanol have been reported [58]. Phytase was also used to treat organic material to increase the availability and to reduce the precipitation of salts in agrogas production [59], to reduce the phytic acid and its salts in ethanol [60], and in the improvement of iron absorption in human diets based on cereals [61].

The interaction of this enzyme with the ground is also very important because large amount of phosphate inositol are present, this may impoverish the soil and any crops grown on it. Studies conducted by Menezes-Blackburn et al. [62] have used phytase in soil fertilization, while Naz et al. [63] studied the P concentration available in several soil profiles with or without phytase produced by *A. niger*, and found that high free phosphate was observed in soil supplemented with phytase. The application of phytase in soil stabilization will be described in Section 5.4 of this chapter.

5.4 SOIL STABILIZATION

5.4.1 INTRODUCTION

Phytases play an important role in soil stabilization due to their capability to enhance the bioavailability of phosphate. This specific group of enzymes degrades

the phytic acid during seed germination. Phytases catalyze the stepwise hydrolysis of phytic acid through a series of reactions producing orthophosphate (which contains the Pi) and lower phosphate esters of myo-inositol [64]. Once dephosphorylated, phytate no longer holds the P present in its chemical structure, making it bioavailable to plants and soils systems. Consequently, it loses its capability to complex with other nutrients such as divalent minerals, starch, and proteins. Therefore, phytases are a key component and central subject of research in the soil stabilization context.

Given the importance of soil P for plant nutrition in both natural an agricultural ecosystems, there is an urgent need to develop procedures that provide accurate information for the stabilization of organic P compounds in soils.

Phosphate is an essential macronutrient for all living organisms, whether in animal nutrition, in plant growth, or in livestock production. In natural ecosystems, phosphorus is returned to the soil and converted to inorganic phosphate (Pi) via biological and chemical processes, after which it is available for a new cycle of plant growth. It is due to the great importance of Pi as a macronutrient for the development of agriculture and the ability of phosphate compounds to bind minerals, that there is an increasing interest in understanding the factors that underlie phosphate uptake and bioavailability in soil stabilization [64].

Phytic acid or it salts, phytates, are introduced into soil through plants residues, animal manure fertilization, organic enrichment, and bioremediation. About 51 million tons of phytate is found every year in commercially produced fruits and crop seeds, representing about 67% of the annual worldwide phosphate application in fertilizers. Phytate is not only bound to inorganic surfaces in soil, but can also be bound, similar to orthophosphate, to humic surfaces via Fe or Al bridges. Humic-metal-phytate complexes may be transported in the soil solution to the roots where hydrolysis and uptake of the liberated P may occur [65]. Great interest is being shown in manipulating phytic acid content in grains [66].

Naturally, phytic acid and its corresponding enzyme, phytase, form a cycle in the soil of agricultural ecosystems (Fig. 5.2): (a) first, the available Pi is assimilated by plant roots from the soil's solution and is distributed to all the other parts of the plant, making possible the appropriate growth and development to maturity (b), where 75% of the entire absorbed amount of Pi is incorporated and stored as phytic acid in the embryo and aleurone parts of the grain. When crops have developed for long enough, the agriculture harvest (c) takes place and provides the mature seeds. These seeds can be used either for agricultural cultivation or for the production of feed for livestock. If employed in crop production (d), they propagate the seed germination and the following plantlet formation (e), closing the natural cycle. On the other hand, the cereal seeds may also be used for the feed of nonruminant animals (f), causing the excretion of large quantities of undigested phytic acid due to the lack of phytases in theses animal's digestive systems. In that case, the resulting manure, which contains high amounts of mainly undigested phytic acid (Pi), is used to fertilize land (g). Because monogastric animals do not naturally have the phytase in their

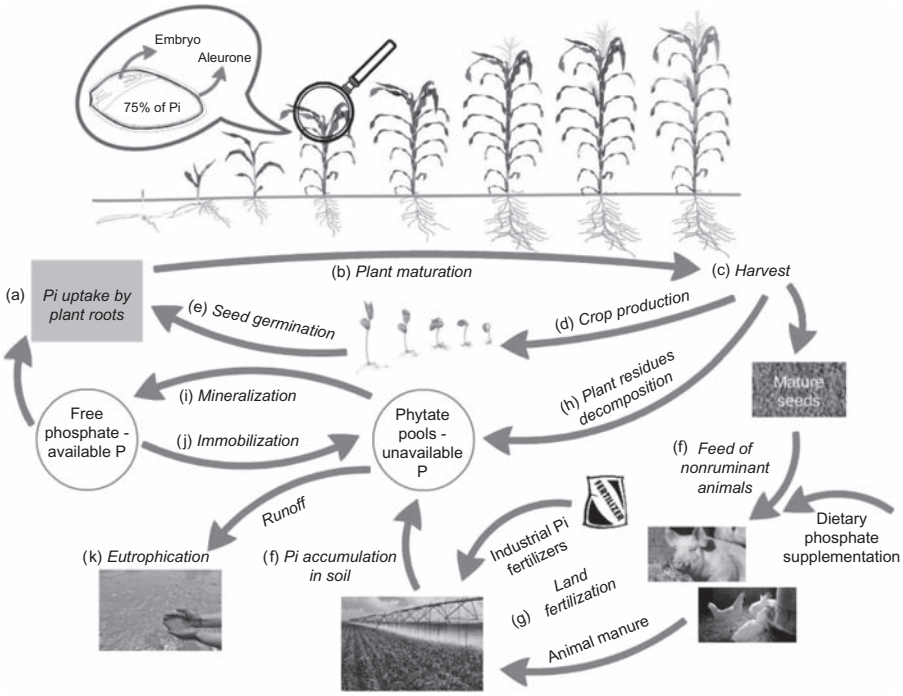

FIGURE 5.2

Cycle of phosphorus (P) in agricultural ecosystems.

digestive system to degrade the phytate and release the available P, a dietary phosphate supplementation is required. Added to that, there is an intense use of industrially produced Pi-fertilizers to ensure agriculture productivity. Hence, more than 80% of the P supplied by fertilizers becomes immobilized in the soil, making it unavailable. Furthermore, the decomposition of plant material residues (h) also contributes to the strong accumulation of this macronutrient in soil, forming a kind of fixed P pools [64]. This immobilization is due to the fact that only a limited amount of the soil Pi is available to the plant owing to its low mobility and high affinity to bind itself with other organic and inorganic compounds, as well as solid particles present in soil. This overload of Pi is not common to the ecosystem and it can follow two different paths: either they accumulate in soil causing a serious environmental problem called eutrophication (k); or they go through mineralization, where they are chemically processed by plants and microbial phytase activity (i), releasing the available Pi from phytate in the phosphate form. The inverse mechanism is called immobilization (i). In the last case where Pi is free, the bigger cycle completes itself, making the absorption of this nutrient by plant roots possible once again.

5.4.2 INFLUENCING FACTORS

The specific reasons for the accumulation of P are not fully clear and understood, and still require a large of research. The biological origin of phytase in soil is varied and phytase activity decreases by the wide range of competing biological, chemical, and physical processes before its interaction with inositol phosphates, either within the solution or the boundary with the solid phase.

Thus, according to George et al. [67], four main factors affect the stability and efficacy of extracellular enzymes in soils. These include: (1) proteinase and microbial-mediated degradation; (2) partial or total deactivation by adsorption onto soil solid particles; (3) deactivation via interaction with polyvalent anions, metal ions, and metabolites; (4) denaturation by soil environmental factors (temperature, pH, water content, light).

In the first hypothesis (1), it is believed that low phytase activity is responsible for the unavailability of Pi to the plant roots, having not hydrolyzed the remaining phytate. However, according to Gerke [65], the second hypothesis (2) that phytate creates strong bonds and chemical complexes with soil particles. This second hypothesis may further limit the acquisition of Pi acquisition (1). Indeed, phytate is strongly bonded and the P sorption is maximum and probably the sorption strength of phytate P to the soil solid phase is much higher, compared to that of orthophosphate P. Considering the results on sorption, immobilization, and fixation of phytate to the soil solid phase reported in the last 20 years, it is possible to state that lack of enzymatic activity of phytases (1) is not a limiting step for the Pi acquisition of higher plants from soil phytate [68]. Thus, regardless of whether extracellular phytases are inhibited by adsorption, immobilizations to the solid phase will presumably limit their interaction with inositol phosphates in soil, particularly if these substrates are also immobilized through adsorption and precipitation reactions [67]. These authors showed that a greater amount of phytase activity was observed in rhizosphere soil solution compared to bulk soil. The changed pH and presence of organic anions and proteins are important influencing factors and, moreover, they represent strong competitors for adsorption sites. Changes in pH might also change the mechanism of adsorption reactions of phytases in soil. They attested that phytases with low pH (isoelectric point) are nearly twice as effective in the degradation mechanism of inositol phosphates in soil solution. It is also clear that the particular biochemical characteristics of phytases originated by different organisms have apparent effects in the chemical conduct in soil environments and consequently for mineralization of inositol phosphates. Hence, increasing pH in acidic soils could be a useful management tool for making phytases more effective in the soil catalysis and environment.

5.4.3 PERSPECTIVES

Increased knowledge of phytic acid biosynthesis, deposition, and degradation, combined with further studies on phytase heat stability and catalytic properties,

should provide biotechnology science with new horizons for improving phosphate quality and mineral uptake in soil stabilization. The discharge of phytases from the roots of plants might prove to be an essential strategy for mobilizing phosphate reserves in the soil. Emergent work will focus on expressing genes in plants for phytase expression with biochemical traits that effect their interaction with soil, such as low pH. This will allow better understanding of the factors that contribute to the biological availability of inositol phosphates in soil and potentially improve the ability of plants to acquire P from this source.

Since the mechanisms by which phytate accumulates in soil as well as the mechanisms P phytate is acquired by plants are not fully understood, Gerke [65] stated that the accumulation of several phosphate esters, including phytate, is related to the strength of bonding to the solid phase. Phytate represents about 45% of the organic P in soil. Commonly, the association between phytate and soil humic substances has an important role; therefore most of the inositol phosphate in soil stands in the high molecular mass fraction. According to the author, the acquisition of P-phytate may show more favorable results by roots exudate, which may increase the solubility of the phytate in the soil solution. Moreover, the excretion of citrate and oxalate by plant roots might also have interesting properties for improving the phytate solubility and consequently its acquisition, making it a promising area for future experimental studies.

For instance, Yan et al. [69] studied the surface complexation and precipitation reactions of phytate on soil minerals. The study indicated that active metal oxides such as amorphous aluminum hydroxide strongly mediate the speciation and behavior of phytate via rapid surface complexation−precipitation reactions, thus controlling the mobility and bioavailability of inositol phosphates in the environment.

Giaveno et al. [70] showed that phytases have a strong affinity for the mineral surfaces, with loss of enzyme activity being highly dependent on the type of mineral. The bioavailability of P-phytate, and therefore the strength of inositol phosphate binding, is mainly governed by the mineral composition of the soil. Rather than the persistence of phytase activity, the previous hypothesis corroborates to the view that retention of inositol phosphates by soil minerals may prevent their degradation.

Despite the advances made until now, there is still a lack of information on some major subjects, such as [71]: (1) quantitative data relating to the release of orthophosphate from different organic P species under field conditions and in a time-frame consistent with the needs of pasture plants; (2) the transition of organic P substrates and phosphatase enzymes from their point of origin, be that the soil matrix or an organism, to the site where decomposition and mineralization actually occurs; (3) the role of the microbial biomass and specific microorganisms in the organic P mineralization process.

All this knowledge will be indispensable for a change in the fertilizer utilization by land managers. With further arguments and real proofs, they will be more assured that accessing organic P can actually contribute to pasture nutrition without negative side effects related to P loss. Consequently, a decreasing application of industrially produced P fertilizers is intended.

Combining new technologies and further scientific discoveries, we will be offered the possibility to understand both the chemistry and biology of the decomposition and mineralization of organic P in soil. These upcoming data could be used to generate innovative hypotheses which will subsequently be tested in model systems. This would be particularly useful in structured experiments where through, e.g., cultivation and the application of organic amendments, a range of soil conditions could be established at one site, minimizing between-plot variation that is attributable to climate, animal management, or soil type [71]. Given the current importance of P to the soil cycle and realizing that it might be a key component in agricultural sustainability, it is believed that there is a compelling argument for a deeper and more innovative and integrated approach to investigating organic P mechanisms in soil stabilization.

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PART

Depolymerizating Enzymes

II

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CHAPTER

Depolymerizating enzymes—cellulases

6

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6.1 INTRODUCTION

Polymer degradation is mainly caused by chemical bond scission reactions in macromolecules. For practical reasons it is useful to subdivide polymer degradation according to its various modes of initiation: thermal, mechanical, photochemical, radiation chemical, biological, and chemical degradation of polymeric materials. Depolimerization is the process that causes changes in macromolecules and plastics, by polymer chain decomposition until monomers or oligomers which proceed at high temperature or with hydrolytic agents.

6.2 CELLULASES

The hydrolysis of cellulose will have in the future a very important economic role and cellulose will be one of the more useful raw materials. That is already the case in the conversion of some lignocellulosic mass into bioethanol [1]. Numerous experimental and review papers have been published on this subject [2,3].

Cellulose is the main vegetable cell wall polysaccharide component. It is the Earth's more abundant organic material and is composed of glucose monomer in links of 1−4-beta chains [4]. For example, in rice straw, yam, cassava, and banana peels, cellulose and hemicellulose represent 75%−80%, while lignin constitutes only 14% [5]. Most of the various aspects of cellulose are considered in view of its lack of toxicity on the one hand and its recalcitrant durable nature on the other [6].

The reducing fossil energy supply, pollution concerns, and economic reasons have stimulated the interest in lignocellulose as a source of fuel. For fuel production, the skeletons of plant cell walls should be hydrolyzed into sugar monomers for fermentation by selected enzymes produced by fungi and yeasts first and then converted into ethanol. Cellulases are the dominant enzymes in lignocellulose depolimerization allowing the release of cellobiose and glucose. These enzymes are also largely applied in detergents, textile and paper industries, food processing, and on a much smaller scale in human and animal nutrition. Cellulases have also been

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used for the improvement of soil quality, allowing increased availability of nutrients for plant growth. Traditionally, straw incorporation is considered an important strategy to improve soil quality and reduce dependence on mineral fertilizers. Exogenous cellulase supplementation to the soil accelerates decomposition of cellulose and therefore the enzyme may be a potential lead to increasing soil fertility. Often in combination with amylase, glucanase, xylanase, and hemicellulose, cellulases are used foremost in the production of fruit juices and other beverages, and in the manufacture of wine, spirits, and alcoholic drinks. As mentioned, cellulases are strongly used in the production of bioethanol as well as washing and cleaning agents, textile production and the paper industry, and as food and feed additives.

Detection of microbial enzymes in natural environments is important for understanding biochemical activities and to verify the biotechnological potential of the microorganisms. A total of 89.6% of isolates from soil, water, and plants produce at least one of the following enzymes: caseinase, gelatinase, amylase, carboxymethyl cellulase, and esterase. There is a predominance of amylase in soil samples, carboxymethyl cellulase in plants, as well as esterase and gelatinase in water [7].

A procedure utilizing cellulase allows rapid and highly reproducible quantitative and complete isolation of protoplasts from carrot suspension culture. Such protoplasts are highly suitable for biochemical studies [8].

The area of bioethanol production is the largest, and research on cellulases, including cellulosome(s), has seen a remarkable development. However, inexpensive commercial production of cellulases still demands strong research efforts for an integrated process such as cellulase production and cellulose-mediated saccharification of biomass in conjunction with the fermentation of released sugars into ethanol in a single step known as consolidated bioprocessing (CBP) [9].

Cellulases also play a primary role in food biotechnology. Macerating enzyme complexes (cellulases, xylanases, and pectinases) are currently used in several agroindustries. They can improve cloud stability and texture, and reduce viscosity of the final products, particularly from tropical fruits. Also, the texture, flavor, and aroma properties of fruits and vegetables improve. A mixture of cellulases, hemicellulases, and pectinases can also be used to obtain olive oil [10]. Cellulase is also useful for therapy of phytobezoars [11].

Waste copier paper is a potential substrate for the production of glucose relevant for manufacture of platform chemicals and intermediates, being composed of 51% glucan. From all cellulase preparations examined, maximum hydrolysis was only achieved with the addition of beta-glucosidase, despite its presence in the enzyme mixtures.

6.3 BETA-GLUCOSIDASES

Thermophilic enzymes are ideal biocatalysts for modern biotechnology because of their thermostability [12], and better yields under extreme operational

conditions. Potential benefits in the hydrolysis of lignocellulosic substrates can be achieved with thermostable cellulases. Higher specific activity decreasing the amount of enzymes, enhanced stability allowing improved hydrolysis performance, and increased flexibility with respect to process configurations, all lead to improvements in the overall economy of the process [13]. The wide range of potential applications of thermophilic cellulases includes food, animal feed, textiles, fuel, chemical industries, paper and pulp industries, waste management, medical/pharmaceutical industries, protoplast production, genetic engineering, and pollution treatment. For example, thermophilic cellulases already play a primary role in food biotechnology. *Sclerotinia sclerotiorum*, a filamentous fungus, produces a complete set of cellulolytic enzymes needed for efficient solubilization of native cellulose [14].

Removal of enzyme inhibitors released during lignocellulose pretreatment is essential for an economically feasible saccharification process. Bioabatement yields improve enzyme hydrolysis similar to that obtained using a chemical (overliming) method for mitigating inhibitors [15]. Solids' loading in enzymatic hydrolysis of lignocellulose is an important factor affecting sugar yield. For example, the increasing concentration of glucose and lignophenolics mainly inhibited the enzymatic hydrolysis of aqueous-ammonia-pretreated corn stover [16].

Mechanistically, cellulase is a family of at least three groups of enzymes, endo-(1,4)-β-D-glucanase (EC 3.2.1.4) exo-(1,4)-β-D-glucanase (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21). The exoglucanase (CBH) acts on the ends of the cellulose chain and releases β-cellobiose as the end product; endoglucanase (EG) randomly attacks the internal *O*-glycosidic bonds, resulting in glucan chains of different lengths; and the β-glycosidases act specifically on the β-cellobiose disaccharides and produce glucose. Cellulosomes located on the cell surface mediate adherence of anaerobic cellulolytic bacteria to the substrate, which thereafter undergo a supramolecular reorganization, so that the cellulosomal subunits redistribute to interact with the different target substrates.

Anaerobic fungi harbor a rich reservoir of undiscovered cellulolytic enzymes and enzyme complexes that can potentially transform the conversion of lignocellulose into bioenergy and biochemical products. *Bacillus amyloliquefaciens* 1067 DSMZ, *Bacillus megaterium* 9885 ATCC, *Paenibacillus curdlanolyticus* 10248 DSMZ, and *Paenibacillus polymyxa* 842 ATCC produce higher enzyme activities as compared to other bacterial cultures grown on different substrates [17].

Research on regulation of cellulases and hemicellulases gene expression may be very useful for increasing the production of these enzymes in their native producers. Production of many of these enzymes is coordinately regulated, and induced in the presence of the substrate polymers. In addition to induction by mono- and oligosaccharides, genes encoding hydrolytic enzymes involved in plant cell wall deconstruction in filamentous fungi can be repressed during growth in the presence of easily metabolizable carbon sources, such as glucose. Carbon catabolite repression is an important mechanism to repress the production of plant cell wall-degrading enzymes during growth on preferred carbon sources. Amore

et al. [18] reviewed the recent advancements in elucidation of molecular mechanisms responsible for regulation of expression of cellulase and hemicellulase genes in fungi. Most of the bacteria that encode cellulases are soil and marine saprophytes, many of which encode a range of enzymes for cellulose hydrolysis and also for the breakdown of the other constituents of plant cell walls such as hemicelluloses and pectins. Surprisingly, cellulases are present in pathogenic organisms that are usually considered as nonsaprophytic, such as *Mycobacterium tuberculosis*, *Legionella pneumophila*, *Yersinia pestis*, and *Escherichia coli* [19].

Phasmid cellulases showed high homology to endogenously produced glycoside hydrolase family 9 (GH9) endoglucanases (EG) from insects, especially to those of termites, cockroaches, and crickets [20].

6.4 CELLULOSE–LIGNIN COMPLEX

In plants, cellulose is generally associated with lignin and the complex is called lignocellulose [21]. Lignin is the most abundant renewable aromatic polymer on Earth and is composed of nonphenolic (80%–90%) and phenolic structures. It is essentially a polymerized aromatic substance, very resistant to hydrolysis. In wood, it represents 20%–30% of the plant [22].

Lignin is degraded by secreting enzymes collectively termed "ligninases" of two ligninolytic families: phenol oxidase (laccase), peroxidases, and manganese peroxidase (LiP and MnP, respectively). Basidiomycetes *Coriolus versicolor*, *Phanerochaete chrysosporium*, and *Tinea versicolor* are the most efficient lignin-degrading microorganisms known. Due to their size, oxidative ligninolytic enzymes are not able to penetrate the cell walls. This means that prior to the enzymatic attack, changes in the lignin structure should be initiated by low-molecular weight diffusible reactive oxidative compounds [23]. Pretreatment of lignocellulosic materials to remove lignin and hemicellulose can significantly enhance the hydrolysis of cellulose as well as the optimization of the cellulase enzymes and the enzyme loading. For ethanol production, simultaneous saccharification and fermentation removes glucose, which is an inhibitor to cellulase activity. Accordingly, the yield and rate of cellulose hydrolysis will be increased [24].

The combination of cellulolytic, hemicellulolytic, and pectinolytic activities works synergistically on the recalcitrant lignocellulose complex of sugar beet pulp (SBP) composite which is difficult to degrade. It is important to achieve a high degree of enzymatic SBP hydrolysis with a low enzyme load [25].

6.5 CELLULOSOMES

Most anaerobic microorganisms produce large multienzyme complexes called cellulosomes, which are usually attached to the outer surface of the microorganism.

The intriguing properties of such complexes include their cohesive nature, their many enzymatic components, and a characteristic glycosylated cellulose-binding, "scaffolding" component. The latter appears to serve as a substrate-targeting carrier, which delivers the other (hydrolytic) components to the cellulose. Progress in establishing efficient model systems for in vitro solubilization of purified cellulose or natural cellulosic substrates has been achieved using purified cellulosome preparations, fortified with beta-glucosidase and pectinase. The latter enzymes were required in order to alleviate the phenomenon of product inhibition which reduces the efficiency of the free cellulosome. Such combined enzyme systems are proposed as examples of future tailor-made cellulolytic systems for the degradation of natural cellulosic materials [26]. Cellulosomes are firmly bound to the bacterial cell wall but flexible enough to also bind tightly to microcrystalline cellulose [27].

These bacterial cellulase systems are much more complicated than the structurally fungal cellulases. Integration of cellulosomal components occurs via highly ordered protein−protein interactions between cohesins and dockerins [28]. Cellulosomes and free cellulase differ by the cohesion-containing scaffolding and dockerin-containing enzyme. The cellulosome consists of a multifunctional integrating subunit (scaffoldin), responsible for organizing the various cellulolytic subunits (e.g., the enzymes) into the complex [29].

The scaffoldin contains one or more cohesin units linked to other functional modules. In scaffoldin, these modules include a cellulose-specific carbohydrate-binding module (CBM), a dockerin, and others. The overall architecture of the cellulosoma depends on the arrangement of the scaffoldin modules and the specificity of the cohesin and/or dockerin [30].

Much of our understanding of its catalytic components, architecture, and mechanisms of attachment to the bacterial cell and to cellulose, has been derived from the studies of *Clostridium thermocellum*, a microorganism in which the cellulosoma complex has been identified [31]. Known bacteria producing cellulosome are quite numerous: *Acetivibrio cellulolyticus*, *Bacteroides cellulosolvens*, *Clostridium cellulolyticum*, *C. thermocellum*, *Ruminococcus flavefaciens*, *Clostridium cellulovorans*, etc. [32]. The architecture of cellulosomes is similar among these species.

Cellulolytic bacteria and fungi have been shown to use two different approaches to degrade cellulose. Most aerobic microbes secrete sets of individual cellulases, many of which contain a CBM, which act synergistically on native cellulose. Most of the cellulosomal enzymes lack a CBM, but the cohesin subunit, to which they are bound, does contain a CBM. The cellulases present in each class show considerable overlap in their catalytic domains, and processive cellulases (exocellulases and processive endocellulases) are the most abundant components of both the sets of free enzymes and of the cellulosomal cellulases. For example, analysis of the genomic sequences of two cellulolytic bacteria, *Cytophaga hutchinsonii*, an aerobe, and *Fibrobacter succinogenes*, an anaerobe, suggest that these organisms must use a third mechanism. This is because neither of these

organisms, encodes processive cellulases and most of their many endocellulase genes do not encode CBMs. Furthermore, neither organism appears to encode the dockerin and cohesin domains that are key components of cellulosomes.

Thricoderma reesi is the more utilized microorganism for cellulase production [33]. A low-cost of cellulose obtained by any technological method remains a key requirement for producing cellulosic biochemicals and biofuels.

The truly cellulolytic bacteria are extremely efficient hydrolyzers of plant cell wall polysaccharides, especially those in thermophilic anaerobic ecosystems. *Clostridium stercorarium*, a thermophilic ubiquitous soil dweller, has a simple cellulose hydrolyzing enzyme system of only two cellulases. However, it seems to be better suited to the hydrolysis of a wide range of hemicelluloses. *C. thermocellum*, a ubiquitous thermophilic gram-type positive bacterium, is one of the most successful cellulose degraders known by their cellulosome.

Whether cellulosomal systems display greater ability to hydrolyze complex biomass materials (e.g., plant cell walls) than nonaggregated enzymes, and in so doing would be appropriate for improved, commercial bioconversion processes, is under discussion. To sufficiently understand the complex macromolecular processes between plant cell wall polymers, cellulolytic microbes, and their secreted enzymes, a highly concerted research approach is required and adaptation of existing biophysical techniques and development of new science tools must be applied to this system [34].

The assembly of proteins that display complementary activities into supramolecular intra- and extracellular complexes is central to cellular function. One such nanomachine of considerable biological and industrial significance is cellulosome. The *C. thermocellum* cellulosome assembles through the interaction of a type I dockerin module in the catalytic entities with one of several type I cohesin modules in the noncatalytic scaffolding protein. The *C. thermocellum* cellulosome is a complex composed of at least 14–18 different types of components organized around a large, cellulose-binding protein [34a]. More recent structural studies have provided the molecular details of how dockerin–cohesin interactions mediate both cellulosome assembly and the retention of the protein complex on the bacterial cell surface. The type I dockerin, which displays near-perfect sequence and structural symmetry, interacts with its cohesin partner through a dual binding mode in which either the N- or C-terminal helix dominate heterodimer formation. The biological significance of this dual binding mode is the plasticity of the orientation of the catalytic subunits within this supramolecular assembly. The flexibility in the quaternary structure of the cellulosome may reflect the challenges presented by the degradation of a heterogeneous recalcitrant insoluble substrate by an intricate macromolecular complex, in which the essential synergy between the catalytic subunits is a key feature of cellulosome function [35].

An obligatory step in cellulose degradation by anaerobic bacteria is the adhesion of the bacterium to the polysaccharide. As already mentioned, in many anaerobic bacteria the adhesion protein, and the enzymes required for extensive polysaccharide hydrolysis, are organized in the cellulosome complex structure. The Gram-positive anaerobe *Ruminococcus albus* also produces a

cellulosome-like complex. Nevertheless, the bacterium appears to possess other mechanism(s) for adhesion to plant surfaces and genes encoding functions relevant to growth on cellulose are conditionally expressed. A novel form of cellulose-binding protein has been identified and shown to belong to the Pil-protein family, being most similar to the type 4 fimbrial proteins of Gram-negative, pathogenic bacteria. These studies have provided new insights into the adhesion of bacteria to plant surfaces, and call attention to the likely existence of genetically analogous adhesion determinants in both pathogenic and nonpathogenic bacteria [36].

The cohesin−dockerin interaction provides the basis for incorporation of the individual enzymatic subunits into the cellulosome complex. By site-directed mutagenesis, Mechaly et al. [37] found that four amino acid residues of the approximately 70-residue dockerin domain play a role in biorecognition, while additional residues may also contribute to the specificity of the interaction.

The adhesion process of the predominant cellulolytic rumen bacteria *F. succinogenes*, *R. flavefaciens*, and *R. albus* has been divided into four phases by Miron et al. [38]: (1) transport of the nonmotile bacteria to the substrate; (2) initial non-specific adhesion of bacteria to unprotected sites of the substrate that is dominated by constitutive elements of bacterial glycocalyx; (3) specific adhesion via adhesins or ligands formation with the substrate, which can be dominated by several bacterial organelles including cellulosome complexes, fimbriae connections, glycosylated epitopes of CBP or glycocalyx, and cellulose-binding domain of enzymes; and (4) proliferation of the attached bacteria on potentially digestible tissues of the substrate. Each of the phases and its significance in the adhesion process are described. The authors described as factors affecting bacterial adhesion: (1) those related to bacterial age, glycocalyx condition, and microbial competition; (2) factors related to the nature of substrate including, cuticle protection, surface area, hydration, and ionic charge; and (3) environmental factors including pH, temperature, and presence of cations and soluble carbohydrate. Each of the mentioned predominant rumen bacteria has a specific mechanism of adhesion to cellulose.

Cellodextrin transporters (cellodextrin permeases) have been identified recently in fungi. The functions of these transporters in cellulose utilization and cellulose expression suggest a crucial function of cellodextrin transporters in the induction of cellulose expression in *Penicillium oxalicum* by insoluble cellulose [39].

The affinity digestion process for cellulose purification consisting of binding to amorphous cellulose, and amorphous cellulose hydrolysis in the presence of dialysis has been optimized to obtain high-activity recoveries and consistent protein recoveries in the isolation of *C. thermocellum* cellulase [40].

6.6 CELLULASES FROM EXTREMOPHILE MICROORGANISMS

Many hyperthermophilic microorganisms utilize several carbohydrates as potential materials for the conversion of lignocellulosic biomass to bioethanol. Utilized

substrates by hyperthermophilic marine archaea are alpha- and beta-linked glucans (starch, barley glucan, laminarin, and chitin), while hyperthermophilic marine bacteria also utilize hemicellulose (xylans and mannans). Crystalline cellulose is not a substrate for these microorganisms. Among the terrestrial thermophiles, this capacity is limited to a few species. Cellulose hydrolysis is done by primary cellulases, which are different from those associated with the large multienzyme complexes, cellulosomes. Many extremophile catalyzers can be identified in genome sequences and their characterization will allow new opportunities of practical utilization [41].

A recently discovered thermophilic isolate, *Geobacillus* sp. R7, was shown to produce a thermostable cellulase with a high hydrolytic potential when grown on extrusion-pretreated agricultural residues such as corn stover and prairie cord grass. The hydrolytic capabilities of *Geobacillus* sp. R7cellulase are comparable to those of a commercial cellulase. Fermentation of the enzymatic hydrolyzates with *Saccharomyces cerevisiae* ATCC 24860 produced ethanol yields of 0.45−0.50 g ethanol/g glucose with more than 99% glucose utilization. Also, it has been demonstrated that *Geobacillus* sp. R7 can ferment the lignocellulosic substrates to ethanol in a single step that could facilitate the development of a CBP as an alternative approach for bioethanol production with outstanding potential for cost reductions [42].

To produce a wide variety of cellulases and hemicellulases, both fungi and bacteria have been heavily exploited. However, the focus has been more towards the fungi because of their capacity to excrete abundant amounts of noncomplex cellulases and hemicellulases. Recently, this trend has shifted towards the bacteria, due to their higher growth rates, presence of more multienzyme complexes, and their presence in wide variety of environmental niches. Not only can these bacteria survive the harsh conditions, but they often produce stable enzymes which may increase rates of bioconversion processes. The cell membranes of thermophiles contain saturated fatty acids which provide a hydrophobic environment for the cell and maintain the cell rigidity at elevated temperatures. Further, the hyperthermophilic archaea have lipids linked with ether on the cell wall and formation of a monolayer rather than bi-layer has been suggested for stability of membranes at high temperatures. Recently, tetraether membrane lipids were reported in a thermoacidophilic euryarchaeota *Candidatus aciduliprofundum boonei* from deep sea hydrothermal vents. In addition to the structural adaptations of cell wall and cell membrane, DNA of thermophiles contains reverse DNA gyrase, which enhances the melting point by producing positive super coils in the DNA imparting temperature stability. In *Sulfolobus solfataricus* a small DNA binding protein, Sso7d, not only imparts thermostability to the DNA but also promotes the annealing of complementary strands above the melting point and the ATPase-dependent rescue of the aggregated proteins. Thermophiles are reported to have a zigzag structure of surface layer proteins which are thermostable and resist denaturation and proteolysis. Certain specialized proteins, known as “chaperons,” are produced by these organisms, which help to refold the proteins to their native form and restore their functions.

Besides the above strategies, thermophilic bacteria, actinomycetes, and archaea tolerate high temperatures by increased electrostatic, disulfide, and hydrophobic interactions in their proteins. Certain thermophilic enzymes are stabilized by certain conformational changes. However, certain metals, inorganic salts, and substrate molecules are also reported to impart the thermostability. Based on the thermal behavior of these enzymes, the Equilibrium Model has been described to reveal the effect of temperature on enzyme activity by reversible active−inactive transition states. Due to the increasing demand for highly thermostable industrial enzymes, certain computation and bioinformatic tools have been designed, which can predict protein rigidity and stability. Protein stabilization can be carried out by site-directed mutagenesis, and genes shuffling. The fungus *Hypocrea jecorina* (anamorph *Trichoderma reesei*) produces a complete set of cellulases classified as cellobiohydrolases (CBH), EG, and ß-glucosidases.

The cellulolytic fungus *Trichoderma* sp. was and is still considered as the best source of cellulases, however, the major bottleneck with *Trichoderma* cellulases is that they produce very low β-glucosidase activity in culture supernatants and the enzyme is subject to product inhibition. Some species of thermophilic fungi degrade cellulose rapidly but their culture filtrates have low cellulase activity. This was contradicted by reports that the thermophilic fungi *Sporotrichum thermophile* and *Talaromyces emersonii* produced cellulase activity almost comparable to that of the mesophilic fungus *H. jecorina* (anamorph *T. reesei*). Characterization of *H. jecorina* (anamorph *T. reesei*) cellulase and xylanase promoters has recently been evaluated.

Cellulolytic rates of some thermophilic fungi, *Chaetomium thermophile*, *S. thermophile*, and *Thermoascus aurantiacus* has been observed to be two or three times greater than that of *Trichoderma viride*. Carboxymethyl cellulose activity of *Paenibacillus tarimensis* is very high in the pH range 3.0−10.5 and 9 mM to 5 M NaCl. Their cellulases, stable and highly functional under harsh conditions, appear to be promising candidates for application in detergents, textiles, paper/pulp industries, as well as treatment for saccharification of lignocellulose [43].

In nature, fungi tend to produce more cellulases than bacteria, however, cellulases produced by bacteria are better catalysts as they encounter less feedback inhibition. Moreover, bacteria grow rapidly compared to fungi, allowing for higher recombinant production of enzymes. The capacity of bacteria inhabiting wide variety of environmental niches such as high temperature, alkaline pH, and acidic pH help these strains to produce biocatalysts which are stable under harsh conditions found in the bioconversion processes leading to increased rates of hydrolysis and finally efficient product recovery. The bacterial cellulases have very high activities against crystalline celluloses such as cotton or avicel and are also more thermostable and are able to perform in an alkaline pH in comparison to fungal cellulases. The cellulolytic property has been reported in *Bacillus* strains and in thermophilic anaerobic bacterium *C. thermocellum*.

In the characterization in detail of 21 cellulose-degrading enzymes derived from a thermophilic microbial community it was found that 70% of them were

[C2mim][OAc]-tolerant. A comparison of optimum temperature and [C2mim][OAc]-tolerance demonstrates that a positive correlation exists between these properties for those enzymes with an optimum temperature >70°C, further strengthening the link between thermotolerance and IL-tolerance for lignocellulolytic glycoside hydrolases [44].

Brine shrimp (*Artemia salina*) belong to a group of crustaceans that feed on microalgae and require a cellulase enzyme that can be used in obtaining bioproducts from marine algae. Analysis of the purified enzyme by molecular spectrometry showed no match to known cellulases, indicating this enzyme could be a novel halophilic cellulase that can be used for the production of bioproducts from marine macroalgae [45].

Currently, industrial demand for microbial production of cellulases is being met by production methods using submerged fermentation (SF) processes. Thermophiles for cellulase production organisms use generally genetically modified strains of *H. jecorina* (anamorph *T. reesei*). Although the high cost of production in SF systems due to increased fermentation time with low productivity, has resulted in a shift towards the solid state fermentation systems (SSF) but the advantage of better monitoring and handling are still associated with the submerged cultures. Though there are reports on cellulases production by SSF, the large-scale commercial processes are still using the proven technology of submerged fermentation (SmF) because the SSF is still noncompetitive. The appropriate technology, operation controls, and improved bioreactor design may make it viable (e.g., the enzyme in SSF crude product after concentration can be directly used in agrobiotechnological applications in silage or feed additives, lignocellulosic hydrolysis, and for processing of natural fiber). SSF may become a competitive method for the production of cellulases as it offers numerous advantages such as high productivity, relatively high concentrations of the products, and less effluent generation. When comparing cellulase production in SF and SSF systems it was shown that there was a 10-fold reduction in the production cost in SSF compared to SF.

The extracellular cellulase activity of *Haloarcula* sp. G10 is an endoglucanase for soluble cellulose. Optimal enzyme activity was found to be at 60°C, pH 9.0 and 17.5% NaCl. High activity and stability over broad ranges of temperature (40−80°C), pH (7.0−10.0), and NaCl concentration (12.5%−27.5%) is observed, showing thermostable, alkali-stable and halostable properties of the cellulase. Significant inhibition by EDTA, phenylmethylsulfonyl fluoride, and diethyl pyrocarbonate reveal it is a metalloenzyme with serine and histidine residues essential for enzyme catalysis. The EG show high activity and stability in the presence of nonpolar hydrophobic organic solvents with log Pow ≥ 0.88. The cellulase from *Haloarcula* sp. G10 maybe an ideal choice for applications in industrial processes under harsh conditions [46].

Much of the high cost of enzymes is due to the low catalytic efficiency and stability of lignocellulolytic enzymes, especially cellulases, under conditions that include high temperatures and the presence of residual pretreatment chemicals, such as acids, organic solvents, bases, or ionic liquids. Improving the efficiency

of the saccharification process on ionic liquid-pretreated biomass will facilitate reduced enzyme loading and cost. Thermophilic cellulases have been shown to be stable and active in ionic liquids but their activity is typically at lower levels. Cel5A-Tma, a thermophilic endoglucanase from *Thermotoga maritima*, is highly active on cellulosic substrates and is stable in ionic liquid environments. Twelve mutants with 25%–42% improvement in specific activity on carboxymethyl cellulose and up to 30% improvement on ionic-liquid pretreated switchgrass were successfully isolated and characterized from a library of 20,000 variants. Interestingly, most of the mutations in the improved variants are located distally to the active site on the protein surface and are not directly involved with substrate binding [47].

Biofuel production from plant-derived lignocellulosic material using fungal cellulases is facing cost-effective challenges related to high-temperature requirements. A cold-adapted cellulase named endo-1,4-β-glucanase (EF-EG2) from the earthworm *Eisenia fetida* has been identified. The amino acid sequence shares sequence homology with the endo-1,4-β-glucanases of *Eisenia andrei* (98%), *Pheretima hilgendorfi* (79%), *Perineresis brevicirris* (63%), and *Strongylocentrotus nudus* (58%), which all belong to glycoside hydrolase family 9. The enzyme exhibited significant activity at 10°C (38% of the activity at optimal 40°C) and was stable at pH 5.0–9.0, with an optimum pH of 5.5 [48].

There have been attempts to produce cellulase through fed batch instead of batch processes which help to override the repression caused by accumulation of reducing sugars.

Pectinolytic and cellulolytic enzymes (Pectinase 62L, Pectinase 690L, and Cellulase CO13P) were used to evaluate the solubilization of carbohydrates and low-molecular-weight flavonoids from bergamot peel, a major by-product of the essential oil industry.

6.7 OBTAINING BIOACTIVE INGREDIENTS AND "NONCLASSICAL" USES OF CELLULASES

The demand for new and novel natural compounds has intensified the development of plant-derived compounds known as bioactives that either promote health or are toxic when ingested. Enhanced release of these bioactives from plant cells by cell disruption and extraction through the cell wall can be optimized using mono- or poly-component enzyme preparations. However, the biotechnological application of enzymes is not currently exploited to its maximum potential within the food industry [49].

Research on the functions and effects of polyphenols has gained considerable momentum in recent times. This is attributed to their bioactivities, ranging from antioxidant to anticancer properties. But their potential is seldom fully realized since their solubility and stability is quite low and their bioavailability is

hampered due to extensive metabolism in the body. Biotransformation of polyphenols using enzymes, whole-cell microbes, or plant cell cultures may provide an effective solution by modifying their structure while maintaining their original bioactivity. Lipase, protease, cellulase, and transferases are commonly used enzymes, with lipase being the most popular for carrying out acylation reactions. Among the whole-cell microbes, *Aspergillus*, *Bacillus*, and *Streptomyces* sp. are the most widely used, while *Eucalyptus perriniana* and *Capsicum frutescens* are the plant cell cultures used for the production of secondary metabolites emphasizing the development of green solvents and identification of different sources/approaches to maximize polyphenol transformation for varied applications [50]. The optimum enzyme-extraction process of total flavonoids from *Psoralea coryl* folia by cellulose-assisted technique is obtained with the following parameters: pH value 4.9, temperature 46°C, time 150 minutes, and enzyme amount 7.2 mg/g. With this simple and feasible technique, the extraction rate of total flavonoids increases by 28% compared with ultrasonic extraction [51].

Pectinolytic and cellulolytic enzymes (Pectinase 62L, Pectinase 690L, and Cellulase CO13P) were used to evaluate the solubilization of carbohydrates and low-molecular-weight flavonoids from bergamot peel, a major by-product of the essential oil industry. The enzymes were characterized for main-chain and side-chain polysaccharide hydrolyzing activities and also against pure samples of various flavonoids previously identified in bergamot peel to determine various glycosidase activities. The addition of Pectinase 62L or 690L alone, or the combination of Pectinase 62L and Cellulase CO13P, was capable of solubilizing between 70% and 80% of the bergamot peel, and up to 90% of the flavonoid glycosides present were cleaved to their aglycones. Cellulase CO13P alone solubilized 62% of the peel but had no deglycosylating effect on the flavonoid glycosides. Over a 24-hour time course, a rapid release of cell wall carbohydrates was observed after treatment with Pectinase 62L, with a concurrent gradual hydrolysis of the flavonoid glycosides. Size-exclusion chromatography of the solubilized extract showed that after 24-hour incubation, the majority of the solubilized carbohydrates were present as monosaccharides with a smaller proportion of oligosaccharides [52].

Ultrasonic-assisted extraction is suitable for fast and effective extraction of flavonoids from *Glycyrrhiza inflata*. They have bacteriostatic bioactivities against *Staphylococcus aureus*, *E. coli*, *Pseudomonas aeruginosa*, and *Bacillus subtilis* [53].

It was found that commercial cellulose preparations from *T. viride* showed transglucosylation activity toward (+)-catechin and (−)-epigallocatechin gallate (EGCG) using dextrin as a glucosyl donor. The enzyme exhibiting transglucosylation activity toward (+)-catechin and EGCG was identified as alpha-amylase homolog (TRa2), which showed high amino acid sequence identity to functionally uncharacterized alpha-amylase homologs from other ascomycetes, which also produced some (+)-catechin and EGCG glucosides [54].

Mixtures of 1% cellulase plus 1% hemicellulase plus pectinase, 0.5% glucose and lactic acid bacteria were tested for efficacy of enzyme-assisted ensiling in the recovery of polyphenols from rosemary and sage, following storage. The enzyme

treatment resulted in silages with the lowest pH values, lowest fiber content, highest water-soluble sugar content-, and highest polyphenol recovery, leading to an increased polyphenol recovery from rosemary and sage, by 100% and 20%, respectively.

A novel enzyme-involved approach to improve the extraction of flavonoids from *Ginkgo biloba*, in which the enzyme is employed not only for cell wall degradation, but also for increasing the solubility of target compounds in the ethanol-water extractant has been recently described [55]. The utilization of enzymatic bifunctionality using enzymatic modification of target compounds and facilitation of cell wall degradation, provides a novel approach for the extraction of natural compounds from plants. *Penicillium decumbens* cellulase, a commercial cell wall-degrading enzyme with high transglycosylation activity, was found to offer far better performance in the extraction than *T. reesei* cellulase and *Aspergillus niger* pectinase under the presence of maltose as the glycosyl donor. *P. decumbens* cellulose could transglycosylate flavonol aglycones into more polar glucosides, the higher solubility of which led to improved extraction. The extraction yield was 28.3 mg/g of dw, 31% higher than that under the preoptimized conditions, and 102% higher than that under the conditions without enzymes.

Xie et al. [56] developed a cost-effective and environmentally friendly approach to improve the extraction of active ingredients from plants, in which a bifunctional enzyme was employed for not only facilitating cell wall degradation but also increasing the bioactivity of target compounds in the extract. In the aqueous extraction of flavonoids from *Glycyrrhizae radix*, *T. viride* cellulase, a commercial cell-wall-degrading enzyme, was found to efficiently deglycosylate liquiritin and isoliquiritin, which are of high aglycone content but low bioactivity, into their aglycones that have much higher physiological activities for dietary and medicinal uses. The same approach was expanded to the extraction of flavonoids from *Scutellariae radix* using *P. decumbens* naringinase, where enhanced production of more bioactive bacalein and wogonin was achieved via enzymatic deglycosylation of bacalin and wogonoside.

Wheat bran, a by-product of the flour industry, is believed to be a raw material for the production of feruloylated oligosaccharides (FOs) because of its high content of conjoint ferulic acid (FA). Studies were carried out to identify edible mushrooms that are able to release FOs from wheat bran. All of the six tested mushrooms (*Pleurotus ostreatus*, *Hericium erinaceum*, *Auricularia auricula*, *Cordyceps militaris*, *Agrocybe chaxingu*, and *Ganoderma lucium*) were found to release FOs, and *A. chaxingu* had the highest yield, reaching 35.4 μM in wheat bran broth. Enzyme detection showed that these species secreted extracellular enzymes during fermentation, including cellulase and xylanase. *A. chaxingu* secreted a significant amount of xylanase (180 mU/mL), which was responsible for the release of FOs from wheat bran, while *H. erinaceum* secreted FA esterase which could disassemble FOs [56].

The content of icaritin and genistein in herba is very low; preparation with relatively large quantities is an important issue for extensive pharmacological studies

and practical application. Jin et al. [57] tested a method of enzymic hydrolysis of flavonoid glycosides/β-cyclodextrin inclusion complex to increase the hydrolysis rate. The solubility of icariin and genistein were increased almost 17 times from 29.2 to 513.5 μg/mL at 60°C and 28 times from 7.78 to 221.46 μg/mL at 50°C, respectively, demonstrating that the inclusion complex could significantly increase the solubility of flavonoid glycosides. The reaction time of icariin and genistin decreased in comparison to the preparation without complex by 68% and 145%, respectively.

Following extraction, several bioactive compounds may be obtained from green tea waste including polyphenols and amino acids. It was shown that an *A. niger* cellulose treatment of green tea waste increased the extractability of various nutritional and functional components after pretreatment with various extraction solvents such as cold water, hot water, sulfuric acid, hydrochloric acid, and methanol. Cellulase from *A. niger* can thus increase the extractability of green tea waste when combined with certain solvent pretreatments and accordingly, the residual functional compounds and essential nutrients from cellulase-treated green tea waste have the potential to be applied in the production of new functional foods [58].

When subjected to enzymatic hydrolysis, two flavonol triglycosides, camelliaside A (CamA) and camelliaside B (CamB), of tea seed extract (TSE) were subjected to enzymatic hydrolysis using five kinds of glycosidases: beta-galactosidase (Gal) induced selective hydrolysis of CamA. Pectinase (Pec) and cellulase induced hydrolysis of CamB. For Gal and Pec only kaempferol diglycoside (nicotiflorin, NF) was produced. The combination of glucosidase and Pec of TSE produced nicotiflorin with high specificity [59].

Enzyme-assisted extraction (cellulase, endo-β-1,4 xylanase and pectinase) and pressurized liquid extraction (water and ethanol was employed maintaining 150°C as extraction temperature) applied for extraction of natural compounds from lemon balm (*Melissa officinalis*) showed that the first method enhanced the total phenolic content and the antioxidant capacity compared to a nonenzymatic control whilst pressurized liquid extracts present higher amount of phenols and antioxidant capacity than enzyme-assisted extracts [60].

The cellulase-assisted extraction and antibacterial activity of water-soluble polysaccharides from the dandelion *Taraxacum officinale*. The yield of polysaccharides from dandelion (PD) reached 20.67% (w/w). The sugar content of PD was 95.6% (w/w), and it displayed high antibacterial activity at a concentration of 100 mg/mL against *E. coli*, *B. subtilis,* and *S. aureus*. PD may be a viable option for use as a food preservative [61].

Spent coffee ground (SCG), a present waste stream from instant coffee production, represents a potential feedstock for mannooligosaccharides (MOS) production. MOS can be used in nutraceutical products for humans/animals or added to instant coffee, increasing process yield and improving product health properties. The steam pretreatment of SCG improved the enzymatic digestibility of SCG by a commercial Japanese cellulase cocktail (Acremonium, Bioshigen Co. Ltd,

Japan) thus reducing the required enzyme dosage for MOS release. Combined enzymatic hydrolysis of untreated or steam-pretreated (150, 190, and 200°C for 10 minute) SCG with mannanase and cellulase cocktail resulted in 36%–57% (based on mannan content) of MOS production with a degree of polymerization of up to 6. The untreated material required at least 1% of both mannanase and cellulase loading. The optimum mannanase and cellulase loadings for pretreated SCG hydrolysis were between 0.3% and 1% and 0.4% and 0.8%, respectively. Statistical analysis suggested additive effects between cellulase cocktail and mannanase on MOS release, with no indication of synergism observed [62].

Oil palm empty fruit bunch (OPEFB) contains abundant cellulose and hemicelluloses and can be used as a renewable resource for fuel and chemical production. The polyhydroxybutyrate (PHB) producer, *B. megaterium* R11 could accumulate PHB up to 51.3% of its cell dry weight from both glucose and xylose. Tryptone was identified as its best nitrogen source. PHB content and production reached 58.5% and 9.32 g/L, respectively, for an overall OPEFB sugar concentration of 45 g/L reaching, respectively, 51.6% and 12.48 g/L for OPEFB hydrolysate containing 60 g/L sugar with a productivity of 0.260 g/L per hour [63].

Determination of levels of total phenolics, condensed tannins, acid detergent fiber, pepsin/cellulase digestibility, and nitrogen in mature leaves of 26 individuals of the ant-plant, *Barteria fistulosa*, showed no relationship with concentrations of phenolics or fiber and only a weak relationship with digestibility and nitrogen. By contrast, light intensity strongly influenced levels of phenolics, notably condensed tannins, so that mature leaves of individuals growing in direct sunlight were less digestible and appeared to be of lower quality as food for herbivores than did mature leaves of individuals in shaded positions [64].

The polysaccharide yield, polysaccharide content, protein content, and infrared spectra were determined, and antioxidant activities of five polysaccharides obtained from *Agaricus blazei* Murrill (ABM) through different extraction methods including hot water extraction, single enzyme extraction (pectinase, cellulase or papain) and compound enzymes extraction (cellulase:pectinase:papain) and antioxidant activities showed that they have antioxidant activities in a concentration-dependent manner. Compared with other methods, the compound enzymes extraction method was found to present the highest polysaccharides yield (17.44%) and compound enzymes extracts exhibited the strongest reducing power and highest scavenging rates on hydroxyl radicals, DPPH radicals, and ABTS radicals. On the contrary, the hot water extraction method had the lowest polysaccharides yield (11.95%). Thus, data obtained with in vitro models suggested that ABM extracts are natural antioxidants and compound enzymes extraction is an appropriate, mild, and effective extracting method for obtaining the polysaccharide extracts from *Agaricus blazei* Murrill [65].

A potent strain of *Trichoderma harzianum* was isolated and used for the efficient deinking of photocopier waste papers. Cellulase and xylanase activities of *T. harzianum* were found to be at maximum at pH 5.5 and temperature 55–60°C with good stability (even up to 6 hours). The crude enzyme was evaluated for the

deinking of photocopier waste papers without affecting the strength properties, with improved drainage as an additional advantage. The crude enzyme-deinked pulp showed 23.6% higher deinking efficiency and 3.2% higher brightness than chemically deinked pulp. Strength properties such as tensile, burst indices, and folding endurance were also observed to improve by 6.7%, 13.4%, and 10.3%, respectively, for enzyme-deinked pulp whilst the tear index was decreased by 10.5% [66].

6.8 HYDROLYSIS OF CELLULOSE IN HUMANS AND ANIMALS

Beta-links of cellulose are not hydrolyzed by endogenous enzymes of humans and animals, with the known exception of termites [67] and some other insects of species from the orders *Dictyoptera*, *Orthoptera*, and *Coleoptera* [68,69].

Lignocellulose—the dry matter of plants, or "plant biomass"—digestion is of increasing interest in organismal metabolism research, specifically the conversion of biomass into sugars. Termites efficiently decompose lignocelluloses, and studies on lignocellulolytic systems may elucidate mechanisms of efficient lignocellulose degradation in termites as well as offer novel enzyme sources, findings which have significant potential industrial applications. Recent progress in metagenomic and metatranscriptomic research has illuminated the diversity of lignocellulolytic enzymes within the termite gut [67].

However, and despite the presence of many carbohydrolytic activities in insects, the involved mechanisms in cellulose hydrolyze are not well known. Cellulase genes are absent from the genomes of *Drosophila melanogaster* or *Bombyx mori*, whilst other insects such as those mentioned above produce their own cellulases. Recent studies using molecular biological techniques have brought new insights into the mechanisms by which the insects and their microbial symbionts digest cellulose in the gut. DNA sequences of cellulose and associated genes, as well as physiological and morphological information about the digestive systems of cellulase-producing insects, may allow the efficient use of lignocellulose biomass on one hand as a sustainable energy source [70] and on the other hand as food and feed supply. Only recent data have supported any direct role for the symbiotic bacteria in the gut of the termite in cellulose and xylan hydrolysis. The metagenomic analysis of the *Nasutitermes* species show the presence of a large and diverse set of bacterial genes for cellulose and xylan hydrolysis [71].

In monogastrics, part of the ingested fiber is converted in the hindgut to SCFAs. SCFAs represent about 15%−30% of the energy supplied by the gastrointestinal tract in the pig [72]. Fermentation in the large intestine is similar to that of the rumen, being less understood. *Bacteroides succinogenes* and *R. flavefaciens* of the pig are similar to those in the rumen and represent similar density of

population. Accordingly, there is a significant potential for lignocellulose and hemicellulose hydrolysis in the pig large intestine. Better knowledge is needed on the interactions of the cellulolytic microorganisms among them and of the microbiota with the host [73].

In horses, the digestibility of neutral detergent fiber (NDF) and acid detergent fiber (ADF) in the total gastrointestinal tract was significantly lower for a high-grass-intake diet than for a low one. The cumulative disappearance of NDF and ADF from the ileum to the left ventral colon attained 90% of total tract digestion regardless of hay intake. There was no difference between treatments in the total volatile fatty acid (VFA) concentration, VFA proportions, or the specific activity of carboxymethyl cellulase and xylanase in each hindgut segment. The fibrolytic activity for the cecum and right ventral colon (RVC) were greatest and decreased beyond the left ventral colon (LVC). A clear relation between mean retention time and fiber digestion was observed in the total gastrointestinal tract, but the segments where a difference in hindgut segment was detected because of the intake were not the same as those with the fiber digestibility depression. The reduction in total tract fiber digestibility with increased feed intake was clearly shown to occur between the cecum and RVC, which were the main segments for fiber digestion [74].

6.9 CELLULASES AS FEED ADDITIVES

Direct-fed cellulases as monocomponent additives are generally not very successful in improving monogastric animals' (pig and poultry) performance (digestibility, feed conversion ratio, and daily weight gain) [75,76], as are other microbial enzymes such as phytases [77,78,79] as well as xylanases in poultry [80,81]. Several reasons can be suggested for the very low performance of direct-fed cellulases: duration of the contact of the enzyme with the digesta within the gastrointestinal tract (in monogastric species, the hydrolysis process with the available cellulases take at least three- to five-times longer than that of digesta flow), gastrointestinal pH, particularly in the stomach, gastrointestinal denaturation and proteolytic activity, etc.

Allowance to pigs and poultry of cellulases in combination with other beta-glucanases are said to have positive effects on the zootechnical parameters of both species [82,83]. Combinations of cellulases with other glucanases are considered by some authors to improve performance in dairy cows and buffalo [84,85] whilst others were unable to observe such positive effects [86,87,88]. For example, in dairy cows, the apparent ruminal digestibilities of dry matter (DM), organic matter, NDF and ADF as well as milk yield and composition were not positively affected by the cellulase and other fibrolytic enzymes [87].

Applications of cellulases and hemicellulases in the feed industry have received considerable attention because of their potential to improve feed value and performance of animals. Pretreatment of agricultural silage and grain feed by cellulases or xylanases can improve its nutritional value. The enzymes can also eliminate antinutritional factors present in the feed grains, degrade certain feed constituents to improve the nutritional value, and provide supplementary digestive enzymes such as proteases, amylases, and glucanases. For instance, the dietary fiber consists of nonstarch polysaccharides such as arabinoxylans, cellulose, and many other plant components including resistant dextrins, inulin, lignin, waxes, chitins, pectins, β-glucan, and oligosaccharides, which can act as antinutritional factors for several animals such as swine. where the cellulases hydrolyze the antinutritional factor, cellulose, in the feed materials into easily absorbent ingredient thus improving animal health and performance. β-Glucanases and xylanases have been used mainly in the feed of poultry species to hydrolyze nonstarch polysaccharides such as β-glucans and arabinoxylans.

A combination of cellulase, hemicellulose, and pectinase has been shown to improve the in vitro (as well as in vivo) digestibility of a corn-soybean meal broiler diet; the birds' zootechnical performance is improved by effectively degrading indigestible cell constituents and thus enabling the protein of the broiler feed to become more digestible [89].

Most low-quality feedstuffs contain higher levels of cellulose, small amounts of protein and fat, and relatively high ash contents, when compared with high-quality feedstuffs. Cellulases can be used to improve silage production for cattle feeding, which involves enhancement of the digestibility of grasses containing large amounts of potentially total digestible nutrients and energy values together with only small amounts of water-soluble carbohydrates. The forage diet of ruminants, which contains cellulose, hemicellulose, pectin, and lignin, is more complex than the cereal-based diet of poultry and pigs. Enzyme preparations containing high levels of cellulase, hemicellulase, and pectinase have been used prior to feeding treatment to improve the nutritive quality of forages. Nevertheless, the results with the addition of enzyme preparations containing cellulase, hemicellulose, and pectinase to ruminant diets are somewhat inconsistent.

The Rusitec system was used by Giraldo et al. [90] to investigate the effects of pure cellulases from *Trichoderma longibrachiatum* and *A. niger* on ruminal microbial growth and fermentation of a grass hay and concentrate substrate. All enzyme treatments augmented methane production, but none of them altered the methane:SCFA ratio as well as the daily flow of solid-associated microorganisms. Both treatments enhanced in vitro fermentation by increasing substrate fiber degradation, SCFA production, and ruminal microbial growth showing a similar effect of both hydrolases.

Animal feedstock production processes generally include heat treatments that inactivate potential viral and microbial contaminants as well as several antinutritional factors. Application of thermophilic cellulase in feedstock production is

compatible with pathogen reduction and also has the potential to enhance digestibility and nutrition of the feed, thereby facilitating a combination of heat treatment and enzyme transformation in a single step. Furthermore, these enzymes can cause partial hydrolysis of plant cell walls during silage and fodder preservation. Spraying certain doses and combinations of enzymes (carboxymethyl cellulase and xylanase) directly onto forages (corn silage and alfafa hay) prior to feeding can improve milk yields but enzyme sources and dose levels are of critical importance. Kung et al. and Yu et al. [91,92] suggested that the addition of a multienzyme cocktail (FA esterase, xylanase, cellulase, endoglucanase [I, II], and beta-glucanase) to oat hulls, wheat straw, or alfalfa hay before feeding enhanced degradation of dry matter.

Extrusion technology has been used successfully to improve the nutritive value of full-fat oilseeds by exposing more peptide bonds to enzymatic hydrolysis. However, the fibrous portion of the seeds is largely intact after extrusion. Therefore, application of carbohydrase mixtures targeting nonstarch polysaccharides might further improve the nutritive value of extruded full-fat oilseeds. Ayoade et al. [93] have shown that enzyme supplementation increase the nutritive value of extruded soy-bean meal in pig feed. Similar conclusions were drawn by Emiola et al. [82], using xylanase-rich blends of enzymes added to wheat distillers dried grains with solubles. It should be underlined that mono-component cellulase addition to pig and poultry diets remains without effects on performance as well as on the availability of nutrients from diets containing high levels of nonstarch polysaccharides [94,95].

Ruminants need cellulolytic ruminal bacteria to digest cellulose. However, these bacteria cannot resist the low ruminal pH that modern feeding practices, based on cereals and leguminous cakes, can induce. The cellulolytic bacteria are unable to grow on cellobiose at low pH, pH sensitivity is a general aspect of growth and not just a limitation of the cellulases activity. Acid-resistant ruminal bacteria have evolved the capacity to let their intracellular pH decrease, maintain a small pH gradient across the cell membrane, and prevent an intracellular accumulation of SCFA anions. Cellulolytic bacteria cannot grow with a low intracellular pH, and an increase in pH gradient leads to anion toxicity. *Prevotella ruminicola* cannot digest native cellulose, but it grows at low pH and degrades the cellulose derivative, carboxymethyl cellulose. The *P. ruminicola* carboxymethyl cellulase cannot bind to cellulose, but a recombinant enzyme having the *P. ruminicola* catalytic domain and a binding domain from *Thermomonspora fusca* is able to bind and has cellulose activity that is at least 10-fold higher. Assuming that the barrier effect allows the survival and colonization of the modified microorganisms in the rumen, gene manipulation can be interesting in converting *P. ruminicola* into a ruminal bacterium that can digest cellulose at low pH [96]. It has been demonstrated [97], that carboxymethyl cellulase, xylanase, and amylase activities, and the concomitant in situ dry matter degradability, are higher in goats than in sheep, and in Korean native cows than in dairy cows.

6.10 PERSPECTIVES FOR THE USE OF CELLULASES IN FOOD/FEED APPLICATIONS

The production of sugars and bioethanol from cellulosic materials is still a costly process, and requires, for positive environmental effects, the production of strong and low-cost enzymes. Today, cellulosic ethanol is twice as expensive as gasoline, and the proportion is similar for the production of bioactives. It is expected that the production cost of cellulases will be reduced from 5 to 1 US cents per liter between now and 2020. The world leaders in the domain (Novozymes, Genencor, and Iogen) would like to reduce the price of cellulases within a few years.

Several microorganisms can be used as providers of cellulases. As already mentioned, *T. reesei* is today a paradigm for the commercial-scale production of different plant cell wall-degrading enzymes, mainly cellulases and hemicellulases. These enzymes have a long history of safe use in industry and well-established applications are found within several processing industries. However, when these enzymes are to be used for the saccharification of cellulosic plant biomass to simple sugars which can be further used as biorefinery products, and thus compete with chemicals produced from fossil sources or from agricultural activities, additional efforts are needed to reduce costs and maximize yield and efficiency of the produced enzyme mixtures. Among other approaches to this end is the use of genetic engineering to manipulate the biochemical and regulatory pathways that operate during enzyme production and control enzyme yield [98].

The Earth' available stocks of lignocellulosic biomass are tremendous and are distributed all around the world. Thus, the sustainability of the valorization of cellulose by cellulases seems clear. More economical feeding can most probably be obtained by improving pretreatment and enzymatic hydrolysis of cellulose. Furthermore, application in human nutrition may be also possible.

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CHAPTER

Laccases—properties and applications

7

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7.1 INTRODUCTION

Lignocellulose is the most abundant complex of polymers on Earth and it is continuously produced. It is estimated that a ton of cereals harvested is accompanied by the production of two to three tons of lignocellulose-rich residues. The cost-effective transformation of nonfood lignocellulose to starch could revolutionize agriculture and reshape the bioeconomy, while maintaining biodiversity, minimizing agriculture's environmental footprint, and conserving fresh water. This transformation would not only promote the cultivation of plants chosen for rapid growth rather than those optimized for starch production, but it would also efficiently use marginal land for the production of the biomass required to meet the increasing needs for renewal chemicals and agrofuels. The cost-effective release of soluble fermentative sugars from lignocellulosic materials through enzymatic hydrolysis is essential in a new generation of lignocellulosic biorefineries.

Lignin is present in all vascular plants making vegetables firm and crunchy, and supplying food and feed fiber. It serves also to regulate the transport of liquid in the living plant. Lignin is an organic substance binding the cells, fibers, and vessels in lignified elements of plants. It is a complex aromatic heteropolymer composed by radical polymerization of guaiacyl, syringyl, and *p*-hydroxyphenyl units linked by β-aryl ether linkages, biphenyl bonds, and heterocyclic linkages. It is extremely resistant to degradation [1] and has many internal H bonds. In wood it is bonded in complex and various ways to carbohydrates hemicelluloses and cellulose. Lignin is actually not a single compound; all are complex, amorphous, three-dimensional polymers that have in common a phenylpropane structure, i.e., a benzene ring with a tail of three carbons. In their natural unprocessed form, they are so complex that none of them has ever been completely described, and they have molecular weights that may reach 15,000 or more. Although most of them contain certain carboxylic acids, lignins are not acids and as they occur in nature (protolignins) have engrouped into several types, characteristic of

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hardwoods, softwoods, and grasses. Within each type there is a much variation: lignins differ from species to species, and from one tissue to the next in the same plant, even within different parts of the same cell wall (Fig. 7.1).

Biomass appears to be a promising alternative and a renewable source for fuels and chemicals. Significant achievements have already been made in the production of ethanol from biomass, primarily starch and sugar-rich components. Lignin can account for up to 40% of the dry biomass weight. Most of the biorefinery schemes focused on utilizing easily convertible fractions—cellulose and hemicellulose—while lignin remains relatively underutilized compared to its potential. After cellulose, it is the most abundant renewable carbon source on Earth. Between 40 and 50 billion tons per year are produced worldwide, mainly as a waste product. Efficient lignin utilization requires its depolymerization to low-molecular-weight phenolics and aromatics that can then serve in the chemical

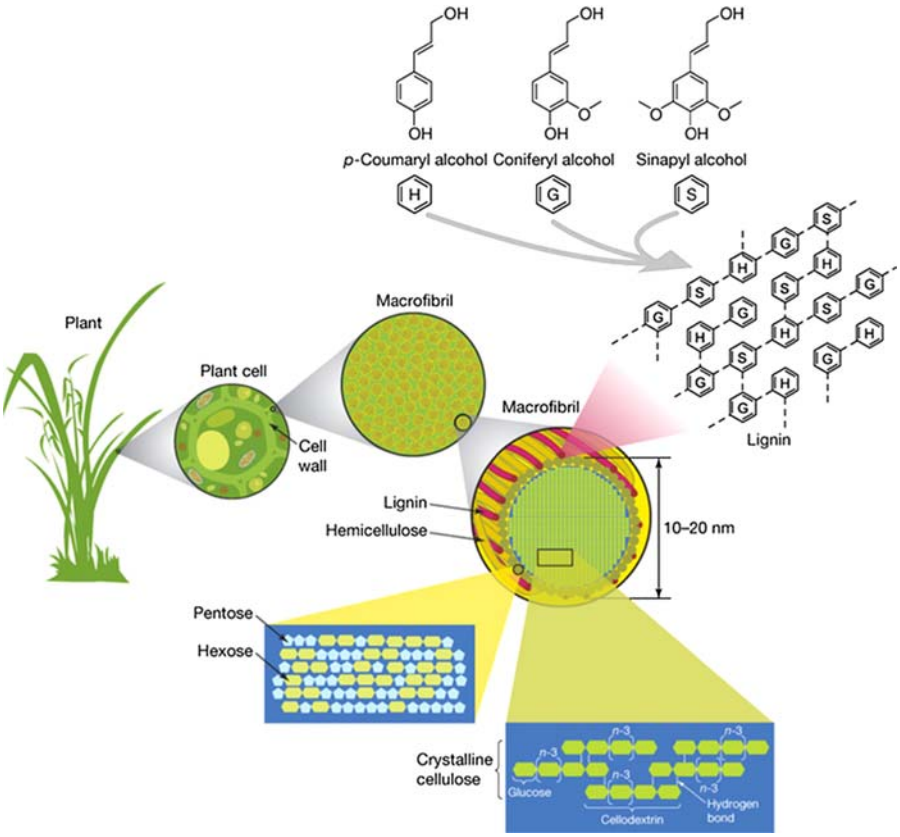

FIGURE 7.1

Schema of a plant cell wall.

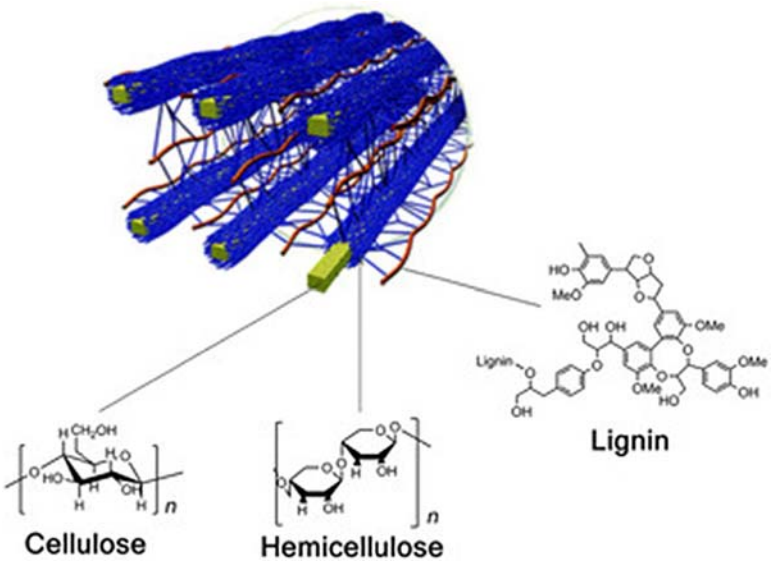

FIGURE 7.2

Schema of lignocellulose.

syntheses of high-value products. There are two ways of the degradation of lignin—chemical and enzymatical. An important advantage of the enzymatic approach is the lack of yield loss associated with the thermal decomposition of lignin, and the opportunity to direct lignin biodegradation utilizing selective microorganisms and/or enzymes. Furthermore, the biocatalysis takes place at lower energy input and reduces the environmental impact (Fig. 7.2).

7.2 LIGNOLYTIC ENZYMES—LACCASES

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are multicopper oxidases, together with different proteins such as plant ascorbic oxidase, mammalian ceruloplasmin, or Fet3p ferroxidase from *Saccharomyces cerevisiae*, and others [2]. They catalyze the oxidation of various substrates with the simultaneous reduction of molecular oxygen to water [3]. Laccase is one of the oldest enzymes ever described. Laccase activity has been found in plants, some insects [4], and a few bacteria [5]. However, most biotechnologically useful laccases (i.e., those with high redox potentials) are of fungi origin. Over 60 fungal strains belonging to *Ascomycetes*, *Deuteromycetes*, and especially *Basidiomycetes* show laccase activities. Among the latter group, white-rot fungi such as *Phanerochaete chrysosporium* are among the highest producers of laccases, but litter-decomposing and ectomycorrhizal fungi also secrete laccases [6].

To date, more than 150 laccases have been isolated and characterized. Based on the literature, general characteristics of these enzymes can be given with the reservation that the majority of laccases were isolated from wood-destroying white-rot fungi, whereas plant laccases are much less studied. The number of isozymes found depends on conditions of cultivation, in particular on the presence of an inducer in the medium. The fungus *Pleurotus pulmonarius* produces three laccase isoforms, two of which (lcc1 and lcc2) are constitutive and the isoform lcc3 can be detected only when the fungus is cultured in the presence of inducers, whereas the fungus *Marasmius quercophilus* strain 17 produces three constitutive and four inducible forms of the enzyme (Fig. 7.3).

The ability of laccases to attack and degrade lignin in conjunction with laccase mediators is currently viewed as one of the fundamental potential applications for greater lignin utilization. In lignin, links with cellulose and hemicellulose create a barrier to lignocellulosic enzymes. Lignin resists to microorganisms, but some basidomycetes, as well as some bacteria, are able to degrade it efficiently by employing different agents and catabolic pathways in a combination of lignocellulosic enzymes, organic acids mediators, and accessory enzymes [7]. Several fungi produce lignin degradating enzymes such as ascomycetes (e.g., *Trichoderma reesei*) and basidiomycetes (e.g., *P. chrysosporium* and *Fomitopsis palustris*). Also some anaerobic species can hydrolyze lignin in the gastrointestinal tract of ruminants, using a complex mixture of enzymes (Fig. 7.4).

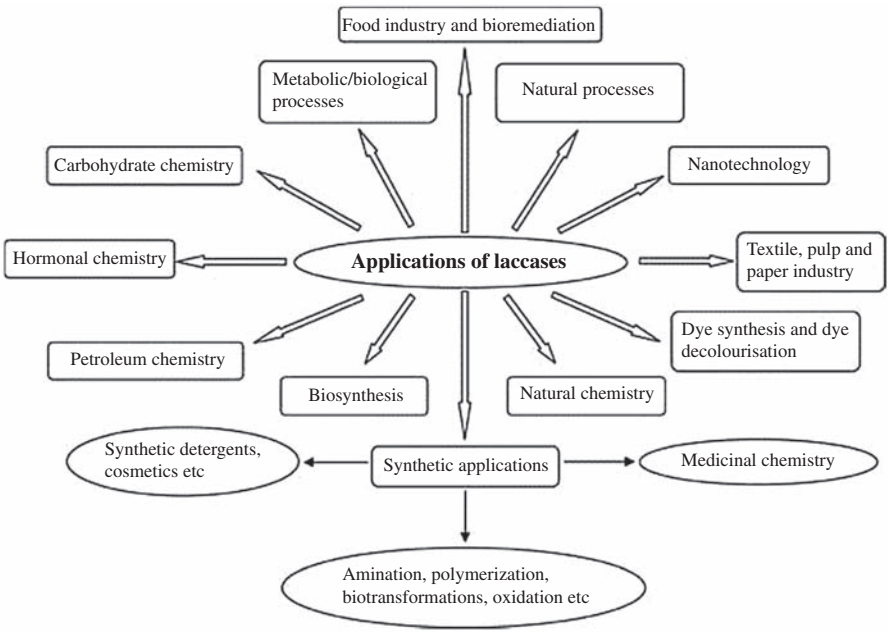

FIGURE 7.3

Applications of laccases.

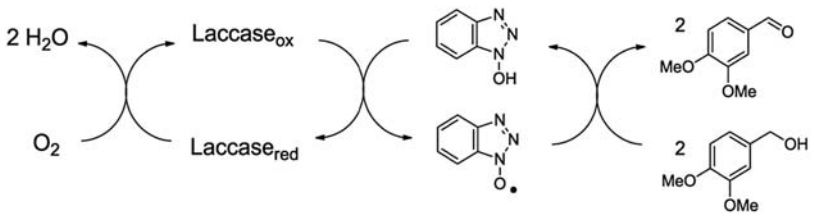

FIGURE 7.4

Hydrolysis by laccase.

Selective lignin degradations have potential applications in biotechnology when lignin elimination is fundamental for obtaining cellulose. It is not possible to define the precise structure of lignins since all show a certain variation in their chemical composition. However, the definition common to all is a dendritic network polymer of phenyl propene basic units. There are two principal categories of lignin: those which are sulfur bearing and those which are sulfur free. The sulfur-bearing lignins are those which have to date been commercialized; these include lignosulphonates (world annual production of 500,000 tons) and kraft lignins (under 100,000 tons p.a.).

Commercial production of several biochemicals and cellulosic ethanol increase the transformation of lignin to value-added products. Recent advances in lignin valorization efforts are related to the discovery of genetic variants in native populations of bioenergy crops, and direct manipulation of biosynthesis pathways have produced lignin feedstocks with favorable properties for recovery and downstream conversion. Advances in analytical chemistry and computational modeling detail the structure of the modified lignin and direct bioengineering strategies for future targeted properties. Refinement of biomass pretreatment technologies has further facilitated lignin recovery, and this, coupled with genetic engineering, will enable new uses for this biopolymer, including low-cost carbon fibers, engineered plastics and thermoplastic elastomers, polymeric foams, fungible fuels, and commodity chemicals [8].

The lignolytic enzymes are classified as peroxidases (lignin, manganese, and versatile peroxidase) and laccases. The broad substrate specificity of laccases and their ability to utilize atmospheric oxygen as electron donor instead of hydrogen peroxide used by peroxidases makes these enzymes promising candidate(s) for diverse industrial applications. Laccases are a family of multicopper oxidases that bear a close resemblance, particularly in amino acid sequence, to ascorbate oxidase (found in cucumbers) and mammalian plasma protein ceruloplasmin. Those present in bacteria, fungi, plants, and insects are the largest subgroup of these enzymes. They were first described in the sap of *Rhus vernicifera* the Japanese lacquer tree by Yoshida in 1883 and demonstrated in fungi [2,9] in 1896 and by Kunamneni et al. [10–12] in the white-rot fungus *Trametes versicolor.* They have also more recently been discovered in bacteria. Their physiological function is

still under intense investigation, although they are implicated in the synthesis and/or degradation of the biopolymer lignin, wound response mechanisms, and the morphogenesis of microorganisms. They have a broad specificity that enables them to catalyze the one-electron oxidation of a range of substrates. These substrates, usually phenolic in nature, provide electrons for the concomitant four-electron reduction of atmospheric O_2 to water. Catalysis takes place via the four copper centers which are classified according to their electronic properties. For example, the type I (T1) Cu shows a characteristic sulfur to Cu(II) charge transfer absorption band at around 610 nm.

The use of the term "laccase" is sometimes confusing since some authors use this name only for those enzymes synthesized by plants and fungi showing high activity on phenolic compounds, while others use this term in a broader sense to refer to any enzyme able to oxidize a laccase substrate. A fungal enzyme, first described as a laccase, was recently found to also oxidize Mn [13]. Accordingly, the term "laccase" sometimes makes reference to the enzymatic activity of a protein, although proteins with such activity can play different physiological roles or oxidize other kinds of compounds.

Currently the catalytic properties of laccases are being exploited for a range of technological applications such as the bioremediation of soils and water and the development of environmentally friendly processes in biorefenery [14]. Furthermore, the laccases' hydrolytic activity on plant biomass aimed at animal as well as at human nutrition has a tremendous potential. Much work has gone into the sequencing, cloning, and mutagenesis of laccase genes from microrganisms. Recently several crystal structures containing the full complement of Cu atoms have been reported.

Anaerobic gut fungi isolated from herbivores produce a large array of biomass-degrading enzymes that synergistically degrade crude, untreated plant biomass, and are competitive with optimized commercial preparations from *Aspergillus* and *Trichoderma*. Compared to these model platforms, gut fungal enzymes are unbiased in substrate preference due to a wealth of xylan-degrading enzymes [15] *Pleurotus ostreatus* is an edible mushroom and a model for delignification applications of woody and nonwoody feedstocks. The available genome allows proteomic studies for identifying enzymes. Oxidoreductases are strongly induced when *P. ostreatus* grows on woody and nonwoody lignocellulosic substrates. One laccase occupied the first position in both secretomes, and three more were overproduced together with one vector protein (VP) and one manganese peroxidase (MnP), suggesting an important role in lignocellulose degradation. Preferential removal of lignin vs carbohydrates was shown by 2D NMR, in agreement with the secretomic results [16]. *Bacillus megaterium* has a good ability to degrade lignin in tobacco straw when fermented in liquid state [17]. Polyphenol oxidase from the marine bacterium *Marinomonas mediterranea* (MmPPOA) is a membrane-bound, blue, multicopper laccase of 695 residues. It possesses peculiar properties that distinguish it from known laccases, such as a broad substrate specificity (common to tyrosinases) and a high redox potential. The recombinant

rMmPPOA-695-His has kinetic properties on catechol higher than that for known laccases, a very high thermal stability, and a strong resistance to NaCl, DMSO, and Tween-80. These properties are required for specific, targeted industrial applications [18].

Both fungi and bacteria have been observed to metabolize lignin. However, their differential reactivity with this substrate indicates that they may utilize different chemical strategies for its breakdown [19].

The activities of four kinds of enzyme are basis to the mechanism of macromolecular lignin depolymerisation. The fungal enzymes comprise lignin peroxidase (with a relatively high redox potential), MnP, an alkyl aryl etherase, and laccase. The peroxidases and laccase, but not the etherase, are expressed extracellularly by white-rot fungi. Some white-rot fungi secrete both kinds of peroxidase but no laccase, while others that are equally effective express extracellular laccase activity but no peroxidases [20].

In the food industry, laccases are used for the elimination of undesirable phenolic compounds in baking, juice processing, wine stabilization, and bioremediation of wastewater. In the beer industry, laccases not only provide stability but also increase the shelf life of beer. For making a fruit juice stable, laccases are commonly used. Phenol compounds and their oxidative products present naturally in the fruit juice give color and taste to the juice. Color and aroma change when polymerization and oxidation of phenolics and polyphenol take place. These changes are due to the high concentration of polyphenol and referred to as enzymatic darkening. Laccases treatment removes phenol as well as substrate−enzyme complex with the help of membrane filtration, and color stability is achieved, although turbidity is present. For improving the texture, volume, flavor, and freshness of bread, a wide range of enzymes are used. When laccases are added to the dough, the strength of the gluten structures in the dough and the baked products is improved: product volume increases, crumb structure improves, and softening of baked products takes place. At crushing and pressing stage, the high concentration of phenolic and polyphenolic compound play an important role in the wine production. The high concentration of polyphenol obtained from the stems, seeds, and skins, which depend on the grape variety and vinification conditions, contributes to the color and astringency. Due to the complex event, polyphenol oxidation occurs in musts and wines resulting in the increase in color and flavor change which is referred to as maderization. Catalytic factors, polyphenol removal, clarification, polyvinylpolypyrrolidone, and high doses of sulfur dioxide are utilized to prevent maderization. Polyphenol removal is selective and results in undesirable organoleptic characteristics. Laccases treatment is feasible, increasing storability and reducing processing costs.

Since laccases catalyze the electron transfer reactions without additional cofactors, they can also be used as biosensors to detect various phenolic compounds, oxygen, and azide. As biosensors, laccases can detect morphine, codeine, catecholamine, estimate phenol or other enzymes in fruit juice and plant flavonoid. During oxidation by laccases, oxygen is reduced, through the transfer of

four electrons, to water. A distinctive feature of tyrosinases and laccases is that the latter is considered not to oxidize L-tyrosine [20].

Most laccases studied so far have been isolated from plants and fungi [21,22]. In fact, the name laccase derives from the first description of an enzyme of this group in the lacquer tree [23]. In plants, laccase activity has been associated with lignosynthesis [24]. In contrast, fungal laccases produced by white-rot fungi show a lignolytic capacity, which is increased by the use of small molecules that act as mediators [25]. Laccase activity has also been detected in bacteria. It was first isolated in *Azospirillum lipoferum*, in which it was associated with pigment synthesis [26]. It is now accepted that multicopper oxidases are widely distributed in bacteria, where they may be related to different physiological processes such as copper resistance [5]. The first archaeal laccase was characterized in 2009 from *Haloferax volcanii* [27].

7.3 SELECTION, PRODUCTION, AND PROPERTIES OF LACCASES

Using molecular oxygen as the final electron acceptor, laccases release only water as a by-product, and as such, laccases are eco-friendly, versatile biocatalysts that have generated an enormous biotechnological interest. This group of enzymes has been used in different industrial fields from food additives and beverage processing to biomedical diagnosis, and as cross-linking agents for furniture construction or in the production of biofuels, as well as in nanobiotechnology. Their capacity to transform complex xenobiotics makes them useful biocatalysts in enzymatic bioremediation [28].

To improve the bioprocessing of lignocellulosic biomass, more effective degradation methods of lignin are in demand. Such enzymes have been thoroughly studied for fungi, but only in recent years have studies been performed for bacterial lignolytic enzymes. Two major classes of bacterial lignin-modifying enzymes (LME) are DyP-type peroxidases and laccases [29].

Fungi such as *Myceliophthora thermophila* M77 appear to decompose natural biomass sources quite well. Its secretomes produce on natural biomass displayed a comprehensive set of enzymes involved in hydrolysis and oxidation of cellulose, hemicellulose-pectin, and lignin [30].

Neurospora discreta is able to degrade up to twice as much lignin in sugarcane bagasse as the well-known white-rot fungus *P. chrysosporium* and produced nearly 1.5 times the amount of lignin degradation products in submerged culture. *N. discreta* is a promising alternative to white-rot fungi for faster microbial pretreatment of agricultural residues [31].

Lignocellulolytic enzyme activities of selective fungi *Daedalea flavida* MTCC 145 (DF-2), *Phlebia radiata* MTCC 2791, and nonselective fungus *Flavodon flavus* MTCC 168 are effective for the pretreatment of cotton stalks, particularly

DF-2. The glucose yield improves more than twofold from 20-day DF-2 pretreated cotton stalks after enzymatic saccharification [32].

A novel thermostable pH-versatile laccase from *Sporothrix carnis* CPF-05 with molecular weight of 56 kDa has been purified by ion-exchange and gel filtration chromatography. The laccase is mildly inhibited by urea, sodium azide, and surfactants, while it exhibits tolerance to organic solvents and has a broad substrate specificity. Characteristics of laccase from *S. carnis* CPF-05 indicate its suitability for a variety of industrial processes [33].

The knowledge of the functional potential of snow microbial communities is of outstanding interest. It was found that Antartica bacteria (*Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Deinococcus-Thermus*, *Planctomycetes*, *Verrucomicrobia*), *archaea* (*Euryarchaeota*), and *eukarya* (*Basidiomycota*, *Ascomycota*, *Cryptomycota,* and *Rhizaria*), produce among other enzymes (lipase, protease, amylase, β-galactosidase, and cellulase), LMEs. This highlights their potential to be involved not only in snow chemistry but also as a source for the selection of cold-tolerating laccases [34].

The use of bacterial laccases for industrialized applications is limited because of their low expression level and catalytic efficiency. L386W/G417L CotA, a bacterial laccase from *Bacillus pumilus*, engineered through presumptive reasoning and rational design approaches to improve catalytic efficiency and thermostability, has catalytic efficiency 4.3-fold higher than that of wild-type. Another mutant, L386W/G417L/G57F, has a half-life of 0.54 hour when incubated at 90°C for 2 hour. Further molecular modification of CotA-laccase may improve efficiency for the physical and chemical treatment of dye wastewater [35]. *Penicillium subrubescens* has the capacity to degrade crude lignocellulose, as well as polysaccharides, and metabolize their monomeric components with activity levels comparable to those of *Aspergillus niger*. The strain FBCC1632 of *P. subrubescens* has a strong potential as a producer of industrial enzyme cocktails [36].

The simultaneous saccharification and fermentation (SSF) of bagasse sulfite pulp to produce L-lactic acid by *Bacillus coagulans* strain CC17 require approximately 33.33% less fungal cellulase than do separate hydrolysis and fermentation. CC17 can coferment cellobiose and xylose without any exogenous β-glucosidase in SSF and has the potentially low-cost and highly-efficient fermentation process [37].

Reducing sugars can be produced from lignocellulosic materials in two steps: solid-state cultivation of fungi for deconstruction, followed by enzymatic hydrolysis using cellulolytic enzymes. *Trichoderma longibrachiatum* has been implemented for fungal autolysis and further enzyme hydrolysis of fermented solids [38]. The phylogenetic composition and lignolytic capacity of Caatinga (Brezil) soil have been established by sequence-based screening. *Proteobacteria* and *Actinobacteria* dominate the bacterial community. A total of 7275 genes were identified encoding 37 glycoside hydrolases families. The large repertoire of lignocellulolytic enzymes gives perspectives for mining potential candidates of biochemical catalysts for biorefinery [39]. Laccase from *Pycnoporus sanguineus*

CS43 was immobilized onto Immobead-150 and Eupergit-C by covalent binding. Immobilized enzyme systems show increased thermostability and better mechanical properties than free laccase, and possess significant potential for wastewater treatment [40]. *Trametes villosa* is a promising species for ligninolytic enzymes production. There are several publications on *T. villosa* applications for lignin degradation regarding the expression and secretion of laccase (Lac), MnP and recently in *T. villosa* CCMB561 the identification and characterization of lignin peroxidase (LiP), a relevant enzyme for the efficient breaking down of lignin [41].

Lignin and lignin derivative biopolymers have several properties, such as high thermal stability, antioxidant properties, biodegradability, antimicrobial actions, adhesive properties, etc., and thus they can be extensively used in a wide range of areas such as coatings and paints, manufacturing of plastics and resins, for rubber packaging, for fuel production, etc. Derivatives of lignin such as sulfonate, phenolic, organosol, Kraft, and sodium sulfonate lignin have good mechanical and physicochemical properties [42].

Lignin-poor cellulose diets fed to *Tsaitermes ampliceps* reduce *Spirochaetes* and increase *Proteobacteria* and *Bacteroides* comparatively to feeding lignin-rich cellulose ones. The hydrolysis is different according to the basis of the diets: wood > corn stover > filter paper [43]. It can be suggested that *T. ampliceps Spirochaetes* have the potential for the selection of lignolytic enzymes aimed at the hydrolysis of highly resistant lignin [43].

Lignocellulosic biomasses can be processed to produce both energy and bioproducts. Nature offers an arsenal of tools through microorganisms to accomplish lignin valorization or degradation; an increasing number of projects dealing with these tasks have been described recently [44]. Transfer of the new insights to the industrial scale and shaping the enzymes to tolerate associated adverse conditions has now shown first success, thus optimizing the economy of cellulosic ethanol, other biofuel and chemicals production [45].

Recently, designer cellulosomes have been developed to incorporate foreign enzymatic activities in cellulosomes so as to enhance lignocellulose hydrolysis further. A dockerin-fused variant of a recently characterized laccase from the aerobic bacterium *Thermobifida fusca* has been obtained and the resultant chimera exhibited activity levels similar to the wild-type enzyme and properly integrate into the cellulosome [46].

From 876 bacterial strains isolated from meager lean coal, 612 belonging to 27 genera in *Actinobacteria*, *Firmicutes*, and *Proteobacteria*, 612 were positive for lignin degradation function. Among them, the dominant lignin-degrading strains were *Thauera*, *Arthrobacter*, and *Rhizobium* [47].

Cellulose organization must be dependent on the organization of other polymers such as lignin. In plants containing high levels of G-lignin, S-lignin, H-lignin, aldehyde-rich lignin, and ferulic acid-containing lignin, along with plants with very low lignin content several mutations—most notably those exhibiting (1) down-regulation of cinnamoyl CoA reductase which leads to cell walls

deficient in lignin and (2) defect of cinnamic acid 4-hydroxylase which greatly reduces lignin content—exhibited significant decrease in the proportion of oriented cellulose fibrils in the cell wall. Changes in lignin biosynthesis lead to significant disruption in the orientation and order of cellulose fibrils in all tissues of the stem and such dramatic phenotypic changes, in mutants with lignin rich in aldehyde or H-units, correlate with the impact the mutations have on the enzymatic degradation of the plant cell wall [48].

A crude enzyme concentrate of *Aspergillus tubingensis* TISTR 3647 containing activities of peroxidase, catechol oxidase and laccase effectively oxidize the phenolic compounds in green tea infusion to theabrownins [49]. Cornstalk can be effectively degraded by *P. ostreatus* and the decrease of cornstalk lignin content potentially improves its suitability for animal feed [50]. A laccase gene lac1 from *Coptotermes formosanus* heterogeneously expressed in insect Sf9 cells shows strong activity toward hydroquinone (305 mU/mg) and 2,6-dimethoxyphenol (2.9 mU/mg). Lac1 could function well from pH 4.5 to 7.5, and its activity was significantly inhibited by H_2O_2 at above 4.85 mM ($P < .01$). This suggests that Lac1 is a phenol-oxidizing laccase like RflacA and RflacB from termite *Reticulitermes flavipes* [51].

The community structure of consecutive gut compartments in each termite species strongly differed, but that of homologous compartments clearly converged, even among unrelated termites. While the alkaline P1 compartments of all termites investigated contained specific lineages of *Clostridiales*, the posterior hindgut compartments (P3, P4) differed between feeding groups and were predominantly colonized by putatively fiber-associated lineages of *Spirochaetes*, *Fibrobacteres*, and the TG3 phylum (wood and grass feeders) or diverse assemblages of *Clostridiales* and *Bacteroidetes* (humus and soil feeders). Bacterial community structure in termite guts is driven by microenvironmental factors, such as pH, available substrates, and gradients of O_2 and H_2 [52].

A novel sequential lignin treatment for the production of low-molecular-weight soluble aromatic products that can be purified and/or upgraded applying further downstream processes has been developed. The method consists of a biocatalytic oxidation step followed by a formic acid-induced lignin depolymerization step using response surface methodology. The biocatalytic step employs a laccase mediator system using the redox mediator 1-hydroxybenzotriazole [53].

Laccase is one of the enzymes that has shown encouraging potential as a biocatalyst in the synthesis of bioactive compounds with new applications in medicine, pharmaceutical, agricultural, and food industries. They are interesting potential industrial catalysts in organic synthesis because they are essentially green catalysts with a diverse substrate range. Their reaction only requires molecular oxygen and releases water as the only by-product. Laccase catalysis involves the abstraction of a single electron from their substrates to produce reactive radicals. The free radicals subsequently undergo homo- and hetero-coupling to form dimeric, oligomeric, polymeric, or cross-coupling products which have practical implications in organic synthesis. Potential laccase applications are organic

synthesis, hair and textile dyeing, polymer synthesis, and grafting processes [54]. Many of the 44 bacterial strains coexisting in natural white-rot-decayed wood promote growth of cocultured *T. versicolor* strain TN6F and accelerated delignification, suggesting that some bacterial strains might promote wood degradation [55]. The presence of ionic liquids (ILs) can either inhibit or stimulate laccase activity, strongly depending on water-miscibility and kosmotropic natures of ILs. In addition, enzyme source, mediator, pH as well as water content are very important factors which influence laccase activity and stability. Strategies based on molecular evolution, enzyme immobilization and/or ILs modification greatly increase the tolerance of laccases against specific ILs. Thus, the use of ILs can spread the laccase applications in biosynthesis, biodegradation, biosensor, and biofuel cells [56].

Thermophilic and thermotolerant microorganism strains have served as the natural source of industrially relevant and thermostable enzymes. Some strains of the *Trametes* genus are thermotolerant. Laccase from *Trametes trogii* LK13 keeps 50% of the initial activity at 80°C for 5 minutes. Organic cosolvent tolerance shows remaining activity of 50% with treatment with organic cosolvent. *T. trogii* LK13 crude enzymes possess high laccase levels (1000 U/g) along with low cellulase (2 U/g) and xylanase (140 U/g) activity. *T. trogii* LK13 is a potential strain to be applied in several biotechnological processes [57].

The purified laccase from *Geobacillus thermopakistaniensis* (formerly *Geobacillus* sp.) SBS-4S is highly tolerant to various halides and organic solvents, having as other laccases from extremophile microorganisms a high and various potential for different industrial applications [58,59].

The molecular determinants of enzyme performance are very important for the design of ad hoc mutants. An approach combining conformational sampling and quick reactivity scoring, was used [60] to shed light on how substrate oxidation was improved during the directed evolution of a *Pycnoporus cinnabarinus* laccase, a relevant class of oxidoreductases. The enhanced activity of the evolved enzyme is mainly the result of substrate arrangement in the active site. Mutations at the active site shift the binding mode into a more buried substrate position and provide a more favorable electrostatic environment for substrate oxidation which seems to be a viable way to the in silico evolution of oxidoreductases.

7.4 APPLICATIONS OF LACCASES

Modern biorefineries focus on lignocellulosic biomass for the production of new biochemicals and agrofuels. Laccases play a major role during lignin degradation and have therefore been intensively researched for potential applications of biomass processing. Laccases can assist the pretreatment of biomass and promote the subsequent enzymatic hydrolysis of cellulose by the oxidative depolymerization of residual lignin on the biomass surface often in combination with mediator

molecules. Studies with lignin model compounds confirm the chemical possibility of a laccase-catalyzed cleavage of lignin bonds, but the strong polymerization activity of laccase counters the decomposition of lignin by repolymerizing the degradation products. Therefore, it is a key challenge to shift the catalytic performance of laccase towards lignin cleavage by optimizing the process conditions. Another field of application for laccases is the detoxification of biomass hydrolyzates by the oxidative elimination of lignin-derived phenolics which inhibit hydrolytic enzymes and are toxic for several organisms [61].

7.4.1 DECONTAMINATING PROPERTIES OF LACCASES AND PRACTICAL APPLICATIONS

The most frequently used technologies for waste disposal are high-temperature incineration and chemical decomposition. However, such techniques have several inconveniences. Strong efforts are currently focused on innovative, environmentally friendly alternatives such as bioremediation.

Laccases act on both phenolic and nonphenolic lignin-related compounds as well as highly recalcitrant environmental pollutants, and they can be effectively used in paper and pulp industries, textile industries, xenobiotic degradation, and bioremediation, and act as biosensors. Since laccases catalyze the electron transfer reactions without additional cofactors, they can also be used as biosensors to detect various phenolic compounds, oxygen, and azide. Recently, laccase has been used as a biocatalyst for the synthesis of organic substances as well as in the design of a biofuel cell. Low-cost laccase production is becoming an important factor determining increased use for treatment of many types of chemical wastes as well as pollutants.

Laccases have demonstrated potential and sometimes are already used for the degradation of antimcrobials (antibiotics and sulfamides) and other drugs (biogenic amines, toxic compounds present in fermented food and beverages [62]. The endophytic fungus *Phomopsis liquidambari* can effectively degrade the xenobiotic sinapic acid, one of the most representative methoxy phenolic pollutants in industrial wastewater [63].

Phenols are also a target for laccases hydrolysis. Phenols are known inhibitors for cellulase and fermentative microorganisms in biorefining processes. The addition of laccase removes the phenolic compounds and subsequently reduces the lag phase of the fermentative microorganism [64]. Laccases are able to catalyze one-electron oxidation of phenolic compounds into radical intermediates that can subsequently couple to each other via covalent bond important reactions in humification process and the transformation of contaminants containing phenolic functionalities [65].

Bisphenol A (BPA) is an endocrine-disrupting chemical ubiquitous in the environment because of its broad industrial use. Extracellular laccase of the most widely cultivated mushroom in the world (i.e., white-rot fungus, *P. ostreatus*)

efficiently degraded BPA. The exposure to BPA has no harmful effects on this edible fungus [66].

Sources of heavy metals in the environment include geogenic, industrial, agricultural, pharmaceutical, domestic effluents, and atmospheric sources. Most of essential heavy metals exert biochemical and physiological functions in plants and animals. However, environments contaminated by heavy metals and trace elements need to be cleared. Several techniques are available to remove such pollution: chemical precipitation, oxidation or reduction, filtration, ion-exchange, reverse osmosis, membrane technology, evaporation, and electrochemical treatment. But most of these techniques are expensive and often ineffective [67].

Microorganisms' uptake of heavy metals can be active (bioaccumulation) and/or passive (adsorption). The microbial cell walls offer many functional groups that can bind heavy metal ions. However, bioremediation technology has some limitations. Several microorganisms cannot break toxic metals into harmless metabolites, and these have inhibitory effects on microbial activity. Engineering of outer membrane proteins of potentially interesting bacteria improving metal binding abilities is one way to enhance their capacity for biotransformation of toxic metals.

There are hundreds of pesticides used worldwide. Some of them, such as chlordecone, have been withdrawn in most countries whilst others, like dichlorodiphenyltrichloroethane (DDT), are banned in some regions of the world and are under strong pressure for banishment in almost all other regions. DDT is, for instance, still used in tropical regions in the fight against malaria.

Microorganisms, have been used to transform pesticides. It is known that part of the soil biota can quickly develop the ability to degrade certain pesticides, when they are continuously applied to the soil. *Pseudomonas* is the most efficient bacterial genus for the degradation of toxic compounds. Others belong to the species *Aspergillus fumigatus*, *A. niger*, *A. terreus*, *Absidia* and *Rhizopus microsporus* var *corymberifera microsporis*. Lindane-degrading capabilities of *Ganoderma lucidum* GL-2 strain make it a potential candidate for managing lindane bioremediation at contaminated sites [68].

The oxidizing capacity of laccases on highly recalcitrant environmental pollutants, such as pesticides, makes them very interesting agents of environmental protection and remediation. For example, optimized laccase from *T. versicolor* has a high capacity for degrading pesticides such as chlorpyrifos, chlorothalonil, pyrimethanil, atrazine, and isoproturon [69].

Organochlorines are persistent pesticides that are still present in the environment despite not being used any longer. These chemicals pose a threat to human health, particularly during periods of increased sensitivity, such as gestation, young mental development, and in the induction of dysplasia. Chlordecone is an organochlorine pesticide that was principally used to control the banana root borer in Central and South America and in the Caribbean. Some soils in current and previous banana fields and some waterways are permanently polluted. Chlordecone is an endocrine disruptor with estrogen-like and progestin-like

characteristics both in vitro and in vivo, as well as a potential carcinogen that has been shown to cause hepatic tumors in laboratory rats and mice. The carcinogenic and hormonal perturbation properties of chlordecone and its long biologic half-life raise concerns of long-term effects, such as cancer. Long-term clordecone exposure in adulthood favors the development of prostate cancer, with a particularly high incidence of this disease observed in the French West Indies [70].

Polycyclic aromatic hydrocarbures (PAHs) are important environmental contaminants with strong deleterious health effects and are difficult to be removed and/or neutralized. *Peniophora incarnata* KUC8836, a white-rot fungus, produces lignolytic enzymes managing the hazardous effects of PAHs [71]. The thermotolerant white-rot fungal strain RYNF13 of *Trametes polyzona* completely degrades phenanthrene as well as 90% of fluorene and 52% of pyrene, and is superior to most other white-rot fungi [72].

A promising biocatalyst SsoPox has been isolated from the archaeon *Sulfolobus solfataricus* and is emphasized in the light of recently positive effects, and the hyperthermostable enzyme appears to be particularly attractive for external decontamination purposes with regard to both its catalytic and stability properties [73].

Considering that colorants such as azo dyes can be degraded physicochemically by oxidation OPs), a majority of the enzymes that are being investigated for their dye degradation potential belong to the enzyme class oxidoreductases. These enzymes are involved in electron transfer reactions. Azo dyes account for $>70\%$ of the global industrial demand (~ 9 million tons). Owing to their genotoxic/carcinogenic potential, the annual disposal of $\sim 4{,}500{,}000$ tons of dyes and/or degraded products is an environmental and socioeconomic concern. The majority of studies on microbial dye-degradation focus on decolorization with least attention towards detoxification. In the absence of ecosystem-based studies, the results of laboratory-based studies on dye degradation, metabolite production and their genotoxic impact on model organisms are used to predict the possible fate and consequences of azo dyes/metabolites in the environment. Many of the azo dyes in common use today have highly charged substitutes such as sulfonate. These resist enzymatic attack and for the most part are poorly absorbed from the intestinal tract, providing poor access to the liver, the major site of the mixed-function oxidase system. White-rot fungi produce various isoforms of extracellular oxidases including laccase, which is involved in the degradation of lignin as well as in that of various xenobiotic compounds and particularly dyes [74].

Waste-water from textile and leather industries, particularly in developing countries requires treatment in an environmentally safe manner. Many studies have shown that a variety of cultured bacteria have efficient enzymatic systems that can carry out complete mineralization of dye chemicals and their metabolites (aromatic compounds) over a wide range of environmental conditions.

Pulp and paper industrial effluent is rich in recalcitrant compounds and causes pollution. For the treatment of such compounds, activated sludge process is frequently used. A bacterial mixture of *Klebsiella* sp., *Alcaligens* sp., and

Cronobacter sp. efficiently remove the pollutants from the wastewater of pulp and paper mills [75]. Pulp and paper mills are some of the main water- and energy-intensive industries responsible for large-scale water pollution. Paper manufacturing releases chlorinated lignosulphonic acids, chlorinated resin acids, chlorinated phenols (trichlorophenol, trichloroguicol, tetrachloroguicol, dichlorophenol, dichloroguicol, and pentachlorophenol), and chlorinated hydrocarbon into the effluent wastewater. They are the major contaminants formed in the effluent of pulp and paper mills. Some compounds in the effluents are resistant to biodegradation and can accumulate in the aquatic food chain. Considerable interest has been focused on the use of biotechnology in pulp bleaching, as large numbers of microbes and the enzymes produced by them are known to be capable of preferential degradation of native lignin and complete degradation of wood. Certain white-rot fungi can delignify kraft pulps increasing their brightness and their responsiveness to brightening with chemicals [76].

Waste from cattle, pigs, and poultry intensive husbandry is a valuable source of nutrients and renewable energy. However, most of the waste is still collected in lagoons or left to decompose in the open, which poses a significant environmental hazard. The air pollutants emitted from manure include methane, nitrous oxide, ammonia, hydrogen sulfide, volatile organic compounds, and particulate matter, which can cause serious environmental concerns and health problems [77].

Panus tigrinus strains (M609RQY and M109RQY) by their lignolytic enzymes have degradation potential in abundant agroindustrial wastes under solid-state fermentation conditions [78]. The huge capacities of laccases to reduce several types of organic and nonorganic pollutants strongly suggest deep evaluation of their activities on one side toward such pollutants and on the other side on the reduction of lignocellulosic content of farmed animals manure.

Mycotoxins are secondary metabolites produced by fungi, particularly the genus *Penicillium, Aspergillus,* and *Fusarium*, having adverse effects in humans and animals. Several hundreds of mycotoxins have been identified. Aflatoxin, ocratoxin, fumonisin, deoxinivanlenol, and zearalenone are the most toxic [79]. Mycotoxins toxicity is chronic with teratogenic and carcinogenic effects resulting from regular ingestion [80]. Mycotoxins are small molecules, almost water-insoluble and resistant at 250°C. They can contaminate several foodstuffs such as peanuts, almonds, nuts, corn, rice, wheat, soya beans, etc. In several African and Asian countries, the problems created by aflatoxins to human health are an enormous issue. In Benin and Togo, aflatoxins induce heavy retardation in the growth of children. The techniques more often used to protect farmed animals from mycotoxicosis are based either on the use of adsorbants (hydrated natrium aluminosilicates, zeolite derivatives, bentonite, etc.), or of chemicals (acids, bases—ammonia and caustic soda, oxidants—hydrogen peroxide, chlorides, etc.) [81,82].

Biological methods utilize lactic bacteria, bifidobacteria, and other microorganisms able to hydrolyze the mycotoxins (*Corynebacterium rubrum*) or to

convert them (*Rhizopus*) [83]. An enzyme, fumonisin esterase obtained from *Komagataella pastoris*, has been recently introduced onto the market (FUMzyme) for detoxification of fumonisin B1 in swine and poultry feed [84]. It should be remembered that the more efficacious form of fight against mycotoxins is to utilize adequate agronomic and storage techniques.

Details on decontamination by laccases can be obtained in Ref. [85] and in Chapter 17 of this book—Enzymatic decontamination of antimicrobials, phenols, heavy metals, pesticides, polycyclic aromatic hydrocarbons, dyes, and animal waste.

7.5 SYNTHESIS OF BIOACTIVE COMPOUNDS BY LACCASES

Laccases are enzymes that have shown encouraging potential as biocatalysts in the synthesis of bioactive compounds with new applications in medicine, pharmaceutical, agricultural, and food industries. They are interesting potential industrial catalysts in organic synthesis since they are essentially green catalysts with a diverse substrate range. Their reaction only requires molecular oxygen and releases water as the only by-product. Laccase catalysis involves the abstraction of a single electron from their substrates to produce reactive radicals. The free radicals subsequently undergo homo- and hetero-coupling to form dimeric, oligomeric, polymeric, or cross-coupling products which have practical implications in organic synthesis. Potential laccase applications are organic synthesis, hair and textile dyeing, polymer synthesis, and grafting processes [86].

Besides catalyzing catabolic and depolymerization processes, based on reaction conditions, laccases can also carry out synthetic processes including the oxidization of aromatic compounds followed by heteromolecular coupling with cosubstrates or simple oligomerization [87]. The main compounds that have been synthesized by laccase-catalyzed reactions include flavonoids, HCAs, and other phenolics.

The antioxidant activity of flavonoids derives from the B-ring, which is important for the H-transfer, and 2−3 double bond ensuring electron delocalization. Moreover, in vitro studies have demonstrated the importance of the 3-OH group for the antioxidant capacity. Rutin has been oxidized by a laccase from *Myceliophtora thermophyla* to produce flavonoid polymers [88]. The same result was achieved by using *Pycnoporus coccineus* and *P. sanguineus* laccases as biocatalysts. Oxidized poly-rutin showed enhanced antioxidant, antiinflammatory, and antiaging capacities compared to the rutin monomer [89].

Laccase-mediated oxidation has been shown to be a good strategy for developing functionalized polymers with enhanced antioxidant and antimicrobial activities. Oxidation of tannic acid by a laccase from *T. versicolor* resulted in a variety of products including gallic acid, gallic acid dimers, partially gallic acid-esterified glucose, and glucose, while oxidation of quercetin offered an oligomeric derivative [90]. Laccase-mediated oxidization was also performed in organic solvents, due to their advantages as media in biocatalysis. The products may serve as lead

for the development of new drugs and as nutraceuticals, showing antioxidant activity comparable to resveratrol and its analogs [91].

The rapidly increasing problem of antimicrobial-drug resistance requires the development of new antimicrobial agents. The laccase-catalyzed amination of dihydroxy aromatics is a new and promising method to enlarge the range of currently available antibiotics. Methyl-1,4-hydroquinone and 2,3-dimethyl-1,4-hydroquinone aminated with amino-β-lactam structures allowed us to obtain 14 novel cephalosporins, penicillins, and carbacephems. All were stable in aqueous buffer and resistant to the action of β-lactamases. They inhibited the growth of several Gram-positive bacterial strain,s including multidrug-resistant *Staphylococcus aureus* and *Enterococci* [92].

7.6 FOOD APPLICATIONS OF LACCASES

Laccases have great potential to be used as processing tools in the food industry, as food additives in food processing, and can make food processing more economical and environmentally friendly [93]. To realize this potential it would require more efficient laccase production systems and better understanding of their mechanisms of action. Redox potentials of laccases from common laccase-producing fungi are reported as 450 mV for *M. thermophila*, 750 mV for *P. cinnabarinus*, 780 mV for *Botrytis cinerea*, and 790 mV for *T. villosa* [94]. It is known that browning is one of the major defects in beverages. Color stability is found to be greatly increased in fruit juices after treatment with laccases. Naturally occurring phenolics and their oxidation products are found to be present in many fruit juices, which add color and taste to them. The natural cooxidation reactions and polymerization of phenols and polyphenols result in undesirable changes in aroma and color. The color change is observed as enzymatic darkening, which increases due to a higher concentration of polyphenols naturally present in fruit juices. Ribeiro et al. and Giovanelli and Ravasini [95,96] used laccase in combination with filtration in the stabilization of apple juice. Laccase treatment causes removal of phenols with higher efficiency compared to other methods, such as activated coals [97]. Compared to conventional treatments, such as addition of ascorbic acid and sulfites, laccase treatment has also been found to be more effective for color and flavor stability [98].

In the bread-making process, it is a practice to add dough-improvement additives to the bread dough, which results in improved flavor, texture, volume, and freshness of the bread/dough. Laccase exerts an oxidizing effect on the constituents of dough which then improves the strength of gluten structures in baked products. Use of laccase in dough results in an improved crumb structure, an increased volume, stability, strength, softness of the baked product, and reduced stickiness. Laccase reduces the dough extensibility in both flour and gluten dough, and increases the resistance of dough to its maximum [99]. The addition

of laccase to oat flour leads to an increased loaf specific volume and addition of proteolytic enzymes to it; simultaneously crumb hardness and chewiness is reduced which results in significant improvements in the texture quality of oat bread. The phenolic compounds are reduced by the induction of laccase, which acts as an inhibitor of fermentation [100,101].

Wine stabilization is one of the main applications of laccase in the food industry as an alternative to physical-chemical adsorbents. Musts and wines are complex mixtures of different chemical compounds such as ethanol, organic acids (aroma), salts, and phenolic compounds (color and taste). Polyphenol removal must be selective to avoid an undesirable alteration in the wine's organoleptic characteristics. Laccase presents some important requirements when used for the treatment of polyphenol removal in wines, such as stability in acid medium and reversible inhibition with sulfite. Additionally, a laccase has been commercialized for preparing cork stoppers for wine bottles. The enzyme oxidatively reduces the characteristic cork taint and/or astringency, which is frequently imparted to aged bottled wine [10].

The storage life of beer depends on different factors [102] such as haze formation, oxygen content, and temperature. The former is produced by small quantities of naturally occurring proanthocyanidins, polyphenols that generate protein precipitation and, therefore, the formation of haze. This type of complex is commonly found as chill-haze and appears during cooling processes but may redissolve at room temperature or above. Even products that are haze-free at the time of packing can develop this type of complex during long-term storage. Thus, the formation of haze has been a persistent problem in the brewing industry. The use of laccases for the oxidation of polyphenols as an alternative to the traditional treatment has been tested by different authors [103]. However, laccases have also been used for the removal of oxygen at the end of the beer production process.

The physicochemical deterioration of food products is a major problem related to the evolution of storage and distribution systems and influences the consumer's perception of the product quality. Thus, different uses of laccase have promoted odor control, taste enhancement, or reduction of undesired products in several food products [104].

A crude laccase from *Coriolus versicolor* is used to improve the flavor and taste of cacao nib and its products. Bitterness and other unpleasant tastes are removed by the laccase treatment, and the chocolate manufactured from the cacao mass tastes better than the control. Oil products may be deoxygenated by adding an effective amount of laccase. The color of tea-based products can be enhanced when treated with laccase from a *Pleurotus* species, and chopped olives in an olive–water mixture were treated with laccase from *T. villosa*.

The sugar beet pectin is a functional food ingredient that can form thermoirreversible gels. These types of gels are very interesting for the food industry as they can be heated while maintaining the gel structure. Laccases are more efficient as gelling agents in luncheon meat and milk than peroxidase. In addition, in many countries it is prohibited to add hydrogen peroxide to food products,

making it impossible to use peroxidases as gelling agents. Hence, it is more realistic to add laccase to food products.

The use of laccases for improving the sensing parameters of food products is not limited to treatment processes but is also involved in diagnosis systems. In this regard, different amperometric biosensors based on laccases have been developed to measure polyphenols in different food products (e.g., wine, beer, and tea).

7.7 FEED APPLICATIONS OF LACCASES

Ruminants dispose of an alloenzymatic provided by the rumen microbes and they can live by obtaining nutrients which are not available by endogenous digestion, as is the case in monogastric species [105]. These latter obtain their nutrients without any other physiological means except the endogenous hydrolases and eventually the enzymes added to the feed [106].

In ruminant nutrition, there is an increasing interest in ingredients that do not compete with human nutrition. Ruminants are specialists in digesting carbohydrates in plant cell walls, therefore lignocellulosic biomass has potential in ruminant nutrition. The presence of lignin in biomass, however, limits the effective utilization of cellulose and hemicellulose. Currently, most often chemical and/or physical treatments are used to degrade lignin. White-rot fungi are selective lignin degraders and can be a potential alternative to current methods which involve potentially toxic chemicals and expensive equipment. Research is conducted on fungal pretreatment of lignocellulosic biomass for ruminant feeds. White-rot fungi colonize lignocellulosic biomass, and during colonization produce enzymes, radicals, and other small compounds to breakdown lignin. The mechanisms of how these fungi degrade lignin are not fully understood, but fungal strain, the origin of lignocellulose, and the culture conditions have a major effect on the process.

Ceriporiopsis subvermispora and *Pleurotus eryngii* are the most effective fungi to improve the nutritional value of biomass for ruminant nutrition. However, conclusions on the effectiveness of fungal delignification are difficult to draw due to a lack of standardized culture conditions and information on fungal strains used. Methods of analysis between studies are not uniform for both chemical analysis and in vitro degradation measurements. In vivo studies are limited in number and mostly describe digestibility after mushroom production, when the fungus has degraded cellulose to derive energy for fruit body development. Optimization of fungal pretreatment is required to shorten the process of delignification and make it more selective for lignin. In this respect, future research should focus on optimization of culture conditions and gene expression to obtain a better understanding of the mechanisms involved and to allow the development of superior fungal strains to degrade lignin in biomass [107].

7.8 LACCASES AND VALORIZATION OF PLANT BIOMASS

Enormous quantities of organic wastes from agricultural and food processing industries which are lignocellulosic in nature are generated annually [108]. A large proportion of these wastes are dumped or left to rot near the factories, burnt, or shredded and composted, and used for landfill or improving soil quality [109]. However, with the application of appropriate bioconversion technology, these wastes represent a valuable resource, especially for areas with agricultural-based economies.

Edible mushroom cultivation represents one of the most economically viable processes for the bioconversion of these lignocellulosic wastes, which include diverse materials such as cereal straws, bagasse, sawdust, cotton wastes, banana leaves, coffee grounds, and sisal wastes [110,111]. Utilization of insoluble lignocellulosic substrates by these fungi depends on their capability to synthesize the relevant hydrolytic (cellulases and hemicellulases) and oxidative (ligninolytic) extracellular enzymes [112].

In Tanzania, high quantities of solid sisal wastes are produced annually as only 2% of the plants are currently used for sisal fiber production, while the remaining 98% are commonly disposed of in the raw into the environment [113].

Some fungi such as *M. thermophila* M77 have the capacity to produce a set of enzymes, represented by secretomes, involved in the hydrolysis of pectins, cellulose, hemicellulose, and lignin [30]. Laccases utilize molecular oxygen as final electron receptors, and thus only produce water as final subproduct. Accordingly, they are neutral from an environmental point of view, having an enormous potential for biotechnological applications. They are already utilized in several industries and also as bioremediation tools [28].

The reduced sugar yield in lignocelulose hydrolyzates is mainly due to the presence of lignin which prevents the accessibility of cellulosic compounds to the enzymes. The pretreatment of plant biomass with laccases, instead of utilizing the classical physical and chemical methods appear as an alternative to be developed [114].

In recent years, more than 150 laccases have been isolated from fungi, bacteria, and archea, including from extremophiles. Most of them have been well characterized and expressed. It is expected that improved enzymes will be soon available.

In monogastric nutrition laccases can be used either as feed pretreatment or as feed additive. Taking into account the short duration of the gastrointestinal content transit in the small bowel, in broilers [115] as well as in pigs [116], insufficient for a reasonable action of the enzyme, it appears that pretreatment will be the preferable technique. In any case, the use of lignolytic enzymes should be the simplest and most economic method to improve the digestibility of lignocellulosic biomass in monogastric animals.

Laccases are promising biocatalysts with many possible applications. The immobilization of enzymes offers several improvements for enzyme applications

because the storage and operational stabilities are frequently enhanced. Moreover, the reusability of immobilized enzymes represents a great advantage compared with free enzymes. Different methodologies of enzyme immobilization have been reported for laccases, such as adsorption, entrapment, encapsulation, covalent binding and self-immobilization [117].

7.9 CONCLUSIONS AND PERSPECTIVES

Laccases have been found to play a major role during lignin degradation and have therefore been considered with regard to potential applications for biomass valorization. Laccases can assist the pretreatment of biomass and promote the subsequent enzymatic hydrolysis of cellulose by the oxidative modification of residual lignin on the biomass surface. Alone or in combination with mediator molecules, laccases are able to catalyze the depolymerization of lignin. They have also a strong polymerization activity that counters the decomposition of lignin by repolymerizing the degradation products [61]. Therefore, it is a key challenge to shift the catalytic performance of laccases towards lignin cleavage by optimizing the process. Other important applications of laccases are the detoxification of biomass hydrolyzates and bioremediation of pollution by several compounds.

The biotechnological significance of laccase enzymes has led to a drastic increase in the demand for these catalysts in recent times. Laccases are promising to replace the conventional chemical processes of several industries. However, one of the limitations to the large-scale application of the enzyme is the lack of capacity to produce large volumes of highly active enzyme at an affordable cost. These problems can be solved with the use of recombinant organisms or screening for natural hypersecretory strains.

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CHAPTER

Amylases

8

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8.1 THE IMPORTANCE OF CARBOHYDRATES AND AMYLASE IN HUMAN NUTRITION

Starch is a major storage form for carbohydrates in nearly all green plants, making it the most abundant biopolymer in nature in addition to cellulose. Plant foods containing high quantities of starch were essential in pushing the course of human evolution in Pleistocene as digestible carbohydrates were necessary to accommodate the increased metabolic demands of a growing brain [1]. Available carbohydrates comprise 40%−75% of modern dietary energy intake [1a], of which starch is the most abundant. Starch is digested slowly and incompletely in raw crystalline form, but more rapidly after cooking. Modern humans require a reliable source of glycemic carbohydrate to support the normal functioning of our brain, kidney medulla, red blood cells, and reproductive tissues. The brain alone accounts for 20%−25% of adult basal metabolic expenditure [2]. In addition to the demands of the brain, red blood cells require approximately 20 g of glucose per day directly from the bloodstream [3]. Under normal circumstances, a glucose requirement of approximately 170 g/day is met by a mixture of dietary carbohydrate and gluconeogenesis from noncarbohydrate sources, such as the glycerol moiety of fats, some amino acids (e.g., alanine), or absorbed propionate from gut fermentations of dietary carbohydrates [4].

Considerable amounts of information in agreement with or contradicting the viewpoint are available when debating whether dietary carbohydrates are actually essential for human nutrition. During starvation or, in the absence of dietary carbohydrate, gluconeogenesis alone is usually not sufficient and the brain begins to utilize ketones, a by-product of high levels of fat oxidation and body reserves of glycogen being limited, provides glycemic carbohydrate for only 18−24 hours of fasting to sustain basic physiological functions of the body [5,6]. Under these conditions, about 80% of the brain's energy needs can be met from ketones but to maintain normal brain function in individuals adapted to an essentially carbohydrate-free diet there remains an absolute requirement for 30−50 g of dietary glycemic carbohydrate per day to fill the gap between gluconeogenic capacity and the brain's requirement for

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glucose [5]. In more prolonged fasting, or under starvation conditions, there is considerable loss of tissue protein, which is broken down out of necessity to provide the amino acids for gluconeogenesis. Indeed, death due to starvation is as much a consequence of critical loss of tissue protein to supply energy to the brain as it is the reduction in the body's fat stores. In an evolutionary context, large stores of glycogen must be generated in order to provide sources of glucose for periods of sustained fasting or hardship. To build these reserves, the diet must consistently provide energy surplus to basal metabolic requirements. There is a limit, considered to be 35%–40%, to the amount of energy requirements humans can derive from proteins; above this ceiling protein toxicity can occur, which can cause death quite rapidly [1,7].

A daily carbohydrate intake of about 50–100 g is considered essential to prevent ketosis in adults, and is consistent with a more realistic recommendation for the practical minimal requirement of 150 g/day of glycemic carbohydrate intake beyond the ages of 3–4 years as brain size increases rapidly and at least one-third of dietary energy should be supplied from carbohydrates [8]. The additional needs for pregnancy and lactation are not taken into consideration in this calculation [1]. Pregnant females have a minimal requirement for 70–130 g/day of preformed glucose or glucose equivalents to maintain optimum cognitive function in the mother (at peak lactation, mammary glands require an additional 70 g glucose/day for synthesis of lactose, the main sugar in milk) and to nourish the fetus; thereby, fetal growth is directly correlated with maternal glucose concentration [1,9] (Fig. 8.1).

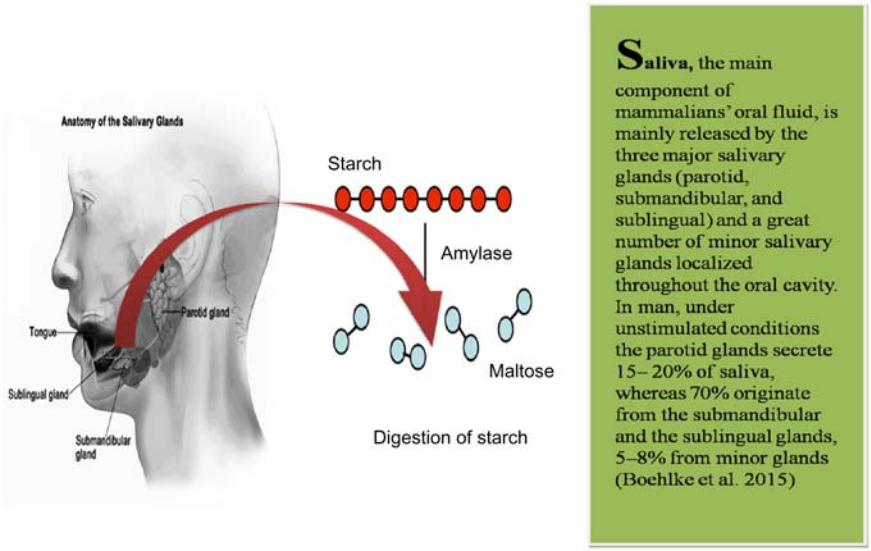

FIGURE 8.1

Mechanism of salivary amylase working on complex starch macromolecules.

Starch is composed of amylose, a mostly linear polymer of α(1→4)-linked anhydro-glucose unit and amylopectin, a highly branched polymer via additional α(1→6)-linkages. Cellulose, in contrast, has linear β-(1→4)-linked anhydro-glucose chains with strong inter- and intramolecular hydrogen bonding, and it has been elucidated that small amounts of cellulosic materials (pure cellulose or wheat bran) have inhibitory effects on starch digestion [9a].

Normally, utilization of plant foods containing starch by plants or other organisms requires initial solubilization of the starch and conversion to dextrins and oligosaccharides by amylases. Various organisms have developed different strategies for starch breakdown, but all involve an initial attack by an amylase to enable the organism to convert insoluble starch (or glycogen) into low-molecular-weight, water-soluble dextrins and sugars, perhaps concomitantly with another enzyme such as a dextrinase or debranching enzyme, followed by a glucosidase which converts the dextrins or oligosaccharides to glucose [10].

Many types of amylases are found throughout the animal, vegetable, and microbial kingdoms. They have evolved along different pathways. Generally they are classified into two types; the α- and β-amylases varying into two distinct mechanistic classes: those hydrolyzing the glycosidic bond with net inversion of anomeric configuration (α-amylase), and those doing so with net retention [11]. The history of amylases began in 1811 when the first starch-degrading enzyme was discovered by Kirchhoff. This was followed by several reports of digestive amylases and malt amylases. It was much later in 1930, that Ohlsson suggested the classification of starch digestive enzymes in malt as α- and β-amylases according to the anomeric type of sugars produced by the enzyme reaction. α-Amylase (1,4-α-D-glucan-glucanhydrolase, EC. 3.2.1.1) is a widely distributed secretary enzyme [12].

Amylases are among the most effective enzymes known. For porcine pancreatic α-amylase and sweet potato β-amylase the turnover numbers are of the order of 1000 per second per molecule of enzyme earning a revered status in present-day biotechnology with applications ranging from food, fermentation, textiles to paper industries, constituting a class of industrial enzymes having approximately 25% of the world enzyme market ([13–17]).

8.1.1 AMYLASES: UNITY IN DIVERSITY

Amylase was among the first enzyme to be discovered and isolated, and for last millennium its various roles have been widely researched and discussed [18–20]. Some of these enzymes exist in multimeric forms. Sweet potato β-amylase is a tetramer [21,22]. Porcine pancreatic α-amylase is apparently synthesized as a single chain of 56,000 daltons, but it undergoes some proteolysis to give smaller associated fragments with retention of activity [23]. Amylases from different tissues of a single animal species are very similar, apparently as a consequence of gene duplication and mutation, as well as tissue-specific regulation and expression [24]. The human amylase gene cluster is a highly repetitive, copy number-variable region that has undergone expansion in gene number specifically in the human lineage [25].

Conversely, the α-amylases from various bacteria, especially extremophiles, e.g., *Bacillus subtilis* (Refs. [12,26]), are surprisingly similar in their product specificity to the α-amylases of germinating cereals [27]. Also, the animal α-amylases give a set of branched oligosaccharides essentially identical to those obtained by the action of the fungal α-amylases, e.g., Taka amylase [28].

Carpenter et al. [29] discussed in detail the diversity expressed in terms of Copy Number Variation (CNV) which measures the true range of variation, in particular, by the human amylase-producing gene cluster AMY1 as well as pancreatic amylase gene clusters AMY2A and AMY2B. Their findings suggest that the AMY2A deletion allele has a frequency of about 7% in Europeans, so that about 1 in 200 people will lack this gene product completely, opening the scope for further studies probing insights to uncover new patterns in human amylase variation and implying a potential role for AMY2 CNV in functional associations as well as its phenotypic consequences. Previously, Perry et al. [25] reported that Higher AMY1 copy numbers and protein levels likely improve the digestion of starchy foods, and may buffer against the fitness-reducing effects of intestinal diseases. Interestingly, they also noted that more AMY1 copies can be found in *Homo sapiens sapiens* than in our nearest living relative, the chimpanzees (Fig. 8.2). Before the expansion of agriculture and domestication of animals started, wild plant and wild animal parts represented the main components of the preagricultural hominid diet [30]. Consequently, the population of hunters and gatherers was not highly affected by caries mainly because food components such as dairy products, cereals and refined sugar were not available [31,32].

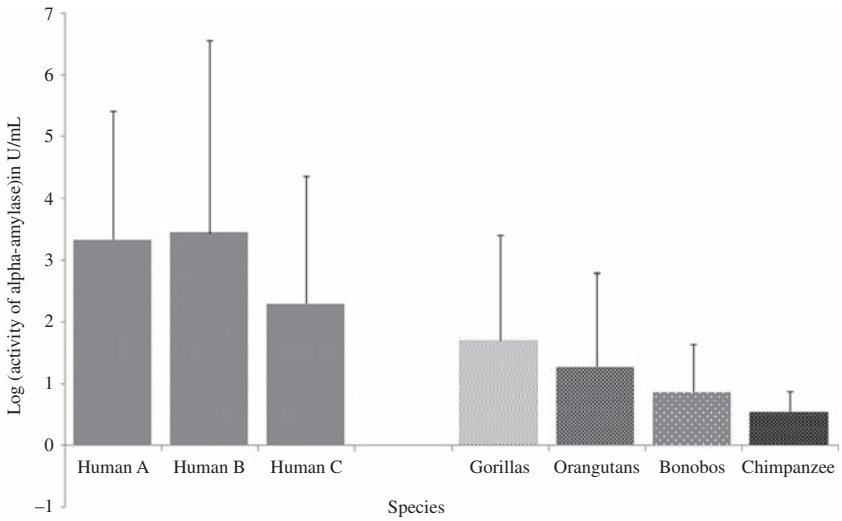

FIGURE 8.2

Comparison between α-amylase activity in humans and closely related primates.

8.1.2 MODE OF ACTION

A large number of reviews do exist dealing in detail with the mode of action of amylases [11,33–38], and α-amylases in particular [12] as well as their exemplary thermal adaption mechanism as found in extremophiles [26]. α-Amylase interacts with its substrate, starch, through a specific binding mechanism, involving five contiguous α (1→4)-linked glucose residues, a high-affinity and highly specific interaction that may be blocked by active site-specific inhibitors, e.g., maltose oracarbose [26a]. Amylases of this groups act via the anomeric inversion mechanism where one residue acts as a general acid and the other as a general base.

Amylases can be further divided into two categories based on their cleaving mechanism as, endo-amylases and exo-amylases. Endo-amylases catalyze hydrolysis in a random manner in the interior of the starch molecule. This action causes the formation of linear and branched oligosaccharides of various chain lengths. Exoamylases hydrolyze from the nonreducing end, successively resulting in short end products [11]. An in-depth review of the methods of determination of the α-amylase activity was performed by Gupta et al. [12] including all the traditional and present-day internationally accepted Falling number method and Amylograph/Farinograph test. To determine amylase activity, different specific und nonspecific methods can be used. With the help of electrophoretic and analytical immunochemical procedures such as polyacrylamide gel electrophoresis (PAGE) and enzyme-linked immunosorbent assay, proteins, e.g., α-amylase, can be detected and quantified but it is not a specific method for the salivary enzyme. A very successful method for purifying individual salivary proteins is represented by a combination of PAGE with electrophoretic elution and concentration [32].

α-Amylases are dextrinogenic and can attack the interior of starch molecules. The products retain the α anomeric configuration. β-Amylases act only at the nonreducing chain ends and liberate only β-maltose. Both α- and β-amylases exhibit multiple (repetitive) attacks; i.e., after the initial catalytic cleavage, the enzyme may remain attached to the substrate and lead to several more cleavages before dissociation of the enzyme–substrate complex. The two mechanisms differ in that inverting glycosidases (Fig. 8.3) operate via a direct displacement of the leaving group by water, whereas retaining glycosidases utilize a double displacement mechanism involving a glycosyl-enzyme intermediate. Despite these differences, it is noteworthy that both classes employ a pair of carboxylic acids at the active site. Both inverting and retaining enzymes catalyze glycoside hydrolysis via transition states with substantial oxocarbonium ion character [10,11].

Ptyalin present in human saliva is an α-amylase, requires Cl-ion for activation and optimum pH 6–7. The enzyme hydrolyzes α-(1,4) glycosidic linkage at random, from molecules such as starch, glycogen, and dextrins, producing smaller molecules maltose, glucose, and disaccharides maltotriose. Ptyalin action stops in the stomach when pH falls to 3.0 [39,40]. Other examples of α-amylases are pancreatic amylases which hydrolyze terminal α-(1–4), glycosidic linkages in polysaccharides and oligosaccharide molecules liberating free glucose molecules [35].

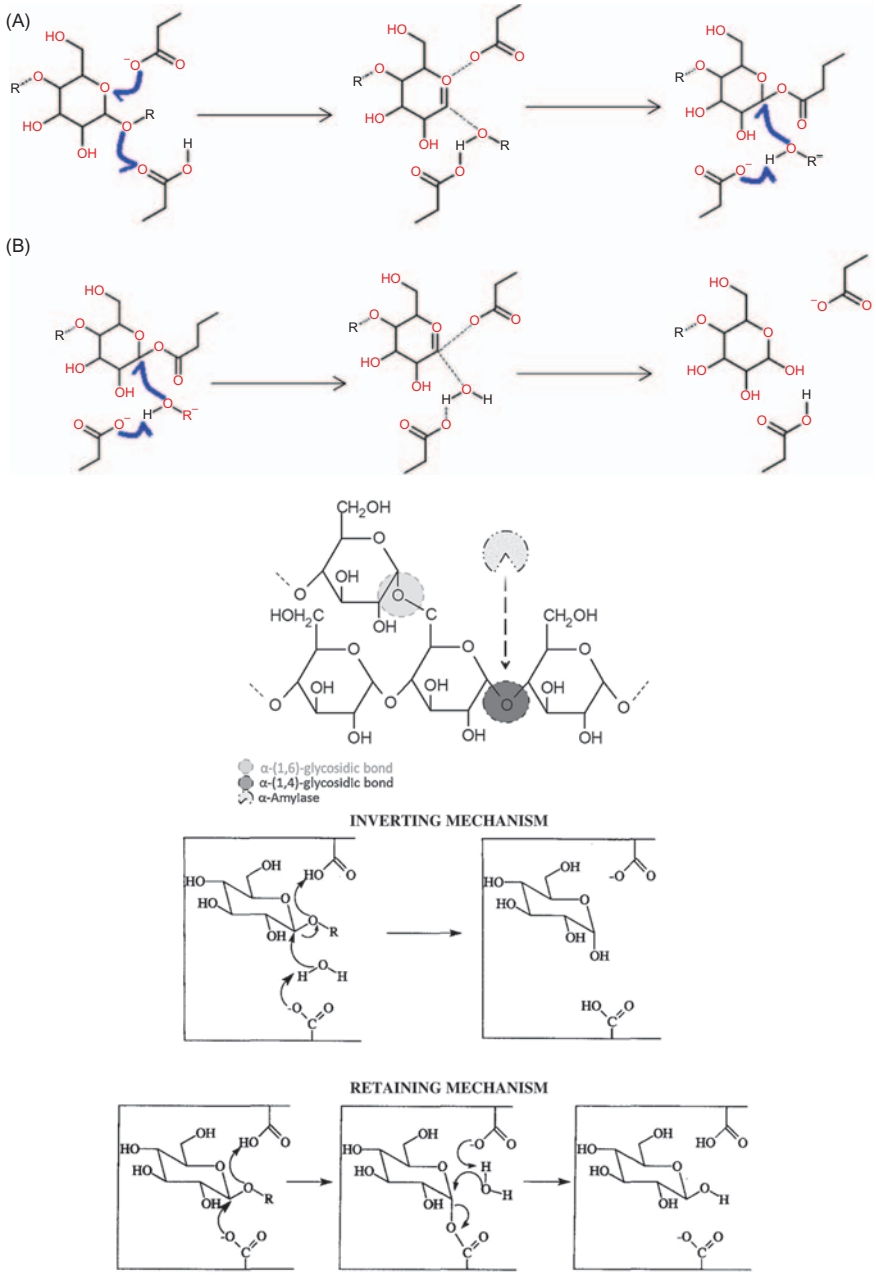

FIGURE 8.3

The proposed mechanisms of action of α-amylases (A) via anomeric inversion and β-amylase (B) via retaining mechanism and the bonds cleaved during the process.

McCarter JD, Withers GS. Mechanisms of enzymatic glycoside hydrolysis. Curr Opin Struct Biol 1994;4 (6):885–892; Boehlke C, Zierau O, Hannig C. Salivary amylase – the enzyme of unspecialized euryphagous animals. Arch Oral Biol 2015;60:1162–1176; Azzopardi E, Lloyd C, Teixeira SR, Conlan RS, Whitaker IS. Clinical applications of amylase: novel perspectives. Surgery 2016;160(1):26–37.

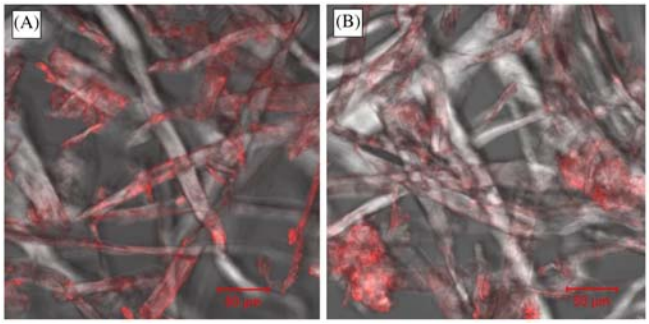

FIGURE 8.4

Representative confocal microscopy images of TRITC-amylase conjugate bound on to the cellulose surface. (A) Before washing; (B) after two successive washes.

Dhital, S., Gidley, M. J., & Warren, F. J. (2015). Inhibition of α-amylase activity by cellulose: Kinetic analysis and nutritional implications. Carbohydrate polymers, 123, 305–312.

α-Amylases display the highest specificity towards starch followed by amylose, amylopectin, cyclodextrin, glycogen, and maltotriose, and a pH optima of 2–12 while showing a huge range of thermal tolerance from 25–30°C as in *Fusarium oxysporum* amylase up to 100–130°C for archaebacteriea like *Pyrococcus furiosus* and *Pyrococcus woesei*, respectively [12,41,42]. The attachment of α-amylases to cellulose has been studied in detail by Dhital et al. (2015) and their confocal microscopy images of tetramethylrhodamine (TRITC)-amylase conjugate in the presence of cellulose shows apparently homogenous binding of the amylase to the cellulose surface (Fig. 8.4).

Out of various other utilizations in a myriad number of industrial processes ranging from pharmaceauticals including surgery [43] to textiles, as reviewed in depth by Godfrey and West [43a], the use of amylases in the food-producing sector needs a special emphasis. To date, the α-amylases used in baking have been cereal enzymes from barley malt and microbial enzymes from fungi, and bacteria after due confirmation of their GRAS status [44]. Supplementation of flour with exogenous fungal α-amylase having higher activities is common in the present day modern and continuous baking process [45]. α-Amylase supplementation in flour not only enhances the rate of fermentation and reduces the viscosity of dough (resulting in improvements in the volume and texture of the product, but also generates additional sugar in the dough, which improves the taste, crust color and toasting qualities of the bread. One of the new applications of α-amylase in the industry has been in retarding the staling of baked products, which reduces the shelf life of these products [12,44,46].

8.2 AMYLASES IN ANIMAL NUTRITION

Carbohydrates represent a sizeable portion of the animal diets and are usually given in bulk owing to their low price and easy availability. To digest the bulk amount of

the carbohydrates in diet the animals must have the amylases working on the complex starch macromolecules to break them into assimilative fractions. Boehlke et al. [32] discussed in depth an alternate role of the amylase that is responsible for the pellicle (thin coating layer of high tenacity covering all solid surfaces in the oral cavity) formation in the oropharyngeal region of unspecialized euryphagous animals. This process is rapid and instantenous with amylase acting as the harboring enzyme for the colonizing bacteria and thereby protecting the enamel of the tooth. Apart from the misconception that pellicle or in advanced form, caries, are detrimental to the oral hygiene of euryphagous animals, they have myriad number of beneficial roles in the physiology of digestion of humans and animals such as being a physiological protective coating, a lubricant to the tooth surface, serving as a barrier and buffer to demineralizing agents, [47] and immobilizing several antibacterial biomolecules of profound significance [48]. He further concluded that in contrast to carnivores and most herbivores, omnivores have considerable amounts of amylase in their saliva (Fig. 8.4, Table 8.1). Focusing on ruminants, negligible levels or an absence of α-amylase was determined. Presence and activity probably differ depending on the species-specific diet. Animals feeding on unripe fruits, seeds, roots, and bulbs exhibit higher activity of salivary α-amylase than species consuming ripe fruits, insects, and vertebrates (Fig. 8.5).

8.2.1 PORCINE AMYLASES

Focusing on farmed animals, the domesticated pig (*Sus scrofa domesticus*) is omnivorous like other pigs, and a number of authors obtained similar but individually slightly variable values for the activity of salivary α-amylase. Ohya et al. [49] and Rajasingham et al. [50] detected a higher activity in domesticated pig than in the pure herbivorous horse (*E. ferus caballus*). In contrast to horses, whose natural diet contains comparably little cereals, pigs feed naturally on starch-containing corns and roots. Beside this, salivary α-amylase activity increased significantly but variably among animals as a result of stress and particularly in pigs, α-amylase was described as a noninvasive stress biomarker [32].

8.2.2 AMYLASES IN FISH NUTRITION

Carbohydrate constitutes one of the three prime components of the fish feeds that are used as energy sources to support growth. Although carbohydrate is not indispensable in fish feed, it constitutes an inexpensive source of energy. Protein is the costliest item in any feed and its share is comparatively higher in aqua-feed. Thus, replacing dietary protein with carbohydrate or lipid not only reduces production costs, but also solves the problem of the discharge of nitrogenous effluent from the culture system. In the absence of carbohydrate there is increased utilization of proteins and lipids as energy sources. In many species a dietary carbohydrate supply appears to be necessary, as it improves growth and, especially, protein utilization [51–53].

Table 8.1 Summary of Salivary α-Amylase Activities in Species Categorized Into the Three Nutrition Types

Nutrition Type	Species	α-Amylase Activity
Carnivorous	Domesticated dog (*Canis lupus familiaris*)	−
	Domesticated cat (*Felis silvestris f. catus*)	−
Herbivorous	Common wombat (*Vombatus ursinus*)	+
	Koala (*Phascolarctos cinereus*)	−
	Red kangaroo (*Macropus rufus*)	+
	Domesticated goat (*Capra hircus*)	−
	Holstein Friesian cow (*Bos taurus taurus*)	−
	Cattle (*Bos primigenius f. taurus*)	+
	Horse (*Equus ferus caballus*)	−
	Rabbit (*Oryctolagus cuniculus f. domestica*)	−
	Elephant (*Loxodonta africana*)	−
Omnivorous	Domesticated guinea pig (*Cavia porcellus*)	+
	Domesticated brown rat (*Rattus norvegicus*)	+
	Domesticated pig (*Sus scrofa domesticus*)	+
	Hedgehog (*Erinaceus europaeus*)	+
	Mouse (*Mus musculus*)	+
	Bank vole (*Myodes glareolus*)	+
	Domesticated hamster (*Mesocricetus auratus*)	+
	Japanese macaque (*Macaca fuscata*)	+
	Crab-eating macaque (*Macaca fascicularis*)	+
	Common squirrel monkey (*Saimiri sciureus*)	±
	Olive baboon (*Papio anubis*)	+
	Collared mangabey (*Cercocebus torquatus*)	+
	Baboon (*Papio hamadryas*)	+
	Chimpanzee (*Pan troglodytes*)	+
	Rhesus macaque (*Macaca mulatta*)	+
	Gelada (*Theropithecus gelada*)	+
	Bonobo (*Pan paniscus*)	+
	Gorilla (*Gorilla gorilla gorilla*)	+
	Sumatran orangutan (*Pongo abelii*)	+
	Bornean orangutan (*Pongo pygmaeus*)	+
	Human (*Homo sapiens sapiens*)	+

+, α-Amylase Activity; −, Missing α-Amylase Activity.
Source*: Boehlke C, Zierau O, Hannig C. Salivary amylase − the enzyme of unspecialized euryphagous animals. Arch Oral Biol 2015;60:1162−1176.*

It has been proposed that it is necessary to provide an appropriate level of this nutrient in the diet to ensure maximum utilization of other nutrients (Refs. [54,55]). Prather and Lovell [56] reported that, if insufficient nonprotein energy is available, then protein, being the costliest component in the fish diet aimed at

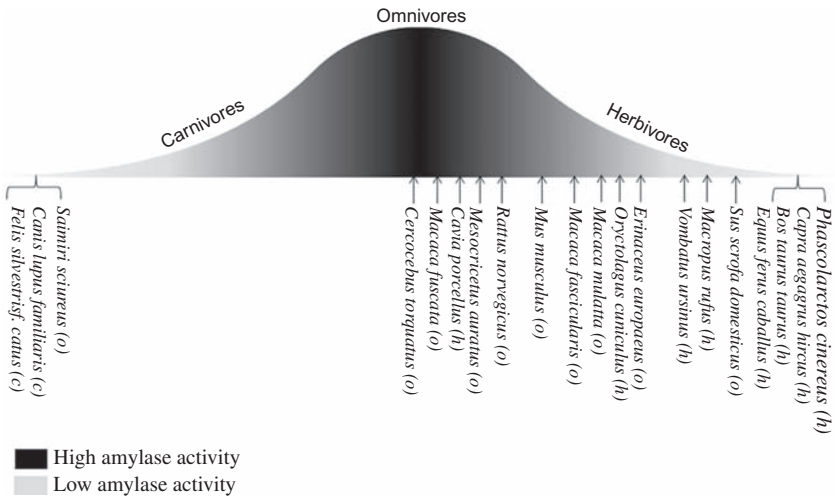

FIGURE 8.5

Comparative assays of amylase activity in between organisms with varied food preferences.

Boehlke C, Zierau O, Hannig C. Salivary amylase – the enzyme of unspecialized euryphagous animals. Arch Oral Biol 2015;60:1162–1176.

augmenting somatic growth, is first used as an energy source rather than for growth purposes. Fish, in general, are diabetic, and thereby, poorly utilize dietary carbohydrate. Furuichi and Yone [57] observed depressed growth and feed efficiency in red sea bream, yellowtail and common carp fed diets high in carbohydrate. A relative inability to metabolize carbohydrates has been reported in several studies in different fish species such as red sea bream, *Pagrus major* [58] and channel catfish, *Ictalurus punctatus* [59]. Carnivorous fish fed with high-starch diets have a poor ability to metabolize the excess glucose [60–62]. However, herbivorous and omnivorous fish have a higher capacity for glucose regulation [55]. Fish with excess glucose are assumed to be under constant metabolic stress [63], which may lead to suppressed immune functions [64]. Stress is well known to affect the susceptibility of fish to infections. Thus, high dietary carbohydrate may increase the incidence of diseases and affect the susceptibility to infectious diseases. This is of prime importance as the prevalence of diseases has jeopardized the aquaculture production in recent years and the changing climate is creating a perfect window for the opportunistic bacteria to invade the immune-compromised fishes.

Dietary modification of carbohydrate may improve its utilization in fish. These modifications are gelatinization of starch (Refs. [65–67]) and supplementation of exogenous α-amylase in the diet (Refs. [68,69]). During the gelatinization process, starch granules are modified in such a way that their susceptibility to enzyme action

increases [69a], making the digestion more complete. Exogenous enzymes are being used in the diets of poultry and pigs. The use of these enzymes has shown that low-cost raw ingredients or even less-processed materials can be used with equal and even better performance than more expensive materials, thereby increasing the wide choice and flexibility for the ingredients for the feed manufacturer [70]. Besides these, addition of exogenous carbohydrase enzymes to aquafeed has also been reported to enhance the utilization of unavailable dietary carbohydrate by Atlantic salmon, *Salmo salar* [71], larval gilthead seabream, *Sparus aurata* [72], and tiger prawns, *Penaeus monodon* [73].

For the production of low-cost feed, more carbohydrate has to be included in the feed. The capacity of fish to digest dietary carbohydrate has been reported to be variable among different species [55,74–76]. The variability may be explained by the wide variations in endogenous digestive carbohydrase activity in different fish [77–79].

Whether or not the limitation of carbohydrate utilization is caused by insufficient endogenous α-amylase is not clear. However, carp are definitely in a better position for carbohydrate utilization because of their omnivore/herbivore feeding habit [55]. Digestibility of native starch is rather low (30%–50%), whereas that of gelatinized starch is higher (50%–90%). This has been shown in trout by Bergot and Breque [65], in carp by Chiou and Ogino [80], in turbot by Jollivet et al. [81], in red sea bream (*P. major*) by Jeong et al. [82], in *Penaeus vannamei* by Davis and Arnold [83] and in European Seabass *Dicentrarchus labrax* by Peres and Oliva-Teles [84]. Pieper and Pfeffer [63] reported a higher digestibility of 95% for gelatinized corn starch in rainbow trout. Gelatinization of starch has been reported to increase the carbohydrate utilization in young carp, *Labeo rohita* [66] and *Catla catla*, significantly [85] as a result of an increase in endogenous α-amylase activity.

Carp represent almost 80%–90% of the freshwater fish species cultured in tropical countries and although 33% of the aquacultured produce still relies on the nonfed farming methods, producing fish food organisms via fertilization and manuring the water column, the onus though slowly, is changing and the world is looking forward to enhanced cultivation of the fed species owing to the predictability in terms of growth in the era of changing climatic conditions every now and then as a result of global warming and greenhouse gas emissions [86]. In recent years, research in aquaculture nutrition has focused on the partial replacement of fishmeal with plant-based protein ingredients to support the globally expanding aquaculture industry and to ensure its sustainability [86a,87]. However, most plant-based feedstuffs have a wide variety of antinutritional factors, which may impair nutrient utilization, interfering with fish performance and health. Attempts to improve the digestibility of plant proteins have included the use of hydrothermal treatments such as extrusion, as well as fractionation of crops to reduce the content of antinutritional factors and increase their protein concentration. While these approaches have been shown to improve protein and energy digestibility of numerous plant protein feedstuffs, the digestibility of many

ingredients remains a bottleneck because of a lack of the enzymes needed for breaking down the complex cell wall structure that encapsulates other nutrients [87,88]. The use of exogenous enzymes as feed additives to improve nutrient digestibility of plant-based feedstuffs has been researched extensively in poultry and swine. In aquaculture, the use of phytase to improve phosphorus utilization has emerged quite readily [89–92]. However, the use of carbohydrase enzymes has not been as nearly as common in aquatic species, despite their promising effects in improving nutrient digestibility by hydrolyzing nonstarch polysaccharides present in plant feedstuffs [74].

Kumar et al. [51] reported 42.43% nongelatinized (NG) corn, when supplemented with 50 mg α-amylase/kg feed fed at a suboptimum level of protein (27%; suboptimal in the sense that the optimum protein requirement is 40% as opined by Mohapatra et al.[66]) significantly enhanced the growth of *L. rohita* fingerlings; however, supplementation of α-amylase did not show any appreciable growth enhancement in the case of a gelatinized (G) corn diet. Furthermore, feed with 28% crude protein (CP) containing either G corn without α-amylase or NG corn with 50 mg α-amylase/kg may be used as the alternative carbohydrate source for *L. rohita* juveniles [52]. It is also noteworthy that exogenous enzyme supplementation via feed has a profound effect on the muscle DNA:RNA ratio of the cultured fishes and as shown by Kumar et al. [53], supplementation of exogenous α-amylase increased the muscle protein/DNA ratio in NG corn fed groups due to increased cell size of the muscle of the treated group (hypertrophy) in agreement with his previous studies [64].

Data on starch hydrolyzing enzyme activities such as α-amylase, alanine amino transferase (ALT), aspartate amino transferase (AST), fructose-1,6-bisphosphatase, glucose-6-phosphatase, strengthens the proposition that NG corn in the diet significantly induced more gluconeogenic and amino acid metabolic enzyme activity, whereas G corn induced increased lipogenic enzyme activity. Increased amino acid catabolic enzyme (ALT and AST) activity was observed either at optimum protein (35%) irrespective of corn type or NG corn without supplementation of α-amylase irrespective of protein level in the diet [93].

8.3 CONCLUSIONS

In contrast to carnivores and most herbivores, omnivores have considerable amounts of amylase in their saliva. It has to be verified whether α-amylase is genetically or epigenetically determined. Besides, amylase can be recognized as a nutritional and evolutionary marker pushing the evolution of hominid species since pleistocene. Although the starch-digesting enzyme has been investigated well in humans and other mammals such as swine and poultry owing to their commercial importance, the complete physiological function of amylase in saliva has not yet been explored completely yet and warrants immediate attention.

Based on the information gathered in this literature, it is clear that research on exogenous carbohydrases supplementation in aquaculture nutrition is not as extensive as in sectors such as swine and avian nutrition. Based on promising results and opportunities found in other nonruminant animals, and favorable effects found in some aquacultured species, it may be significant to devote more attention to this subject, because it could be a useful tool to further reduce the use of fish-meal in fish diets and increase the use of plant-based protein feedstuffs, ensuring aquaculture sustainability.

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PART

NSP Enzymes

III

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CHAPTER

Nonstarch polysaccharide enzymes—general aspects

9

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9.1 INTRODUCTION

The intensification of agricultural systems has converted cereal production to be the most important economic food product worldwide. Generally, in most parts of the world, cereal-based diets are adopted as caloric and protein source. Wheat, maize, rice, barley, sorghum, oats, rye, and millet are highly acceptable cereal grains which provide 56% of the food energy and 50% of the protein [1]. Indeed, the high demand on cereal markets for human consumption within the last decade has resulted in a trend for animal feeds with reduced cereals content and increased coproduct content. This phenomenon has especially been noted in areas where cereals produced are being used locally in value-added processing, including flour milling and ethanol production. Recently, due to increased production and reduced prices, coproducts have become attractive in animal feeds to reduce production costs. Generally, coproducts are lower in starch and higher in crude protein and fiber [2], and have a larger range in nutrient quality. Thus, inclusion of coproducts introduces a risk for a lower growth performance and feed utilization through its impact on animal digestive physiology. The risk of using coproducts in animal feeds can be reduced by exogenous enzymes supplementation. Some of the targets of the supplemental exogenous enzymes are nonstarch polysaccharides (NSPs), which include all the plant polysaccharides other than starch. NSPs are predominantly present as structural polysaccharides in plant cell walls where they are associated and/or substituted with other polysaccharides, proteins, and phenolic compounds such as lignin [3], can comprise up to 90% of the cell wall of plants [4], and usually constitute less than 10% by weight of the grain [5]. The most abundant NSPs in plant cell walls include cellulose, hemicellulose, and pectins; while fructans, glucomannans, and galactomannans belong to the group of NSPs that are not as abundant as cellulose, hemicellulose, or pectins, and serve as the storage polysaccharides [5].

NSPs can be classified into various groups based on their physicochemical properties, e.g., viscosity, water-holding capacity, fermentation, and the capacity to bind organic and inorganic molecules. Besides, based on the reaction with

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water, NSPs are classified as either soluble or insoluble. However, the most preferred and clear classification was proposed by Bailey [6]. Under this division, NSPs fall into three main groups, namely cellulose, noncellulosic polymers, and pectic polysaccharides. Mixed-linked β-glucans, heteroxylans (arabinoxylans and glucuronoarabinoxylans), mannans, xyloglucan, and callose come under the category of noncellulosic polymers; while polygalacturonic acids substituted with arabinan, galactan, and arabinogalactan are included in the group of pectic polysaccharides.

Plant ingredients generally contain a mixture of both soluble and insoluble NSPs in a ratio that varies according to the type and stage of maturity of grains. Soluble NSPs form dispersions when mixed with water and have the ability to increase the viscosity of digesta, while insoluble NSPs do not enhance digesta viscosity, but can be characterized by their fecal-bulking capacity [7]. Cellulose is insoluble whereas the other types of NSPs are soluble or partly soluble. Soluble NSPs slow down the diffusion of digestive enzymes and the absorption of nutrients, and these consequences lead to antinutritive effects in monogastric animals [5]. Nevertheless, NSPs through their high water-binding capacity play an important role in providing bulk to gut contents and allow easy passage through the human intestine [7], thereby playing a crucial role in the correct functioning of the digestive system [5]. Moreover, cereal-based diets contain high levels of soluble NSPs that significantly affect the efficiency of the digestive process, and impair animal performance [8]. For instance, barley incorporation in animal diets is limited by its high content of NSPs, mainly soluble 1,3-1,4-β-glucans, which upon solubilization lead to an increase in digesta viscosity. Thus, an increase in digesta viscosity affects the interaction of the endogenous digestive enzymes with their target substrates, and decreasing animal growth [8,9]. To reduce the negative effects associated with the presence of dietary NSPs, commercial exogenous enzyme mixtures (including NSP-enzymes) are widely used to supplement animal diets. These enzymes can partially hydrolyze NSPs, reduce the viscosity of gut contents, and result in improvements in nutrient digestion and absorption [10–12].

The benefits of NSP-enzymes supplementation to NSPs-rich diets are well documented [8,10,12–19]. Several of these studies have demonstrated improvements of nutritive value, feed utilization, body weight gain, composition and activity of intestinal microbiota, and reduction in excreta volume after supplementation of plant-ingredients-based diets with NSP-enzymes such as cellulases, pectinases, hemicellulases, arabinoxylanases, and β-glucanases. Nevertheless, information on the potential and effectiveness of different types of NSP-enzymes in improving the nutritive values of plant ingredients and their coproducts-based diets (especially NSPs) is still limited and more research is needed to prove the mechanisms of their effectiveness in human and animal nutrition. The recent awareness on the direct link between the supplementation of NSP-enzymes and an array of potential health benefits has boosted consumer attentiveness of good nutrition and increased interest in diets and dietary ingredients enriched in NSPs.

Distiller's dried grains with solubles (DDGS), a coproduct of the ethanol industry, are often used as feed material in livestock and poultry nutrition. Results

of many experiments have indicated, however, that a high dietary level of DDGS can negatively affect the digestibility of nutrients and the performance of monogastric animals due to their high content of NSPs. The efficacy of feed enzymes added to poultry and pig diets containing DDGS is not consistent and depends on many factors [20].

A critical role in host nutrition, health, performance, and quality of the products produced is played by the intestinal microbiota. Feed enzymes can make an impact on gut microbial ecology by reducing undigested substrates and antinutritive factors and producing oligosaccharides in situ from dietary NSP with potential prebiotic effects. Therefore, the microbiota responses to feed enzymes administration, rather than being absolute, are a continuum or a population of responses. The opportunities and challenges for the role of major feed enzymes (carbohydrases and phytase) on the gut health of poultry and swine species with a specific focus on the impact on gut microbiota should be evaluated in detail [21].

Targeting specific NSPs of wheat, barley, or rye with enzyme preparations has proven effective for diets based on these cereals but not for corn- and soybean-meal-based diets, primarily due to the differences in constituent NSPs. The increased use of whole flaxseed in poultry diets represents an additional research area for effective enzyme development to alleviate potential negative effects of constituent NSP components [22].

Besides, the use of NSP-enzymes to enhance nutrient utilization of NSPs, the underlying mechanisms in animal digestive physiology and current gaps in knowledge to advance the use of NSP-enzymes for NSPs' utilization are very interesting for evaluation. Therefore, taking these issues into account, this chapter presents different types of NSP-enzymes, and their definition, classification, production, and various physiobiochemical effects, industrial purposes, and further mechanisms that prove their effectiveness in human and animal nutrition.

9.2 SPECIFIC TARGET COMPONENTS FOR NSP-ENZYMES

Polysaccharides are widespread biopolymers representing the most important group in botanical feed. Carbohydrates constitute a diverse nutrient category ranging from sugars easily digested by the monogastric animals in the small intestine to dietary fiber fermented by microbes in the large intestine [23]. Dietary fiber is divided into insoluble dietary fiber (IDF) including celluloses, some hemicelluloses, and lignin, and soluble dietary fiber (SDF) which includes β-glucans, pectins, gums, mucilages, and some hemicelluloses. The IDF and SDF compounds, apart from lignin, are known collectively as NSPs. They are the plant structural analogs of the skeletal system of the animal kingdom, and in NSPs starch and free sugars are excluded. The agronomic cultivation conditions such as environmental factors prior to harvest and storage conditions after harvest can influence NSP content.

Many NSPs dissolved in water result in viscous solutions. NSPs increase viscosity by directly interacting with the water molecule. At higher concentrations, the molecules of the NSPs interact with each other and become entangled in a network further increasing the viscosity [24].

The NSP content of different feed ingredients is variable. The NSPs in cereal grains are composed predominantly of arabinoxylans (pentosans), glucans, and cellulose. NSP content of rice, jowar, and maize is comparatively low. Cereal byproducts, which are obtained after separating away the starchy portion, have very high values of NSPs.

The insoluble NSPs make up the bulk of the total fiber in diets. It fulfills the satiety of the animals. A small quantity is beneficial in promoting evacuation of gut contents/defecation and maintaining normal gut health. A very high concentration of dietary fiber shortens residence time of digesta leading to reduced digestion and absorption of feed nutrients. Soluble NSPs acts as antinutritive substances in the body. They undergoe rapid fermentation in the intestine and act as a source of energy for anaerobic microbes. They help to propagate harmful pathogens such as *Clostridium perfringens* which causes various diseases in animals and poultry. NSPs are associated with an increase in digesta viscosity in broiler chicken [25]. The viscous property impairs the diffusion and connective transport of lipase, oil, and bile salt micelles within gastrointestinal contents. Dietary fiber increases the secretion of mucous [26]. It increases the resistance for transport of nutrients through the epithelial surface by increasing the thickness of the mucous layer and changing the physiochemical properties of the mucus. The viscous property of NSPs delays digestion and absorption due to encapsulation of nutrients. Beoford and Classen [25] and Choact [27] recently demonstrated that diets rich in insoluble NSP markedly elevated fermentation in the small intestine. NSPs do not only interfere with digestive processes, but also have strong negative effects on the availability of energy and nutrients to animals. Beoford and Classen [25] reported that diets high in insoluble NSPs are very effective in preventing the onset of cannibalism in laying hens. This is due to the bulky nature of fiber and its subsequent effect on digesta transit rate.

Breakdown of complex polysaccharides can be performed by the addition of NSP degrading enzymes. But it is difficult to select suitable enzyme mixtures as feed additives in such diets due to a lack of knowledge of the nutritive value of the enzymes and the idea of the presence of antinutritive factors. Knowledge of the chemical structures of NSPs has permitted the development of enzyme technology to overcome the adverse effects.

9.3 CLASSIFICATION OF NSP-ENZYMES

Based on the NSPs degrading, NSP-enzymes can be classified into three major types, such as (1) cellulose-degrading enzymes (cellulases), (2) noncellulosic

polymer degrading enzymes (xylanase, β-glucanase, β-mananase), and (3) pectic polysaccharide degrading enzymes (pectinases). Recently, many studies have been reported on the function of NSP-enzymes and their applications in animal feeds [5,12,18,19,28]. The types of NSP-enzymes and classification of NSPs found in commonly used plant feedstuffs for animal diets are presented in Table 9.1.

9.4 CELLULOSE DEGRADING NSP-ENZYMES

9.4.1 CELLULASES

Cellulase is one of the cell-wall-degrading enzymes, an NSP-enzyme that degrades cellulose. Cellulose, a polymer of glucose residues connected by β-1,4-linkages, being a principal component of plant cell walls, is the most abundant carbohydrate in nature [29]. It consists of composite forms of highly crystallized microfibrils among amorphous matrixes, thus refusing access to hydrolyzing enzymes. Cellulose is the world's most abundant polymer comprising over 50% of all the carbon vegetation. Cellulose quantity in whole grains can vary from species to species and is largely a consequence of the thickness of the husk and seedcoat; those that contain more cellulose tend to have thicker and stronger cell walls. Seed endosperm cells have only thin cell walls and in a well-filled grain the proportion of cellulose to starch, or other reserve polysaccharide, should be low [30]. Although cellulose is the basic structural component and comprises of about 33% of all vegetable materials, it cannot be degraded by humans and most animals because of the absence of cellulase enzyme, and therefore, does not contribute directly to the nutrition of these animals. However, cellulose is very important for maintaining the structure of cell membranes. The metabolism of cellulose in humans and most animals has long been a focus of interest, but progress has been hindered by a lack of accurate chemical methods for its measurement. Also, native cellulose as present in the plant cell wall behaves differently in the gut. Among mammals, only those that are ruminants can digest cellulose, because they have special bacteria and microorganisms in their digestive tracts containing cellulose; and they are then able to absorb the broken-down cellulose and use as a food source.

Utilization of cellulose as a nutrient source requires the enzyme cellulase that cleaves β-1,4-glycosidic bonds in the polymer to release glucose units [31]. Cellulolytic bacteria and fungi have developed complex forms of cellulase systems which actively convert insoluble cellulosic substrates into soluble saccharides [32]. Cellulase consists of three classes of enzymes: (1) endoglucanase acts on carboxymethyl cellulose, causing random scission of cellulose chains yielding glucose and cello-oligosaccharides; (2) exoglucanase acts on microcrystalline cellulose, communicating an exo-attack on the nonreducing end of cellulose, liberating cellobiose as the primary product; (3) β-glucosidases facilitate hydrolysis of

Table 9.1 Types of Nonstarch Polysaccharides (NSP)-Enzymes and Classification of NSP Found in Commonly Used Plant Feedstuffs for Animal Diets

NSP-Enzyme	NSP-Category	Monomeric Residue	Linkage	Plant-Based Feedstuff(s)
Cellulase	Cellulose	Glucose	β-(1→4)	Cereals (barley, corn, wheat) and legumes (soybean, cottonseed, rapeseed/canola, lupin)
	Noncellulosic polymers			
Xylanases	Arabinoxylans	Arabinose and xylose	β-(1→4)-linked xylose units	Cereals (barley, corn, wheat, oat, sorghum)
β-Glucans	Mixed-linked β-glucans	Glucose	β-(1→3) and β-(1→4)	Oat and barley
β-Mananase	Mannans	Mannose	β-(1→4)	Coffee seed
β-Mananase	Galactomannans	Galactose and mannans	β-(1→4)-linking mannan chains with α-(1→6)-linked galactosyl side groups	Locust bean gum and guar gum
β-Mananase	Glucomannans	Glucose and mannans	β-(1→4)-linked mannan chain with interspersed glucose residues in the main chain	Sugar-beet pulp, lilies, irises
	Pectic polysaccharides			
Pectinase	Arabinans	Arabinose	α-(1→5)	Cereal coproducts
Pectinase	Galactans	Galactose	β-(1→4)	Sugar bean meal, sugar-beet pulp
Pectinase	Arabinogalactans (type I)	Arabinose and galactose	β-(1→4)	Grain legumes (soybean, lupin)
Pectinase	Arabinogalactans (type II)	Arabinose and galactose	β-(1→3,6)	Canola/rapeseed cotyledon

Source: Adapted from Sinha AK, Kumar V, Makkar HPS, De Boeck G, Becker K. Non-starch polysaccharides and their role in fish nutrition – a review. Food Chem 2011;127:1409–1426.

cellobiose to glucose [33]. Extensive basic and applied research on cellulases has revealed their commercial significance and industrial applicability [34]. Cellulases have industrial, medical, and nutritional applications, as well as a role in obtaining bioactives. Recently, the use of cellulase has become one of the important measures to improve livestock and poultry production performance and feed utilization [35,36], as well that of fish species [37–39].

Direct-fed cellulases as monocomponent additives are generally not very successful in improving monogastric animals' (pig and poultry) performance (digestibility, feed conversion ratio and daily weight gain) [40], as are other microbial enzymes such as phytases [41,42] as well as xylanases in poultry [43]. Several reasons can be suggested for the very low performance of direct-fed cellulases: duration of the contact of the enzyme with the digesta within the gastrointestinal tract (in monogastric species, the hydrolysis process with the available cellulases take at least three to five times longer than that of digesta flow), gastrointestinal pH, particularly in the stomach, gastrointestinal denaturation and proteolytic activity, etc.

Allowances to pigs and poultry of cellulases in combination with other β-glucanases are said to have positive effects on the zootechnical parameters of both species [44]. Combinations of cellulases with other glucanases are considered by some authors as improving performance in dairy cows and buffalo [45] whilst others were unable to observe such positive effects [45–47]. For example, in dairy cows, the apparent ruminal digestibilities of dry matter (DM), organic matter, neutral detergent fiber (NDF) and acid detergent fiber (ADF) as well as milk yield and composition were not positively affected by the cellulase or other fibrolytic enzymes [46].

Applications of cellulases and hemicellulases in the feed industry have received considerable attention because of their potential to improve feed value and performance of animals. Pretreatment of agricultural silage and grain feed by cellulases or xylanases can improve its nutritional value. The enzymes can also eliminate antinutritional factors present in the feed grains, degrade certain feed constituents to improve the nutritional value, and provide supplementary digestive enzymes such as proteases, amylases, and glucanases. For instance, the dietary fiber consists of NSPs such as arabinoxylans, cellulose, and many other plant components including resistant dextrins, inulin, lignin, waxes, chitins, pectins, β-glucan, and oligosaccharides, which can act as antinutritional factors for several animals such as swine where, the cellulases effectively hydrolyze the antinutritional factor, cellulose, in the feed materials into easily absorbent ingredient thus improving animal health and performance. β-Glucanases and xylanases have been used mainly in the feed of poultry species to hydrolyze NSPs such as β-glucans and arabinoxylans [47,48].

The production of sugars and agroethanol from cellulosic materials is still a costly process, and requires, for positive environmental effects, the production of strong and low-cost enzymes. Today, cellulosic ethanol is twice as expensive as gasoline and the proportion is similar for the production of bioactives. It is expected that the production cost of cellulases will be reduced from 5 to 1 US cents per liter between now and 2020. The world leaders in the domain

(Novozymes, Genencor, and Iogen) would like to reduce the price of cellulases within a few years.

The Earth's available stocks of lignocellulosic biomass are tremendous and are distributed all around the world. Thus, the sustainability of the valorization of cellulose by cellulases in combination with lignolytic enzymes such as laccases seems clear. More economical feeding can most probably be obtained by improving pretreatment and enzymatic hydrolysis of cellulose. Furthermore, an application in human nutrition may be also possible. Details are presented in Chapter 6, Depolymerizating enzymes—cellulases and Chapter 7, Laccases—properties and applications of the present book.

9.5 NONCELLULOSIC POLYMERS DEGRADING NSP-ENZYMES

9.5.1 XYLANASES

Xylanases are known for their catalytic breakdown of xylans (quantitatively important hemicelluloses), the components of the cereal cell walls [49]. Xylanases are composed of two classes of enzymes, β-1,4-endoxylanase, which hydrolyzes the initial linkage of xylan backbone to xylooligosaccrides, and β-xylosidase which converts the xylooligomers to monomer units (xylose).

Generally, cereals belong to commelinoid monocotyledons, which have type II cell wall architecture, and these cell walls are characterized by the presence of acidic xylans (arabinoxylan) and mixed glucans [50]. Arabinoxylan is a hemicellulose that has a xylose backbone with arabinose side chains [51]. They are comprised of a linear β-(1-4)-linked xylopyranose backbone substituted with arabinofuranose side chains attached via α-(1-3)- and/or α-(1-2)-linkages. Arabinoxylans have been identified in a variety of tissues of the main cereals: wheat, rye, barley, oat, rice, and sorghum [52]. Although these polysaccharides are minor components of entire cereal grains, they constitute an important part of plant cell walls. Thin walls that surround the cells in the starchy endosperm and the aleurone layer in most cereals consist predominantly of arabinoxylans (60%–70%); exceptions are endosperm cell walls of barley (20%) and rice (40%) [52]. Nonendospermic tissues of wheat, particularly the pericarp and testa, also have very high arabinoxylan content (64%) [53]. When the arabinose residues are stripped off the xylan backbone (using oxalic acid), aggregation appears at a xylose-to-arabinose ratio [12]. The loss of arabinose side chains also correlates with a loss in water-binding capacity [12]. When this is absent, the molecule binds less water and becomes less soluble. The degree of arabinose substitution has little influence on the overall semiflexible conformation and hence the viscosity [12]. Not only can arabinoxylans establish covalent cross-links, they may also form "junction zones" by intermolecular hydrogen bonding between unsubstituted regions of the xylan backbone [52]. Such types of interactions of arabinoxylans may be of great importance in determining their conformational changes and solubility properties, and thus their antinutritional activities [54].

Xylanases are composed of two classes of enzymes, β-1,4-endoxylanase, which hydrolyzes the initial linkage of the xylan backbone to xylooligosaccrides and β-xylosidase converts the xylooligomers to monomer units (xylose). Xylanases are used for industrial purposes including polishing of grains [55,56]. Xylanolysis accessory enzymes are also important enzymes for complete degradation of cell wall structures coforming xylanes and arabinoxylanes [19].

9.5.2 β-GLUCANASES

β-Glucanases hydrolyze the 1,4- or 1,3-linkages found in mixed-linked glucans disrupting the integrity of endospermic cell wall. Mixed-linked β-glucans occur exclusively in members of the monocotyledon family Poaceae, to which the cereals and grasses belong, and in related families of the order Poales. Mixed-linked β-glucans are also referred to as (1,3 or 1,4)-β-D-glucans or cereal β-glucans. They are linear, unbranched polysaccharides in which β-D-glucopyranosyl monomers are polymerized through both (1,4)- and (1,3)-linkages [57]. It was reported that in barley, the β-glucans contain approximately 70% (1,4) linkages and 30% (1,3) linkage, in which segments of two or three (1,4) linkages are separated by a single (1,3) linkage [58], whereas in cereals mixed-linkage β-glucans are linear cell wall homopolysaccharides of D-glucopyranose arranged as blocks of consecutive (1,4)-linked β-D-glucose residues separated by single (1,3)-linkages. The resultant structures of β-glucans in cereal contain 58%−72% of β-(1,3)-linked units and 20%−34% of β-(1,4)-linked units [59].

The digestion of cereal grains with β-glucanase leads to maximum release of the higher-quality protein and energy; and this enzyme was used for both industrial purposes for polishing [60], and for experimental purposes as supplements in higher animals and fish [10,61,62].

9.5.3 β- MANANASES

β-Mananase is an NSP-enzyme part of hemicellulase that degrades mannans. Mannans are important constituents of the hemicellulose family fraction in softwoods and show widespread distribution in plant tissues [63] including the cell walls of the coffee seed endosperm [64]. In general, mannans present a structural role in plants, acting as hemicelluloses that bind cellulose [65] and form the molecular basis for the hardness of the plant. Besides functioning as a signaling molecule in plant growth and development [65], they also act as nonstarch carbohydrate reserves in endosperm walls and vacuoles of seeds and vacuoles in vegetative tissues [66]. Mannans have been classified into four subfamilies: linear mannan, glucomannan, galactomannan, and galactoglucomanan [64], and can be degraded by β-mananase. Like the other noncellulosic polymers degrading NSP-enzymes, β-mananase was used as a supplement in plant-ingredients-based animal diets [67−69].

9.5.4 PECTIC POLYSACCHARIDES DEGRADING NSP-ENZYMES

9.5.4.1 Pectinases

Pectinases have attracted attention globally as biological catalysts in numerous industrial processes [70]. Pectinase catalyzes degradation of pectic substances through depolymerization (hydrolases and lyases) and deesterification (esterases) reactions [71], and helps to understand the structure of pectin. Henri Braconnot in 1825 first isolated pectin and described that it is primarily made up of α-(1-4)-linked D-galacturonic acid residues. Pectins can be defined as those polysaccharides that are solubilized from the cell wall by aqueous solutions of chelating agents such as ethylenediaminetetra-acetate or ammonium oxalate [72]. They are partially methylesterified rhamnogalacturonans, in which rhamnose units are inserted. Also, it occurs attached to short side chains of arabinose and galactose [73]. Pectins are a heterogeneous group of glycans and consist of a large amount of uronic acids and their derivatives. Some of the quantitatively important pectins are arabinans, galactans, arabinogalactans, galactouronans, homogalactouronans, and rhamnogalactouronans I and II [74]. In the cell wall of fruits and vegetables, pectin is the predominant component [75]. Among fresh fruits, currants contain the highest percentage of pectin, between 0.9% and 1.5%, but in the case of dried fruits, lemon peel and pulp contain the maximum percentages of pectin at 35.5% and 32.0%, respectively [76].

Pectin is involved in cross-linking cellulose and hemicellulose fibers, thus pectinases help to improve access of cellulases to their substrates [68]. Pectinases mainly include polygalacturonases, pectin esterases, pectin lyases, and pectate lyases with different substrate specificities [77]. Pectinases are efficiently produced by microbes and plants but there is no report on pectinase production by animals [78]. Pectinases act on plant cell walls decreasing the intracellular adhesivity and tissue rigidity [79]. There are numerous reports on different types of pectinase production from different pathogenic fungi and bacteria including pectin methyl esterase, whose isoforms are detected in all higher plants so far examined [46,80], and these microorganisms have been isolated from soils rich in pectic waste, fruit waste-processing areas, sewage of juice centers of different locations, mud and pieces of long stored crop waste from the pectin-producing industry, and agroindustrial residues [81]. For instance, decomposing fruit materials after enrichment have been used to isolate pectinolytic bacteria [18]. Applicability of pectinases is pH dependent and acidic pectinases are mainly used in the beverage industry [82] to remove pectic substances which are responsible for the consistency, turbidity, and appearance of fruit juices [83].

9.5.5 NSP-ENZYMES PRODUCTION

According to Singh et al. [19], the overall strategy of enzyme production (including NSP-enzymes) to its extraction and concentration requires several steps, as presented in Fig. 9.1. Many reports present enzyme production by submerged

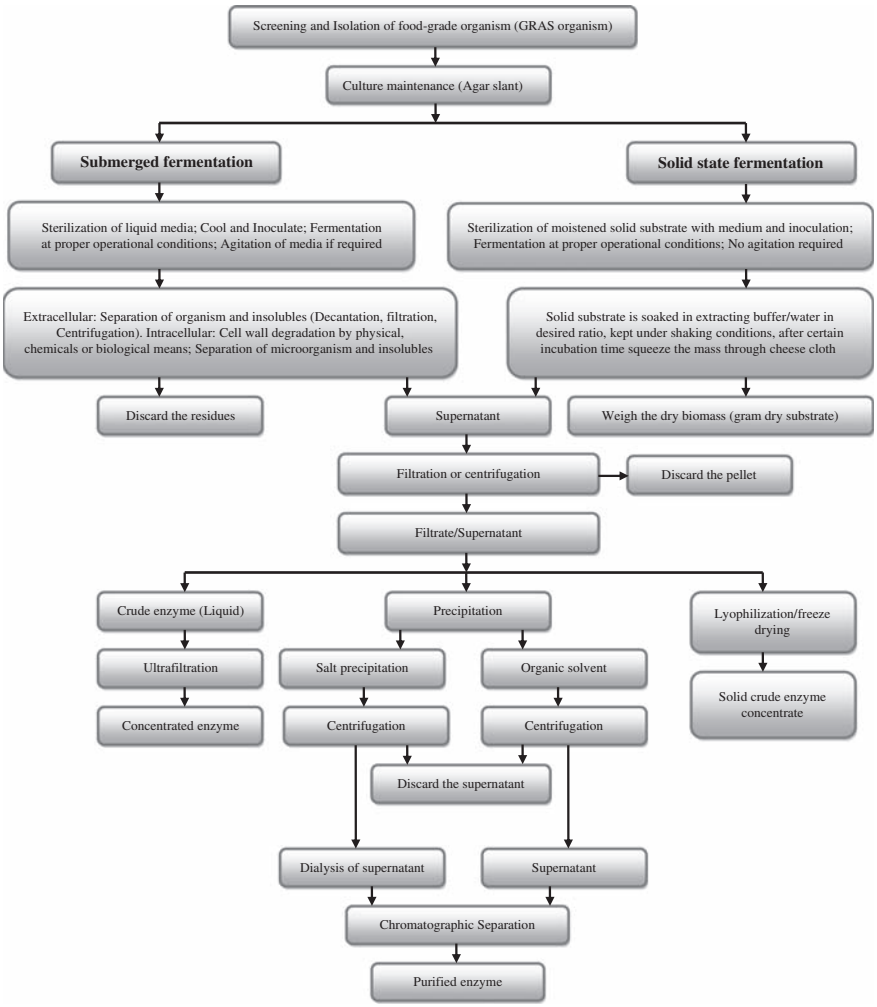

FIGURE 9.1

Process scheme for microbial enzyme production for food processing. *GRAS*, generally regarded as safe.

Adapted from Singh A, Sharma V, Banerjee R, Sharma S, Kuila A. Perspectives of cell-wall degrading enzymes in cereal polishing. Food Biosci 2016;15:81–86.

fermentation, semisolid and solid-state fermentation (SSF). SSF has various advantages for fungal enzyme production because of its better productivity and higher yield of enzymes with lower capital investment [84]. The substrate choice is also a critical factor for microbial growth. Agroresidues rich in cellulosic material (wheat bran, rice straw, paper waste, hull, etc.) are highly explored as the

substrates for cellulase production. Similarly for the xylanase, sugar cane bagasse, wheat bran, corncobs, green gram husk, red gram husk, peanut meal, and rice bran are some of the solid residues evaluated for SSF [19].

9.5.6 PHYSIOBIOCHEMICAL ASPECTS OF NSP-ENZYMES

Some NSPs are present as part of the cell wall, thus shielding substrates from contact with the digestive enzymes, or as part of cell content where their presence may interfere with digestion and absorption due to their chemical nature [85]. Thus, the main function of NSP-enzymes supplementation to plant-ingredients-based diets is to hydrolyze complex NSPs present in plant feedstuffs that nonruminant animals are incapable of hydrolyzing with their endogenous pool of digestive enzymes. Besides, one of the most important benefits of NSP-enzymes supplementation is the reduction in NSP-induced digesta viscosity. NSP-enzymes have also been reported to facilitate a decrease in the degree of polymerization of feed, reducing its viscosity and liberating carbohydrate oligomers, therefore improving nutrient utilization [86]. Studies on the effects of NSP-enzymes in different animals fed diets with plant-based feedstuffs are presented in Table 9.2.

In recent years, there has been interest in the potential use of NSP-enzymes in higher animal and fish diets [16,36,96–98]. Monogastric animals including fish lack the intestinal enzymes for the degradation of NSPs. Thus, the improvement in the digestibility of NSPs is achieved by supplementation of NSP-enzymes in diets. Such an approach has successfully been used in poultry diets, but little work showing the potential of NSP-enzymes in fish is available [37,38,39,93,97]. These interests derive from the high cost of animal production, the availability of new enzyme mixtures, and the potential economic returns to be realized with effective enzyme supplements.

Degrading enzymes (including NSP-enzymes) isolated from plants and bacteria have been used successfully in animal feed industries to enhance starch utilization, and also to overcome the negative effects of the soluble fraction of dietary NSPs associated with plant ingredients [99,100]. Digestibility studies with pigs and poultry have indicated that the soluble components of NSPs present in wheat and barley, predominantly (1-3), (1-4)-β-glucans, and arabinoxylans, may increase the viscosity of the digesta and interfere with enzyme access and activity, and ultimately inhibit the uptake of other macronutrients such as protein and lipids [99,100]. The positive effects of supplementing exogenous NSP-enzymes to ruminant diets have also been reported for some commercial products fed to growing cattle or lactating dairy cows: supplementing an enzyme mixture containing NSP activities to alfalfa hay diets improved the live weight gain of cattle by as much as 35% and feed conversion ratio by up to 10% as a result of enhanced total tract digestion [14]. Application of NSP-enzyme mixture to grass hay also improved total tract digestibility in steers [13]. In high-grain feedlot finishing diets, an 11% improvement in feed conversion ratio was reported, resulting from a 6% increase in weight gain and a 5% decrease in feed intake using a product with high

Table 9.2 Studies on the Effects of Nonstarch Polysaccharide (NSP)-Enzymes in Different Animals Fed Diets With Plant-Based Feedstuffs

Animal	Plant-Based Feedstuff	NSP-enzyme	Effect	References
Growing cattle or lactating dairy cows	• Alfalfa hay, corn or barley • 45% forage	• NSP-enzymes mix • NSP-enzymes mix (β-glucanase and xylanase)	• Improved live weight gain and feed conversion ratio. Enhanced total tract digestion. • Increased intake of digestible nutrients to a greater extent, but did not increase milk yield or milk component yield	Beauchemin et al. [14,15]; Lewis et al. [13] Beauchemin et al. [16]
Piglets	• Barley • Wheat bran, oats, barley	β-glucanase and xylanase	• Decreased the viscosity in the distal part of the small intestine, lowered the plasma urea nitrogen concentration and increased the apparent ileal digestibility of energy and some amino acids • Influenced the distribution of total organic acids in the ileum, indicating a shift in dominating bacteria.	Yin et al. [62] Högberg and Lindberg [87]
Swine	• Wheat, oat and coproducts, • Maize, wheat, barley and rye • Wheat and oat	β-glucanase and xylanase	• Improved nutrient digestibility and voluntary feed intake and thereby reduce the risk of feeding diets high in coproducts. • Played an important role in efficient and effective nutrient use of coproducts. • Increased feed intake and weight gain decreased Gram-negative bacteria overall, but only increased Lactobacilli, Bifidobacteria, and overall biodiversity for the wheat-based diet. • Affected intestine morphology, bacteria populations, and microbial metabolites in the gut content.	Zijlstra et al. [88]; Willamil et al. [89] Reilly et al. [90]; Smith et al. [91]

(*Continued*)

Table 9.2 Studies on the Effects of Nonstarch Polysaccharide (NSP)-Enzymes in Different Animals Fed Diets With Plant-Based Feedstuffs *Continued*

Animal	Plant-Based Feedstuff	NSP-enzyme	Effect	References
			• Confirmed that nutrient digestibility differ among cereal grains, and that enzymes can enhance nutrient utilization in wheat-based diets. • Both cereal grains and enzymes affected components of gut health, including intestine morphology, bacteria populations, and microbial metabolites in the gut content	
Rainbow trout, *Oncorhynchus mykiss*	Soybean, sunflower and rapeseed meal	Enzyme cocktail (i.e., β-glucanase and xylanase)	• Increase in apparent nutrient digestibility, especially lipids. • No effect on fish performance and nitrogen retention.	Dalsgaard et al. [92]
Grass carp (*Ctenopharyngodon idella*)	Duckweed and wheat flour	Cellulase	• Increased the digestive enzyme activities, improved the intestinal flora, and promoted the growth of fish	Zhou et al. [39]
Caspian salmon, *Salmo trutta*	Trout diet (48% protein) with no specified plant ingredient	Multienzyme complex (i.e., xylanase, cellulase, β-glucanase)	• Improved growth performance and feed utilization. • No effects on hematological indices.	Zamini et al. [93]
Vanaraja chicken	Guar mea	NSP-enzymes mix (i.e., xylanase, glucanase, cellulase and mannanase)	• Improved the performance of Vanaraja birds compared to those fed diets without NSP-enzymes.	Rama Rao et al. [94]
Adult cockerels (poultry)	Defatted rice bran (in vitro degradation experiment and in vivo metabolism assay)	NSP-enzymes mix	• Combining of feruloyl esterase with NSP-enzymes and phytase improved both in vitro dry matter degradation and ferulic acid release, and in vivo nutrient digestibility of rice bran-based diets.	Liu et al. [36]

			• It showed a high potential in improving the use of rice bran as poultry feed.	
Broiler (poultry)	Barley	1,3-1,4-β-glucanase and 1,4-β-glucanase	• The 1,3-1,4-β-glucanase, but not 1,4-β-glucanase improved the nutritive value of barley-based diets for broilers.	Fernandes et al. [95]
Broiler (poultry)	Wheat	Xylanase and β-glucanase	• Thymol + carvacrol, in combination or not with an NSP- enzymes, improved growth performance, enhanced nutrients retention, increased total volatile fatty acid, reduced cholesterol and modulated intestinal microbial counts in broilers fed on a wheat-based diet.	Hashemipour et al. [96]

xylanase activity [15]. In another study, supplementation of NSP-enzymes including β-glucanase, xylanase, and endocellulase in a commercial feed increased the intake of digestible nutrients to a greater extent for cows fed the low concentration than for cows fed the high concentration of these NSP-enzymes mixes; however, increased intake of digestible energy due to enzyme supplementation did not increase milk yield or milk component yield, because the experimental cows were in positive energy balance [16].

In monogastric diets, β-glucanases and xylanases have been successfully used to hydrolyze NSPs, such as barley β-glucans and arabinoxylans [103]. In addition, supplementation of β-glucanase (600 units/kg diet) and xylanase (745 units/kg diet) in pig diets containing hull-less barley decreased the viscosity in the distal part of the small intestine, lowered the plasma urea nitrogen concentration, and increased the apparent ileal digestibility of energy and some amino acids [59]. Similarly, Yin et al. [102] reported the beneficial effect of NSP-enzymes in pigs.

Moreover, supplementation of NSP-enzymes during feed production was found to degrade NSPs and markedly improved the digestion and absorption of feed components as well as growth performance in broiler chickens [10]. Broiler chicks fed high-viscosity barley showed lower amylase and lipase activities in the digesta, while supplementation of β-glucanase in the diet increased activities of the two enzymes and of trypsin as well [104]. Similar results were reported with hybrid tilapia, where 1 g/kg of NSP-enzyme (50 FBG (fungal β-glucanase units)/g; Roche Shanghai) increased the activity of amylase in the hepatopancreas and intestine by 11.4% and 49.5%, respectively [28]. However, silver perch fed on diets supplemented with Natugrain-blend containing β-glucanase and β-xylanase at concentrations of 75, 150, and 300 μL/kg containing 30% wheat or dehulled lupin did not show significant effects on dry matter, energy, or protein digestibilities [104]. The missing effect of Natugrain-blend supplementation on nutrient digestibility could be due to the intolerance of silver perch to high levels of galactose and xylose in their blood [105].

Moreover, the inclusion of microbial enzymes in poultry feeds based on guar meal appears to be an effective method of minimizing the deleterious effects of guar gum. Supplementation of β-mananase to a diet containing guar meal reduced intestinal viscosity and alleviated the deleterious effects associated with feeding of guar meal [69]. In addition, supplementation of β-mannanase facilitates higher inclusion levels of guar meal from 25 to 50 g/kg in the diet [70]. Nevertheless, performance and carcass variables did not show significant improvement in a study with broilers fed a diet containing guar meal at 5, 100, and 150 g/kg [68]. However, the study suggested that guar meal could be used below 100 g/kg in broiler rations without any adverse effects. In another study, guar meal at 200 g/kg supplementation resulted in depression in performance; while, supplementation of NSP-enzymes improved the performance of Vanaraja birds compared to those fed 200 g/kg guar meal without enzymes [94].

Furthermore, Reilly et al. [90] studied the effects of oat-based β-glucans or oat-derived β-glucans in a wheat-based diet with or without a β-glucanase cocktail. They reported that intact β-glucans increased hindgut fermentation and Bifidobacteria and Lactobacilli populations in the colon; the supplementation of β-glucanase reduced these effects. Their combined results indicated that intact β-glucans stimulate gut health. Willamil et al. [89] studied maize or wheat–barley–rye diets with or without a β-glucanase and xylanase cocktail. Enzyme increased feed intake and gain for the wheat-based but not the corn-based diets, perhaps due to increased starch digestion in the caecum. Enzyme supplementation decreased Gram-negative bacteria overall, but only increased Lactobacilli, Bifidobacteria, and overall biodiversity for the wheat-based diet. Willamil et al. [89] used the same study design, indicating that enzyme tended to increase absorptive area and reduced cell proliferation and intraepithelial lymphocytes in the gut. The results of these studies confirmed that nutrient digestibility differs among cereal grains and those enzymes can enhance nutrient utilization in wheat-based diets. Both cereal grains and enzymes may affect components of gut health, including intestine morphology, bacteria populations, and microbial metabolites in the gut content [88].

In aquatic animals NSP-enzymes (e.g., cellulase, xylanase, β-glucanase) increased the digestive enzymes activities, enhanced digestion capacity, improved intestinal flora, and promoted growth [37,38,39,92,93]. The cellulase enzyme can be synthesized in herbivorous fish, not by the fish themselves but by certain microorganisms [106]. The endogenous cellulase of these fish species is far from sufficient to fully digest the ingested fiber. Therefore, exogenous cellulase is needed to supplement in the fish diets especially when using plant ingredients. As recent study reported, omnivorous and carnivorous fish may need more NSP-enzymes, thus cellulase application is recommended in aquaculture as a feed additive [39].

9.5.7 NSP-ENZYMES FOR INDUSTRIAL PURPOSES

In addition to the health-consciousness attitude of consumers, the palatability of any food depends on the external appearance of the products. Whole cereal grain after dehusking, despite being the source of various bioactive components, is unacceptable to a major part of the population because of the presence of bran; as a result the cereal-processing industries started removing the bran and germ by mechanical milling processes. However, this resulted in the breakage and loss of a major portion of the aleurone layer. This disadvantage of mechanical milling was overcome by the use of enzymes for biopolishing. One of the industrial purposes of NSP-enzymes is biopolishing. The advantages of NSP-enzymes for biopolishing in the cereal-processing industry are based on two basic aspects: (1) quality control of the cereal, and (2) to modification of the properties of cereal components, to be used as food additives [19].

9.5.8 CELLULASE IN BIOPOLISHING

Enzymatic (including cellulase) treatment of brown rice resulted in a decrease in crude oil and crude fiber content [56]. Besides, cellulase treatment increased nutritional quality of basmati rice [107]. Furthermore, studies on cellulolytic enzymes have demonstrated their biotechnological potential in various industries including food, animal feed, brewing and wine making, agriculture, biomass refining, pulp and paper, textiles, and laundry [108].

9.5.9 NONCELLULOSIC POLYMERS IN BIOPOLISHING

Xylanase, a noncellulosic polymer-degrading NSP-enzyme, was used to polish rice [56]. Enzymatic polishing (including xylanase) of rice increased milling performance and cooking quality [57]. Another noncellulosic polymer-degrading NSP-enzyme, β-glucanase was used in the treatment of barley to enhance the digestibility of nonstarchy polysaccharides for poultry purposes [61].

9.5.10 PECTIC POLYSACCHARIDES IN BIOPOLISHING

Pectinases, pectic polysaccharide-degrading NSP-enzymes, combined with other exogenous carbohydrase enzymes were used for biopolishing of cereals [19]. Microorganisms are naturally endowed with potential to produce numerous enzymes extracellularly, e.g., *Streptomyces* GHBA10 is an efficient producer of pectinase that may be used industrially in extraction and clarification processes, including biopolishing [109]. Samples for isolation of pectinolytic isolates have been soils rich in pectic waste, fruit waste-processing areas, sewage of juice centers of different locations, mud and pieces of long-stored crop waste from the pectin-producing industry, and agroindustrial residues [81].

9.5.11 NSP-ENZYMATIC DEGRADATION MECHANISM OF NSPS

Sinha et al. [55] suggested three mechanisms for the actions responsible for NSP-enzymatic degradation of NSPs, such as (1) disruption of cell wall integrity, (2) reduction of digesta viscosity, and (3) stimulation of bacterial population (Fig. 9.2).

9.5.12 DISRUPTION OF CELL WALL INTEGRITY

In cereals and legumes, the cell wall is constructed mainly of small amounts of cellulose, hemicellulose, and arabinoxylan, with minor β-glucan components. The activity of NSP-enzymes creates “holes” in the cell wall, and these allow water hydration and permit pancreatic proteases and amylases to act, enabling better digestion of the starch and protein [55]. Xylanases, and to a lesser extent cellulases, have been proven to be most effective in broilers [110]. Mannanases and

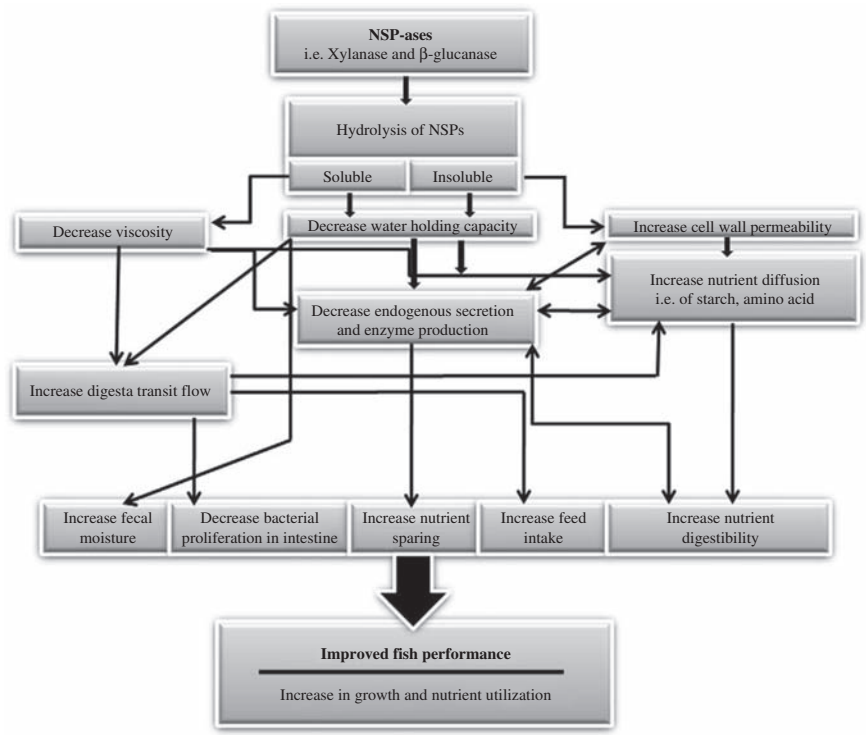

FIGURE 9.2

Modes of action of nonstarch polysaccharide degrading enzymes.

Adapted from Wyatt et al., 2008. C.L. Wyatt, T. Parr, M. Bedford, 2008. Mechanisms of Action for Supplemental NSP and Phytase Enzymes in Poultry Diets. Carolina Poultry Nutrition Conference 2008.

pectinases have shown good results in a soy-based diet [111]. Nevertheless, xylanase and glucanase were more effective in breaking down the insoluble fiber fraction in a cornsoy-based diet [55]. In addition, a study in young pigs suggested that an improvement in digestibility of nutrients in barley-based diets with cellulase supplementation was due to the increased degradation of β-linked components in barley, which made them available to the host animal [63].

9.5.13 REDUCTION OF DIGESTA VISCOSITY

Studies on monogastric animals have shown that reduced digesta viscosity due to NSP-enzyme supplementation is the main factor responsible for the observed enhanced performance response on feeding plant materials rich in NSPs [39,112]. Nevertheless, Partridge [113] demonstrated that pig small intestinal viscosity is relevant for its performance, but it is of a lower order of importance than for broilers.

9.5.14 EFFECTS ON BACTERIAL POPULATION

Inclusion of NSP-enzymes in animal diets breaks down plant cell wall carbohydrates and reduces chain length, producing smaller polymers and oligomers. These fragments further become small enough to act as a substrate for bacterial fermentation that can be beneficial with short-chain fatty acids (SCFA = volatile fatty acids (VFA)) production and altering the bacterial population [55]. Several studies have shown that applications of exogenous enzymes (including NSP-enzymes) significantly alter VFA production and the population profiles of gut-associated microflora [11,87,90]. Recent studies on pig (β-glucanase and xylanase) [90,91], poultry (β-glucanase and xylanase) [96], and fish [39] have reported that supplementation of some NSP-enzymes in a cereal grain and their coproducts-based diets modulated the intestinal microbial counts of their respective animals. Nevertheless, care must be taken while selecting the dietary NSP-enzyme because some products can be overdosed and reduce the size of the oligosaccharides down too far, to monosaccharides. If excess monosaccharides are produced, osmotic diarrhea and/or poor performance may result [114].

9.6 CONCLUSIONS AND PERSPECTIVES

The use of enzymes to hydrolyze NSP has been questioned in large debates and there is no general consensus on which particular enzyme, or enzymes, result in the measurable. The complexity of the potential substrates can partly explain the contradictions. Much of available information on the use of NSP-enzymes can be based on data obtained in experiments not adequately designed to support clear conclusions [115]. Positive effects of NSP-enzymes' addition to monogastric diets are supposed to reduce antinutritional compounds in feed and reduce nutrient encapsulation as well as by lowering viscosity coming from certain NSP compounds such as arabinoxylans and β-glucans.

Gastrointestinal microorganisms have an important role in host nutrition, health, and performance. Dietary added enzymes can have a positive impact on digestive microbial ecology by reducing undigested substrates and antinutritive factors, and possibly by producing oligosaccharides from dietary NSPs with potential prebiotic effects [21]. The potential role of carbohydrases on the gut health of monogastrics with a specific aim on the gut microbiota should be pursued.

There are at least other two fields of research activities to be considered: on the one hand, to clarify the use of multicomplex versus individualized NSP enzymes; and on the other hand, to develop depolymerases aimed on the valorization of nonedible plant biomass, not only for the production of a second generation of agrofuels but also for other applications, particularly in nutrition.

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CHAPTER

Depolymerizating enzymes in human food: bakery, dairy products, and drinks

10

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10.1 INTRODUCTION

Enzymes, the biological catalysts, differ from the chemical catalysts by being much more specific in their functions. When it comes to food, the use of enzymes dates back to much longer before the 19th century when their catalytic abilities were scientifically recognized. Typically, enzymes are used to modify/manipulate biopolymers in order to improve the shelf-life, nutritional value, as well as ease of extraction of edible parts of raw materials. However, not all enzymatic activities are beneficial, for instance, autolysis in vegetables, fat rancidity, and proteolysis in bread dough are among those enzymatic reactions that need to be prevented. The oldest industrial application of enzymes has been traced back to mid-1800 in the brewery industry [1]. Nowadays, more than 80% of the global market of enzymes accounts for industrial applications [2] in which food and feed enzymes represent the major share. These include enzymes used in baking, brewery, beverages, dairy, dietary supplements, as well as fats and oils.

In general, there are three different approaches in the use of food enzymes: (1) to convert the raw material into the main product; (2) as additives to modify a functional characteristic of the product; (3) to control or improve the process. Moreover, enzymes are generally considered as natural products [3]. As the food industry is moving toward clean labels and with the Food and Drug Administration regulations on restricting the use of chemical additives, food enzyme applications have become more important. In this review, the recent applications of food enzymes in different food processing areas such as baking, beverage, specialty foods, as well as in health and wellness products are reviewed, and we present trends and future perspectives of food enzyme industry.

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10.2 SOURCES OF FOOD ENZYMES

Enzymes are biocatalysts for the biochemical reactions that occur in all living organisms, either microbial or higher organisms, in order to support their lives. Food enzymes, more specifically, are dietary proteins present in most foods and feeds, regardless of whether they are fresh or processed. These proteinous molecules are inherently safe and are degraded and metabolized after ingestion [4]. Traditionally, enzymes are isolated from microorganisms, plants, and mammalian tissues. The majority of food and feed enzymes are originated from terrestrial microbes. Marine-origin organisms have been also been identified as good sources of food enzymes [5,6]. Most of these enzymes perform efficiently under mild processing conditions of pH, pressure, shearing, and more importantly temperature; however, their utilizations on a commercial scale are limited mainly due to their cost, limited substrates, and their lower stability when exposed to harsh and large-scale processing conditions [7]. Today, most of the food enzymes are derived from recombinant microorganisms. These microorganisms are not culturable under lab conditions, but are genetically engineered for manufacturing enzymes with improved activity and processability. Several studies have targeted enhancing the performance of enzymes for food and feed industrial applications through improving the enzyme thermostability, identifying a wider pH range for enzymes' activity, and decreasing the need for metal coenzymes [8]. Table 10.1 presents some of the strategies used for improving the performance of common food and feed enzymes. The techniques for enzyme isolation from the above-mentioned origins are extensively explained by Fernandes [8]. Olempska-Beer et al. [4] more specifically reviewed and discussed the information on food-processing enzymes from recombinant microorganisms and described the safety assessment of this technique. Tables 10.2 and 10.3 classify some representative examples of food and feed enzymes and their roles. Some of the other useful marine-origin food and feed enzymes derived from higher organisms are listed in Table 10.4 [8].

10.3 FOOD ENZYMES IN THE BAKING PROCESS

The most common baked foods can be named as bread, cake, cookies, pies, pastries, tortillas, biscuits, and crackers. Indubitably, depending on the physiochemical properties of the final product, these products are different in their formulae. With the starch being the main constituent of the baked products, a typical formula for any backed products can include protein, fat, carbohydrates, sugar, eggs, emulsifiers, milk, and water. Cereal flour is the main source of protein and carbohydrates in the baked goods formula [20], which is also a key source of enzyme substrates in the product. Each of the above-mentioned constituents has a role throughout the baking process and impacts the texture and final properties of the product. Considering bread as a model baked product, it is usually made from

Table 10.1 Strategies Undertaken to Improve the Performance of Enzymes with Food and Feed Applications

Enzyme	Role	Targeted Improvement	Strategy/Comments
α-Amylase	Starch liquefaction	Thermostability	Protein engineering through site-directed mutagenesis. Mutant displayed increased half-life from 15 min to about 70 min (100°C)
	Starch liquefaction	Activity	Directed evolution. After three rounds the mutant enzyme from *Saccharomyces cerevisiae* displayed a 20-fold increase in the specific activity when compared to the wild-type enzyme
	Baking	pH-activity profile	Protein engineering through site-directed mutagenesis
L-Arabinose isomerase	Tagatose production	pH-activity profile	Protein engineering through directed evolution
Glucoamylase	Starch saccharification	Substrate specificity, thermostability, and pH optimum	Protein engineering through site-directed mutagenesis
Lactase	Lactose hydrolysis	Thermostability	Immobilization
Pullulanase	Starch debranching	Activity	Protein engineering through directed evolution
Phytase	Animal feed	pH-activity profile	Protein engineering through site-directed mutagenesis
Xylose (glucose) isomerase	Isomerization/ epimerization of hexoses, pentoses and tetroses	pH-activity profile	Protein engineering through directed evolution. The turnover number on D-glucose in some mutants was increased by 30–40% when compared to the wild type at pH 7.3. Enhanced activities are maintained between pH 6.0 and 7.5
		Substrate specificity	Protein engineering through site-directed mutagenesis. The resulting mutant displayed a threefold increase in catalytic efficiency with L-arabinose as substrate

Source: *Adopted from Fernandes P. Enzymes in food processing: a condensed overview on strategies for better biocatalysts. Enzyme Res 2010;2010:Article ID 862537, 19 pp. doi:10.4061/2010/862537. References used for each row are [9–18].*

Table 10.2 Food Enzyme Classes and Their Roles in Food and Feed Processing

Class	Enzyme	Role
Oxidoreductases	Glucose oxidase	Dough strengthening
	Laccases	Clarification of juices, flavor enhancer (beer)
	Lipoxygenase	Dough strengthening, bread whitening
Transferases	Cyclodextrin	Cyclodextrin production
	Glycosyltransferase	
	Fructosyltransferase	Synthesis of fructose oligomers
	Transglutaminase	Modification of viscoelastic properties, dough processing, meat processing
Hydrolyses	Amylases	Starch liquefaction and saccharification
		Increasing shelf life and improving quality by retaining moist, elastic and soft nature
		Bread softness and volume, flour adjustment, ensuring uniform yeast fermentation
		Juice treatment, low calorie beer
	Galactosidase	Viscosity reduction in lupins and grain legumes used in animal feed, enhanced digestibility
	Glucanase	Viscosity reduction in barley and oats used in animal feed, enhanced digestibility
	Glucoamylase	Saccharification
	Invertase	Sucrose hydrolysis, production of invert sugar syrup
	Lactase	Lactose hydrolysis, whey hydrolysis
	Lipase	Cheese flavor, in situ emulsification for dough conditioning, support for lipid digestion in young animals, synthesis of aromatic molecules
	Proteases (namely, chymosin, papain)	Protein hydrolysis, milk clotting, low-allergenic infant-food formulation, enhanced digestibility and utilization, flavor improvement in milk and cheese, meat tenderizer, prevention of chill haze formation in brewing
	Pectinase	Mash treatment, juice clarification
	Peptidase	Hydrolysis of proteins (namely, soy, gluten) for savory flavors, cheese ripening
	Phospholipase	In situ emulsification for dough conditioning
	Phytases	Release of phosphate from phytate, enhanced digestibility
	Pullulanase	Saccharification
	Xylanases	Viscosity reduction, enhanced digestibility, dough conditioning
Lyases	Acetolactate decarboxylase	Beer maturation
Isomerases	Xylose (glucose) isomerase	Glucose isomerization to fructose

Source*: Adopted from Fernandes P. Enzymes in food processing: a condensed overview on strategies for better biocatalysts. Enzyme Res 2010;2010:Article ID 862537, 19 pp. doi:10.4061/2010/862537.*

Table 10.3 Enzymes Used in Sweet Baked Products

Enzyme	Cakes	Biscuits/ Cookies	Pastries/ Croissants
Endoxylanases	+		
Thermostable 4-α-glucanotransferase	+		
α-Amylase	+		+
β-Amylase, lipase	+		
Xylanase	+	+	+
Proteases		+	+
Transglutaminase, phospholipase, glucose oxidase oxygenase, oxidases, peroxidases			+

Source: *Adopted from Chandrasekaran [19].*

Table 10.4 Examples of Marine Origin Enzymes Isolated From Higher Organisms and Their Potential Application in Food and Feed Processes/ Products

Class	Enzyme	Source
Transferases	Transglutaminase	Muscles of atka mackerel (*Pleurogrammus azonus*), botan shrimp (*Pandalus nipponensis*), carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*), scallop (*Patinopecten yessoensis*)
Hydrolases	Amylase	Gilt-head (sea) bream (*Sparus aurata*), found in Mediterranean sea and coastal North Atlantic Ocean
		Turbot (*Scophthalmus maximus*), found mostly in Northeast Atlantic Ocean, Baltic, Black and Mediterranean seas, and Southeast the Pacific Ocean
		Deepwater redfish (*Sebastes mentella*, fond in North Atlantic)
	Chymotrypsin	Atlantic cod (*Gadus morhua*), crayfish, white shrimp
	Pepsin	Arctic capelin (*Mallotus villosus*), Atlantic cod (*Gadus morhua*)
	Protease	Marine sponges *Spheciospongia vesperia*, found in Caribbean sea and South Atlantic, close to Brazil, and *Geodia cydonium*, found in Northeast Atlantic Ocean and Mediterranean sea
		Mangrove crab (*Scylla serrata*), found in estuaries and mangroves of Africa, Asia, and Australia
		Sardine Orange roughy (*Hoplostethus atlanticus*)

Source: *Adopted from Fernandes P. Enzymes in food processing: a condensed overview on strategies for better biocatalysts. Enzyme Res 2010;2010:Article ID 862537, 19 pp. doi:10.4061/2010/862537.*

a mixture of starch, nonstarch polysaccharides, gluten, lipids, and enzymes. Technically, bread baking includes two phases of dough and backing. During the dough phase, the mixture of flour, salt, sugar, yeast, water, shortening, and other ingredients goes through complex biophysiochemical reactions catalyzed by flour (wheat) enzymes and yeast thus affecting dough rheological properties (i.e., dough extensibility, viscoelasticity, gas holding and water absorption capacity) [21–23]. In this phase, the flour particles are hydrated and sheared while being mixed, the gluten proteins create a transient network with the dispersed starch granules leading to dough development [24]. According to Joye et al. [25], the processability (machinability) of dough, good gas retention, the crumb structure, and bread final volume are affected by the gluten network quality. The air bubbles in the gluten network created during the mixing accommodate the carbon dioxide produced by the yeast fermentation and affect the final crumb structure (CO_2 diffusion into the air bubbles). During the baking phase, the combination effects of temperature, moisture mobility, evaporation, and baking duration result in starch gelatinization, heat-setting of gluten proteins and transformation of the transient gluten network into a continuous network, and expansion of the baked bread [26]. Development of the continuous gluten network could be due the formation of new disulfide cross-links [26–28] and thus addition of α- and γ-gliadins into the glutenin network, which are essential for giving bread the ability to rise during baking [29]. Also, the continued CO_2 production due to yeast fermentation along with water and ethanol evaporations due to heating facilitate baking of the bread from the outside to the inside and thus formation of a baked crumb [30]. Even during the postbacking cooling process, the biophysiochemical interactions between the starch, protein, and lipid biopolymers are continued ,resulting in formation of amylose–polar-lipid complex, which is responsible for crumb softness and plasticity in fresh bread [26,31].

Food processers are always looking for opportunities to improve the process (i.e., to shorten the baking process, to postpone the staling process, or to be able to use different cereal flours, etc.). The use of certain enzymes can facilitate the achievement of these goals. In general, three sources of enzymes are used in the baking process, including endogenous enzymes in flour, exogenous enzymes, usually added to the dough, and the metabolic enzymes of the microorganisms (lactic acid bacteria and yeast) [32]. As mentioned earlier, the main purpose of adding enzymes is to improve the processability or quality of the dough by modifying the dough properties such as rheology, gas retention, and crumb texture, and even to control the level of chemicals formation such as acrylamide [33]. In addition to the exogenous enzymes, other baking aids are also added to the dough or flour as enzyme improvers. An enzyme cocktail might also be added to the dough. The synergistic effect of lactic acid bacteria and several exogenous enzymes (i.e., microbial glucose-oxidase, lipase, endoxylanases, α-amylase, and protease) used for producing sourdough results in increased and accelerated lactic acidification as well as the textural properties of sourdoughs [32]. In an earlier study, bread volume, crumb texture, and in general the baking performance were improved

when endoxylanases, glucose oxidase, and peroxidase were added to the wheat dough [34]. Endoxylanases' tendency to react with multiple substrates resulted in high concentrations of larger molecules of arabinoxylans in the liquid phase of the wheat dough and development of an optimal gluten network [35]. Amylases and hemicelluloses also significantly impacted the fresh bread properties and thus storability of wheat bread, mainly due to the starch degradation and changes in free sugar contents. Formation of low-molecular-weight dextrin and thus water-soluble starch and solids during baking and fermentation favored crumb texture and bread freshness during storage [35]. On the other hand, presence of water-insoluble pentosans induced changes in crumb elasticity, whereas the presence of water-insoluble pentosans correlated with crumb hardness and elasticity during storage. Combined effects of α-amylase and endoxylanase, cellulases and protease, at their optimum inclusion levels, on Canadian short bread and Japanese-style sponge and bread processes resulted in reduced sheeting work requirements, dough softening, increased volume, and softer crumb, which could be associated with water release and its consequent effect on dough physical characteristics [36,37]. The individual effects of cellulase, pentosanase, and α-amylase on completely waxy wheat flour dough resulted in improved bread quality and nutritive values. Addition of cellulase reduced dough mixing properties and increased the crumb softness, while the addition of pentosanase presented similar effects on the dough properties but reduced the crumb softness. Interestingly, inclusion of α-amylase did not show any impact on completely waxy wheat flour dough and bread properties [38]. According to Shafisoltani et al. [39] the combination of α-amylase, xylanase, and glucose oxidase improved dough softening. In one of the most recent studies, the interactional effects of cellulase, xylanase and α-amylase on the rheological properties of Chinese steamed bread dough enriched with 15% wheat bran, also resulted in increased dough extensibility, softening, mixing tolerance index, and stickiness [40]. Making steamed rice breads requires a dough treatment with a combination of one or more maltogenic α-amylases and one or more native starch-degrading enzymes. A steamed bread process can be improved by overcoming the challenges associated with finding effective enzymatic solutions to inhibit the staling process, if an aging resistance agent (ARA) is added to the flour. The ARA comprises 1%−7% amylolytic enzyme, 3%−7% hemicellulase, 3%−7% lipase, 40%−70% emulsifying agent, 15%−40% hydrophilic colloid, and 2%−6% coagulation inhibitor. The bread internal structure was improved, a softened core, high antiaging performance, along with better moisture retention was also achieved [41]. Enzymatic treatment of wheat dough with glucose oxidase resulted in free sulfydryl groups and increased glutenin contents. In addition, changes in the structure of albumin, glutenin, and globulin affected the formation of protein aggregates and ultimately, reduced the specific volume of product [42]. On the other hand, pentosanase led to an increased level of water-soluble pentosans and reduction of pentosans size, and thus increased protein interactions and eventually resulted in breads with higher specific volumes.

As shown, the trend is to use the synergistic effect of complex mixtures of enzymes on the different types of flour or dough. However, it is very important to choose the right enzyme combination with the right inclusion level and acceptable application rate; otherwise, the interactions among the enzymes can result in unexpected process challenges or products [42]. In the next subsections, typical enzymes utilized in food process and their roles are discussed.

10.3.1 HYDROLYSES

One major reaction occurring during bread making is hydrolysis of the large polymers such as carbohydrates, protein, and lipid. Hydrolyses are used as a biocatalysts in the reaction.

10.3.1.1 Carbohydrate hydrolyses

These enzymes include α-amylases, β-amylases, cellulases, β-glucanases, and glucosidases. Amylases are routinely used for starch hydrolysis in bread making and can be subdivided into two main groups due to their site of actions: endoenzyme and exogenzyme α-amylases (EC 3.2.1.1) are the most important endo types which hydrolytically catalyze the breakdown of α-(1,4)-glycosidic bonds from the inner part of the amylose (Fig. 10.1) or amylopectin chain (Fig. 10.2) [43] leading to the creation of several branched α-oligosaccharides of different lengths and α-limit dextrins [44]. As a result, the carbohydrate profile of the hydrolysate can vary depending on the length of the oligosaccharide fragments, the number of

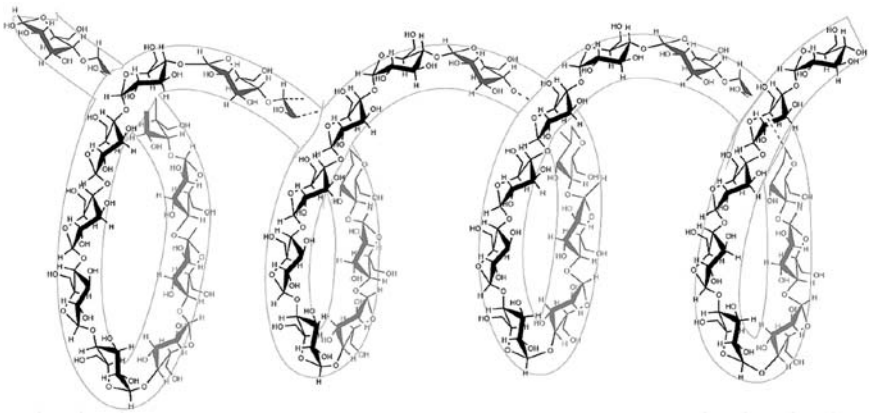

FIGURE 10.1

Amylose chain, assumed as a left-handed spiral due to α(1->4) glyosidic bonds ($n = 500–6000$ α-D-glycopyranosyl units).

Adopted from Miguel MAS, Martins-Meyer TS, Veríssimo da Costa Figueiredo E, Paulo Lobo BW, Dellamora-Ortiz GS. Enzymes in bakery: current and future trends. In: Muzzalupo I, editor. Food industry, under CC BY 3.0 license, InTech; 2013. http://dx.doi.org/10.5772/53168.

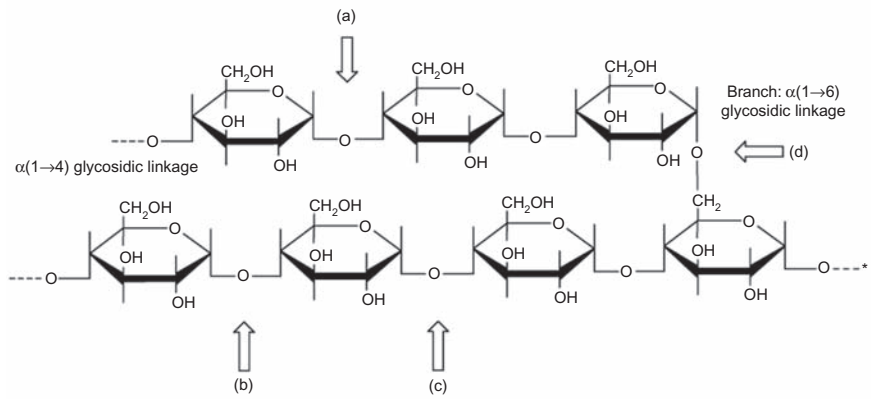

FIGURE 10.2

Partial structure of amylopectin. The amylolytic enzymes action site are: (a) α-amylases, (b) amyloglucosidases, (c) β-amylases, (d) isoamylases and pullulanases.

binding sites, and the location of catalytic regions. The optimum pH is 5, and due to its lower optimum temperature and higher production, it has been extensively applied in the industries of food, beverages, and medicine. α-Amylase is known to be the most common starch converting endoenzyme associated with the glycosyl hydrolases (GH13) family [45,46]. The GH13 family has two parts of endoenzyme and exogenzyme with diverse thermal stability profiles [47] and are usually originated from cereal, fungi, and bacteria, including recombinant microorganisms. On the other hand, the exogenzymes maltogenic α-amylase (EC 3.2.1.133) along with other maltooligosaccharide-forming amylases (EC 3.2.1.60) catalyze releasing maltose from the starch molecule. The α-(1,6)-bonds of the side chains of amylopectin molecule are also hydrolyzed by other debranching enzymes such as pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68) [21]. β-Amylases (EC 3.2.1.2) and glucoamylases (EC 3.2.1.3) belong to the GH14 and GH15 families, respectively. These exohydrolases catalyze hydrolysis of α-(1,4)-D-glucosidic bonds at the nonreducing ends of amylose and amylopectin to remove maltose units from the end of the chain. Their optimal pH is similar to that of α-amylase. None of the amylases (α or β) can cleave the α-(1,6)-glucosidic linkage present in amylopectin. Due to their limited activity on α-(1,6)-linkages, the maximum hydrolysis degree for amylose is 75%−90%, whereas these enzymes would possibly be able to catalyze amylopectin hydrolysis up to 50%−60%. Debranching enzymes along with α-glucosidases may assist α- and β-amylases in a more complete conversion of starch to simple sugars. Glucoamylases also have a limited activity on α-(1,6)-linkages, and theoretically might be able to fully catalyze conversion of starch polysaccharides into β-glucose [21]. Other carbohydrate-debranching enzymes include β-glucosidases, cellulases, and arabinoxylanases.

The functionality of amylases depends mainly on their thermostability, origin, and degraded residuals. For instance, α-amylases of bacterial origin are typically very heat stable, but fungal enzymes lose most of their activity during baking [48]. Thus, selection of a potent strain of microorganism for enzyme production is the initial step. On the other hand, amylases are used for flour standardization and overdosing results in low-quality crumb structure. Liu et al. [40] reported that the addition of α-amylase softened the dough and significantly increased the extensibility as well as stickiness of the dough, but reduced its resistance to extension. They also reported that combination of α-amylase, xylanase, and cellulase induced synergetic effect on rheological properties of regular dough and dough incorporated with wheat bran. As a result, water absorption reduced by 10% and extensibility increased by more than 40% [40]. An excess of α-amylase activity results in starch degradation during dough mixing and fermentation; consequently, the dough becomes sticky and results in low-quality breads that are unacceptable to consumers [49].

In bread baking, fungal α-amylase is initially used to increase the level of fermentable sugars in the dough by increasing the reducing sugar content of the flour, or in other words, to standardize the flour. During the dough stage of bread baking, the α-amylases cleave damaged starch molecules into smaller oligosaccharides and β-amylase converts them into maltose to be easily consumed by yeast during the fermentation. These lighter molecules also reduce dough viscosity during the gelatinization process and improve bread softness. Due to the Maillard reaction, the remaining reducing sugars react with protein molecules of dough and enhance the development of bread flavor and crust color. Bread volume is also improved due to the effect of these enzymes on gas-holding capacity of fermented dough [20].

Retarding the staling of bread is very important in the bread-making industry. Several researches have evaluated the use of starch-degrading enzymes as a means to delay bread staling [45]. Bacterial α-amylases with intermediate thermostability, such as *Bacillus stearothermophilus* mutagenic α-amylase provide antistaling functions through reducing the firming rate of bread crumb [21,26]. This antifirming behavior could be due to the presence of a particular dextrin created during starch disintegration and its interference with retrogradation of the branched amylopectin molecule [50]. It could be also associated with the formation of cross-links between starch and protein [51] over the staling process. Maltogenic amylase is the other enzyme with antistaling properties. It is originated from *B. stearothermophilus* and exhibits distinct actions on amylose and amylopectin [52,53]. As the enzyme cleaves the side chains of amylopectin, it somewhat prevents them from reorganization. It also improves the amylose crystallization by increasing the amylose mobility and restricting the degradation process through its endomechanism. Consequently, the bread firmness is increased at the beginning but gradually is reduced due to amylose rapid aggregation, and thus a weaker network is formed which hampers the cross-linking between starch compounds. Encapsulation was also used to modify the thermal stability of amylases

[54]. The encapsulated α-amylase continuously releases the α-amylase enzyme during and following the baking process. The food-grade lipid enveloping the enzyme provides a thermal barrier and preserves the enzyme activity toward the end of and after the baking process, resulting in an antifirming property. Consumers increasingly prefer wheat bran fortified bread and baked food products due to their health benefits. Bran inclusion into cereal-based foods impacts on the production process, finished food physical properties, as well as the organoleptic quality. Hemdane et al. [49] extensively reviewed the research results for wheat bran in bread making. The specific distribution of different hydrolases used in wheat-bran-fortified breads is illustrated in Table 10.5. These bran-derived enzymes may react with specific components of wheat flour and thus impact the bread-baking process [55]. The antistaling effect of amylases when combined with transglutaminase and xylanase could help in the production of softer and less chewy crumbs, and delay starch retrogradation. These enzyme combinations reacted even more effectively in the presence of glycerol [56].

10.3.1.2 Kinetic and activity of α-amylase

The catalytic activity of enzymes varies depending on the substrate concentration and small molecules termed cofactors (metals and small organic molecules). The rate of catalysis linearly changes with substrate concentration, which indicates the enzyme function. The kinetic properties of an enzymatic reaction can be determined by Michaelis–Menten equation and Lineweaver–Burk plot. Many studies have examined the thermokinetic behaviors of α-amylase in the process of starch hydrolysis for different ranges of temperatures, pH, and selected metal ions as coenzyme. Different analytical methods were also used to assess the kinetics and determine the optimal activity of the enzyme. At similar starch and enzyme concentrations, the lower the value of Michaelis constant (K_m), the higher affinity of the enzyme for the substrate [57].

10.3.2 PROTEASES

Proteases are also largely used in the industrial production of bread and other baked goods such as crackers, biscuits, pastries, cookies, and waffles. The enzymes are used for protein hydrolysis or proteolysis to cleave the peptide bonds. They are classified into two groups of exo- and endoproteases depending on their active site. In general, the proteolytic activity of grains before germination is very low and they can be considered as somewhat inactive [58]. In addition to starch, protein storage of flour (gliadins and glutenins) is also considered as one of the main contributors to physical/rheological properties of dough; however, each of these proteins functions differently. For instance, the less cohesive and elastic properties of the hydrated gliadin fraction of protein make it responsible for dough viscosity and extensibility, while the more cohesive and elastic properties of the hydrated polymeric glutenin allow it to contribute to dough strength and elasticity. Thus, gliadins functions as a “plasticizers” or “solvents”

Table 10.5 Relative Comparison of Enzyme Activities for Different Bran Products

Class	Enzyme	Flour	Coarse Bran	Shorts	Pollard	Germ
Hydrolases	α-Amylase	1–2	1–15	1–6	3–5	1–15
	β-Amylase	1–9	2–3	n.a.	5–6	2–5
	Peptidase	1	0–5	0–5	n.a.	n.a.
	Endoxylanase	1–3	7–32	4–15	n.a.	5
	Xylosidase	1	5–15	5–8	n.a.	n.a.
Oxidoreductases	Lipoxygenase	0–4	3–12	3	8–20	35–50
	Peroxidase	0–1	1–4	3	1–3	6
	Ascorbate oxidase	1–2	3–4	n.a.	n.a.	n.a.
	Dehydroascorbate reductase	1	2–4	n.a.	2–10	3–7
	Polyphenol oxidase	0–3	3–9	12	4–16	3–5
	Superoxide dismutase	1	4	3	n.a.	n.a.
Isomerases	Protein disulfide isomerase	1	10–16	n.a.	16–18	18–19

The absolute values for enzyme activities were expressed relative to the sound flour activity. A common interval was composed for each enzyme. n.a., *not applicable.*
Source: *Adopted from Hemdane et al. [49].*

for glutenins [59]. One advantage of using proteases in the bread-making process is improvement in bread texture and flavor [21]. The acidic peptidases (both endo- and exotypes) play more important roles in protein hydrolysis. Their optimum pH ranges from 4.2 to 5.5. Therefore, the position of the enzyme active site plays a crucial role in the functional properties of the proteases. For instance, for wheat flour, most proteolytic activities occur in more acidic pH, which are related to aspartic proteases (EC 3.4.23) and carboxypeptidases (EC number 3.4.16−3.4.18). Some of these enzymes facilitate the baking process by decreasing the consistency and enhancing the uniformity of the dough, controlling wheat gluten strength, and reducing the mixing time [60] as well as the required energy for mixing. These changes are mainly due to cleavage of the peptide bonds to smaller peptides, weakening the gluten network and gliadin, reducing the gluten elasticity [61] and thus their impacts on the rheology of bread dough (Fig. 10.3) [62]. High-gluten dough can be softened by adding fungal acid proteases to the dough [32]. It was reported that dough elasticity is an important factor in shrinkage of dough after processing [63]. Kara et al. [63] evaluated the effect of high-protease-activity flour along with two different commercial proteases on properties of wheat-gluten-based cookies and postulated that the proteases hydrolyzed the gluten protein from gliadins and glutenins parts and resulted in reduced gluten elasticity and higher spread ratio. The effect of high-protease-activity flours on prolongation of bread shelf-life was also reported by Furcsik [64]. Adding protease to brown rice flour resulted in a significant increase in bread specific volume, improved crumb softness, and reduced chewiness [65]. According to Chandrasekaran [19], this improvement in bread quality could be due to the hydrolysis of albumins and globulins, which are responsible for cellular structure in bread crumb.

10.3.2.1 Asparginase

Asparaginase (EC 3.5.1.1) catalyzes the hydrolysis of L-asparagine to L-aspartic acids. This enzyme plays a crucial role in reducing the risk of carcinogenic acrylamide formation during baking or processing of any starchy foods, such as pastry, potato chips, breakfast cereals, cake, pretzel, bagels, and gingerbread [66].

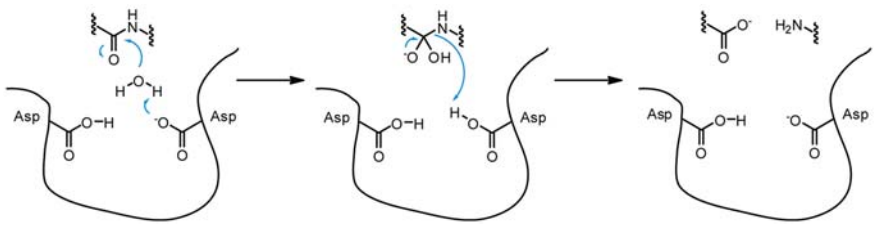

FIGURE 10.3

Proposed mechanism of peptide cleavage by aspartyl proteases.

Adopted from Suguna et al. [62].

Basically, L-asparagine can be converted to Acrylamide in the presence of reducing sugar during any heat processing at above 120°C. Adding asparaginase along with a hydrolytic enzyme could significantly prevent its formation. It was reported that when asparagine and glucose were present, the highest levels of acrylamide could be observed [67]. These authors also proposed a pathway for the formation of acrylamide from amino acids and reducing sugars due to the Maillard reaction, which is responsible for the color, smell, and taste of products prepared at high temperature. However, both studies revealed that this formation did not have any impact on browning and the Maillard reaction itself.

10.3.2.2 Kinetic and activity of proteases

Proteases can be produced from different microorganisms including bacteria, yeast, fungi, and actinomycetes. Fungal expression systems can produce larger quantities of proteases compared to bacterial expression system, which makes them a better choice for commercial applications [68]. Industrial proteases are usually derived from *Aspergillus* species [69]. Several researches have studied the kinetic properties of proteases. Based on their catalytic mechanism, they are typically classified as serine, metallo-, aspartic, and serine peptidases [70]. The kinetic parameters of K_m and V_m for immobilized protease from *Aspergillus* were reported as 5 mg/mL and 370 U/mL, respectively, when the enzyme optimal condition was identified at 85°C and pH 10 [69]. They also found that immobilization improved the thermostability of protease and increased the V_m by 85%. In another study, the K_m and V_m for protease from *Bacillus licheniformis* (BBRC 100053) were reported as 0.626 mM and 0.0523 mM/minute, respectively. They also studied the inhibition constant for different protease inhibitors [71].

10.3.3 HEMICELLULOSES A NONSTARCH HYDROLYSES

Nonstarch hydrolyses are those carbohydrate-degrading enzymes that act on nonstarch polysaccharides. Two different types of these dietary fibers include water-soluble and water-insoluble nonstarch polysaccharides. Hemicellulases hydrolyze hemicelluloses into a diverse group of dietary fibers such as xylan, xyloglucan (heteropolymer of D-xylose and D-glucose), glucomannan (heteropolymer of D-glucose and D-mannose), galactoglucomannan (heteropolymer of D-galactose, D-glucose and D-mannose), and arabinogalactan (heteropolymer of D-galactose and arabinose) [72]. Pentosans or endoxylanase (4-β-D-xylan xylanohydrolase, EC 3.2.1.8) is one of the most important hemicellulases, which is capable of hydrolyzing 1,4-β-D-xylosidic linkages in xylan and arabinoxylans. These glycosidases (*O*-glycoside hydrolases, EC 3.2.1.x) are commonly called xylanase, endoxylanase, 1,4-β-D-xylan-xylanohydrolase, endo-1,4-β-D-xylanase, β-1,4-xylanase, and β-xylanase [73]. Xylanases have potential applications in food and feed processing. In addition to their use in baking processes, they are widely used in beverages, fruit juices, nectars, purees, as well as oils and wine industries (Table 10.6) [73]. For heat-sensitive processes such as flavor and food grade

Table 10.6 Applications of Xylanase in Food and Feed Industries

Industry	Application	Function
Food: fruit and vegetable processing, brewing, wine production, coffee	Fruit and vegetable juices, nectars and purees, oils (e.g., olive oil, corn oil) and wines	Improves maceration and juice clarification, reduces viscosity. Improves extraction yield and filtration, process and product quality (reduce haze)
Food: baking	Dough and bakery products	Improves dough elasticity and strength, and bread texture
Feed: animal feeds	Monogastric (swine and poultry) and ruminant feeds	Decreases the nonstarch polysaccharides contents, reduces the intestinal viscosity, improves the utilization of proteins and starch, improves animal performance, improves digestibility and nutritive value of poorly degradable feeds such as barley and wheat

Source: *Adopted from Collins et al. [73].*

colors, or in the processes where heating is a major cost-bearing stage, cold-adapted xylanases are commonly used. Some industrial researches have demonstrated that cold-adapted xylanases are more efficient in the baking process specifically during the dough-preparation and proofing stages which require $<35°C$ temperature [74]. In contrast, in some other processes such as extrusion and pelleting processes, thermostable xylanase would be beneficial [73]. A wide range of microorganisms (bacteria, archaea and fungi) are capable of producing xylanases [75]. The xylanases, which belong to the GH 10 (glycosyl hydrolase) family are able to release shorter fragments. The pentosanases or endo-1,4-β-xylanases hydrolyze arabinoxylans and arabinogalactans and randomly cleave the xylan backbone from the nonreducing termini of arabinoxylo-oligosaccharides, reduce their molecular weights, and release water-soluble arabinoxylans [76]. After all, the ferulic acid esterases (EC 3.1.1.73) are used to cleave ester linkages between arabinose residues and ferulic acid; then, the α-L-arabinofuranosidases (EC 3.2.1.55) are used to remove the arabinose residues [73,77]. As stated before, in the baking processes, xylanases are widely used to improve the processability and end-product quality. Xylanases are reportedly used to improve the dough stability [73,78,79,80], due to removing the insoluble arabinoxylans content and reducing their interfering effect on the formation of the gluten network [76,77,81,82]. Creation of arabinoxylo-oligosaccharides in bread during this process would be also beneficial to human health [83]. In another study conducted in 2012, the effect of whole-wheat flour enriched with *Penicillium occitanis* Pol6

xylanases (0.12 U/g flour basis) in bread resulted in a significant decrease and increase in water absorption and dough rising, respectively, decreased firmness, increased cohesiveness, and reduced gumminess, as well as remarkably better sensory attributes [84]. The optimum conditions for mesophilic xylanases were found to be 40–60°C and neutral to slightly acidic (for fungal xylanases) pHs [85,86].

10.3.4 PHYTASE

Phytase belongs to the phosphatase family, which catalyzes the hydrolysis reaction of phytate usually found in plant-based food products. Phytase incorporation in cereal-based products contributes to the health benefits of the products. Phytate or phytic acid consists of hexa-phosphorus ester of inositol (1,2,3,4,5,6-cyclohexanehexolphosphoric acid), which is indigestible in human gut. It chelates the major content of phosphorous, as well as other minerals such as Ca, Fe, Mg, and Zn, through binding with inositol (a six-carbon ring molecule), creates a mineral salt (phytin) and thus reduces their bioavailabilities [19]. Phytic acid is present in all seeds and legumes. Among legumes, soybeans have higher levels of phytic acid. The nutritional quality of soy protein is influenced negatively by the formation of complexes with phytic acid, soybean protein, and multivalent metal ions, which could cause deficiency disorders, especially for vegetarians, elderly people, and infants [87]. On the other hand, phytic acid can reduce the digestibility of soy protein by inhibiting pepsin and trypsin enzymes in the gastrointestinal tract. The presence of a relatively large amount of unavailable phosphorus in infant food can cause severe bone mineralization defects [87]. Phytase is capable of cleaving the bound phosphorus and enhancing the bioavailability of these mineral nutrients [88]. In addition to the health benefits of this enzyme, improvements in product quality and final properties were also reported. For instance, in bread making, phytase supplementation could reduce the fermentation time (Fig. 10.4), increase bread specific volume, and reduce the crumb hardness [89]. The authors also reported that the improving effect of phytase on whole-wheat bread could be due to the reactivation of α-amylase by calcium ion, which was released from Ca-phytate. No changes in phytase activity were observed due to the fermentation process (Fig. 10.5). The optimum pH of phytase is 5.15 [90]. However, the decreased pH throughout the proofing stage led to increased enzyme activity. In another research, it was revealed that microbial phytases supplementation of corn-soybean meal-based diets for monogastric animals could increase the feed consumptions and reduce their fecal phosphorous excretion by up to 50% [91]. A schematic model of phytate hydrolysis in catalytic reaction is shown in Fig. 10.6. Wong et al. [87] disclosed a method for phytase-treated acid stable soy protein beverage. Biological production and applications of phytases have been extensively reviewed [87]. The combined effect of fungal phytase and fungal α-amylase, phytase dosage, bran content, and particle size distribution on the remaining amount of phytic acid in the bread as well as processing parameters were evaluated [92].

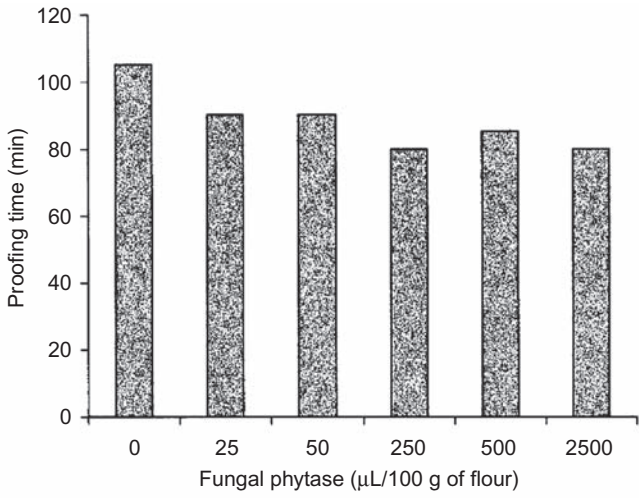

FIGURE 10.4

Effect of adding fungal phytase on proofing time.

Adopted from Haros et al. [91].

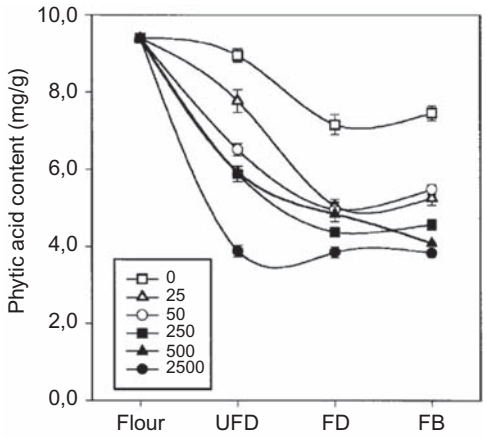

FIGURE 10.5

Effect of adding different fungal phytase dosage (μL/100 g of whole wheat flour) on phytate degradation through all stages of the bread-making process. *FB*, fresh bread; *FD*, fermented dough; *UFD*, unfermented dough.

Adopted from Haros et al. [89].

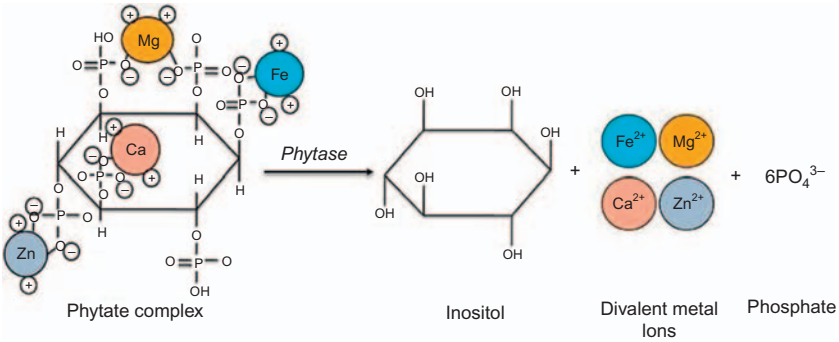

FIGURE 10.6

Schematic representation of phytate hydrolysis in catalytic reaction.

Adopted from Kim et al. [91].

10.3.5 ENZYMES FOR GLUTEN-FREE PRODUCTS

The absence of structure-forming components, i.e., gluten protein networks in gluten-free products, significantly impacts the processability as well as the final product properties. The major challenge is the inability to retain the carbon dioxide during fermentation, which results in low-quality dough and induces challenges in handling and processability of dough, mainly due to the limited viscoelasticity of protein in gluten-free cereals such as corn, rice, and other pseudoplastic grains like quinoa. Another challenge is nutritional deficiency of gluten-free foods mainly due to their low dietary fiber content. Several studies examined the application of starches (i.e., corn, soya, sorghum, and buckwheat) and a mixture of gums with hydrocolloids (rice flour, cassava, and corn) in the formulation of nongluten baked products to resemble the gluten network properties [93–96]. The other approach to improve the quality of gluten-free products is enzymatic creation of protein network mimicking gluten network functionalities [97]. Transglutaminase, glucosidases, and laccases have been used repeatedly in gluten-free products. The incremental inclusion of transglutaminase (0.5%–1.5%) to rice flour resulted in improved viscoelastic properties of this gluten-free dough. In a later study, final properties of batter and products of different transglutaminase-treated flours were compared. The results revealed that comparable improvement might not be achieved with all gluten-free flours, which indicate the dependency of the enzyme on the protein properties such as thermostability, origin, as well as the enzyme concentration, etc. [98]. More specifically, they reported that transglutaminase did not have any improving impact on rheological properties of dough made from oats and sorghum. Interestingly, in a later study, significant changes in viscoelastic behavior of sorghum dough were observed when sorghum flour was augmented with pre-gelatinized cassava starch before adding transglutaminase enzyme [99]. In that respect, protein fortification

of the gluten-free flours along with transglutaminase treatment was studied for creating an improved-structured protein network. Protein ingredients were incorporated in various forms, as a constituent of gluten-free flours (e.g., rice, soy, pea) or in the form of protein concentrates and/or isolates including: protein isolates from pea, soybean, egg albumen, and whey protein [100]; soybean and pea protein [9]; skimmed milk and egg protein [10]. The addition of combined non-gluten proteins in the production of gluten-free foods could improve texture and sensory properties as well as nutritional quality of the product by reducing the deficiency of essential amino acid profile. In this approach, the main role of enzymes such as transglutaminase and oxidases is to create protein cross-linking [11–15]. For example, the catalytic effect of transglutaminase on the reaction between lysine and glutamine residues resulted in a covalent cross-linking of proteins that improved the elasticity of brown rice flour batters and physical properties of the final rice bread; however, the nutritional quality of the bread was reduced. Perhaps, transglutaminase uses almost all the proteins and lysine amino acids for the creation of the cross-linkage! Thus, incorporation of different protein sources could provide resources for the enzyme activity and catalytic reaction, as well as enhance the nutritional score of the product [16].

10.3.6 LIPASES

Lipases (EC 3.1.13) or triacylglycerol acylhydrolases are typically used to catalyze the hydrolysis of ester bonds by attacking triacylglycerols. The products of this enzymatic hydrolysis are mono- and diacylglycerols and free fatty acids. Free fatty acids are very much prone to oxidative rancidity; thus, the lower the activity of lipase enzyme, the smaller the risk of rancidity due to hydrolysis of native lipids and of other fats used in the process [63]. Lipase can be extracted from plants, animals, bacteria, and fungi. The most important applications of lipase in the food industry (i.e., fats and oil industry, dairy industry, and bakery) are catalyzing transesterification, hydrolysis, and esterification. Table 10.7 summarizes the lipase applications in the food industry (adopted from Aravindan et al. [17]). Lipases are stable over the wide range of pH; however, the majority of lipase is stable at or close to neutral pH [18]. The main application of lipases in the food industry is associated with biomaterials cleavage and modifications [101]. One of the most common industrial applications of lipase is flavor development, specifically in dairy, meat, and vegetable industries [17]. They are also widely applied in enzymatic degumming of vegetable oils where phospholipase hydrolyzes phospholipids. This hydrolysis turns the phospholipids into more water-soluble particles, which can be easily washed [17,102]. Enhancing the emulsifying capacity of egg yolk through hydrolysis of egg lecithin is the other common use of this enzyme. Phospholipases have been used to modify the pasteurized and freeze-dried hen's egg yolk [103]. These authors reported that fermentation with phospholipases remarkably influenced the different steps of heat-induced gelation of egg yolk. Two different phospholipases were used (A_1 and A_2). Fermentation of native yolk

Table 10.7 Lipase Applications in Food Processing

Food Industry	Action	Production of application
Dairy foods	Hydrolysis of milk, fat, cheese, ripening, modification of butter fat	Development of flavoring agents in milk, cheese, and butter
Bakery foods	Flavor improvement	Shelf-life propagation
Beverages	Improved aroma	Alcoholic beverages, e.g., sake, wine
Food dressings	Quality improvement	Mayonnaise, dressings, and whippings
Health foods	Transesterification	Health foods
Meat and fish	Flavor development	Meat and fish products, fat removal
Fats and oils	Transesterification, hydrolysis	Cocoa butter, margarine, fatty acids, glycerol, mono- and diglycerides

Source: *Adopted from Aravindan et al. [17].*

using 0.1% phospholipase A_1, the temperature of the first viscosity maximum was increased by 23% (from 79°C to 97°C). In terms of pasteurized yolk, fermentation with phospholipase A_1 improved the homogenous gel-like consistency and the temperature from the first viscosity maximum was further stabilized up to 100°C. Enzymatic modification of fresh egg yolk using phospholipase A_2 was also examined with the aim of producing an enzymatically modified egg yolk powder [104]. Enzymatic reaction led to generation of an egg yolk powder with higher emulsifying activity, emulsion stability, protein solubility, and mayonnaise stability compared to those of the untreated egg yolk powder. In addition, during the enzymatic modification process, the structure of phospholipids and lipoproteins of the yolk was changed due to the cleavage of apo-high-density lipoprotein. The functional properties of the newly modified egg yolk powder were comparable with that of the fresh egg yolk. Role of lipase in flavor development is a proven fact. Lipases synthesized esters from short-chain fatty acids [17]. Lipases are also used for developing value-added products such as cocoa butter replacer, trans-free margarine with enhanced spreadability, low calorie fats, human milk fat replacer for infant formulation aiming to increase the fat digestibility, polyunsaturated fatty acids ω-3 and ω-6 for health and wellness products [105].

10.4 FUTURE PERSPECTIVES

Future application of enzymes in the food industry is foreseen in various trends: (1) exploring new sources of enzymes or producing new enzymes with tailor-made properties for developing specialty processed foods and further employment of mechanized processing; (2) developing new enzymes and enzymatic

applications for improving healthy and nutritious food products; (3) expanding screening to relatively untapped marine and extreme-temperature environments; and (4) answering the consumer demands for natural and chemical-free food products (green-label movement).

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CHAPTER

Perspectives of nonstarch polysaccharide enzymes in nutrition

11

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11.1 INTRODUCTION

In recent years, the role of nonstarch polysaccharides (NSPs) in food has been recognized, and they are now entering the market as low-calorie functional food ingredients and prebiotics. Nutraceuticals or health-promoting food additives are becoming more and more important in food industries and many new functional roles for NSP in health food will be developed in the near future [1].

The application of exogenous enzymes (including NSP-enzymes) as feed additives to improve nutrient digestibility of plants and coproducts-based feedstuffs has been researched extensively in higher animals, and is now broadly used throughout the world as a way to reduce the antinutritional effects of NSP and phytic acid, increasing the utilization of carbohydrates and phosphorus, respectively [2]. As well as in aquaculture, the use of NSPs-enzymes to improve nutrient digestibility and utilization from plant feedstuffs has emerged quite rapidly [3–6].

The main function of NSP-enzymes is to hydrolyze complex NSP present in plant feedstuffs that nonruminant animals are incapable of hydrolyzing with their endogenous pool of digestive enzymes [7]. In fish, the presence of digestive enzymes that specifically hydrolyze the β-glycosidic bonds of NSP seems to be very low or nonexistent [8]. Some of these NSPs are present as part of the cell wall, thus shielding substrates from contact with the digestive enzymes, or as part of cell content where their presence may interfere with digestion and absorption due to their chemical nature [9]. Previous studies have reported improvements of nutritive value, feed utilization, body weight gain, composition and activity of intestinal microbiota, and reduction in excreta volume after supplementation of plant-ingredients-based diets with NSP-enzymes [6,10–19]. However, information on the potential functions and applications of NSP-enzymes in human and animal nutrition particularly in improving the nutritive values of plant ingredients and their by-products-based diets (e.g., NSPs) is limited and still needs further research. More studies are also required to prove the specific molecular mechanisms of their functions in human and animal nutrition. Therefore, this chapter

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reports on the application of NSP-enzymes, mechanisms of their effectiveness in human and animal nutrition, and opportunities for their supplementation in food, beverages, and animal feed, presenting trends and perspectives.

11.2 APPLICATION OF NSP-ENZYMES IN ANIMAL NUTRITION

Enzymes including NSP-enzymes can be applied in various fields, such as for technical use, food manufacturing, animal nutrition, cosmetics, medication, and as tools for research and development. At present, almost 4000 enzymes are known, and of these, approximately 200 microbial original types are used commercially [20]. However, only about 20 enzymes are produced on a truly industrial scale. With the improved understanding of the enzyme production biochemistry, fermentation processes, and recovery methods, an increasing number of industrial enzymes can be foreseeable. There are about 12 major enzyme producers and 400 minor suppliers worldwide. Approximately 75% of the total enzymes are produced by three top enzyme companies, such as US-based DuPont (through the May 2011 acquisition of Denmark-based Danisco), Denmark-based Novozymes, and Switzerland-based Roche. The market is highly competitive, has small profit margins, and is technologically intensive [20].

11.2.1 NSP-ENZYMES APPLICATION IN INDUSTRIES

The use of enzymes including NSP-enzymes in animal nutrition has an important role in current farming systems [21]. Feed NSP-enzymes can increase the digestibility of nutrients, leading to greater efficiency in feed utilization; they can also degrade unacceptable components in feed, which are otherwise harmful or of little or no nutritional value. Another importance of NSP-enzymes is a positive impact on the environment by allowing better use of natural resources and reducing the fecal nutrient level applied to land. For instance, diets based on cereals such as barley, rye, and wheat are higher in NSPs, which can decrease the intestinal methane production when supplemented with NSP-enzymes. Currently, feed NSP-enzymes available commercially by catalytic types are: 3-phytase, 6-phytase, α-galactosidase, glucanase, and xylanase, and mostly for the swine and poultry sectors [22]. Though research interest into the potential value of feed NSP-enzymes has occurred in the field of aquaculture and ruminant nutrition, commercially viable versions have not been produced [23]. Additionally, development of heat-stable, improved specific activity and some new NSP-enzymes, along with rapid, economical, and reliable assays for measuring enzyme activity has always been the focus and been intensified recently [21].

The global market for enzymes including NSP-enzymes (particularly for the use of feed industries) was estimated at around US $344 million in 2007 [20],

and expected to increase by twofold in the coming years. The use of NSP-enzymes as feed additives is restricted in most countries by local regulatory authorities [24]. Thus, applications may therefore vary from country to country. Though some of the geographies have attained saturation, the market for feed NSP-enzymes globally in broad terms, is growing; e.g., regions like the United States, China, and South-East Asia still hold a high growth potential.

11.2.2 NSP-ENZYMES APPLICATION IN FOOD PROCESSING

Enzymes including NSP-enzymes have been used in food production and processing for centuries. Iconic examples include rennet for cheese making, diastase for starch hydrolysis, pectinase for pectin hydrolysis, and amylases and proteases in the production of soy foods [25,26]. Enzymatic preparations obtained from extracts of plants or animal tissues were commonly used in food production, although currently microorganisms are by far the main providers of commercial enzymes for food applications [27]. The main reason could be that microorganisms are easier to work with and to manipulate than mammalian or plant cells, and allow for large-scale and cost-effective production of enzymes. Besides, enzymes from animal origins bring a considerable risk of disease potency both in handling and in manufacturing, which is much more complex and expensive than microbial-based production. The extensive use of enzymes within the scope of the food industry is not only related to their catalytic specificity, ability to operate under mild conditions, and biodegradability, but also to their generally regarded as safe label. To achieve the latter status, the candidate enzyme or enzyme formulation must however comply with a complex set of regulatory constraints [27,28].

Today's consumers demand higher levels of quality in their foods in terms of natural flavor and taste, not only in the United States and Europe, but also in the developing countries where consumption shifts away from staple sources of calories towards life enjoyment. This trend triggered the need for the development of enzymes (NSP-enzymes) applications in food processing. A modest, expected decline of enzyme sales has been seen in the baking industry; while the concepts and need for healthy foods promote the positive growth in the whole food enzyme market [20].

Food enzymes are mainly used in the baking industry, fruit juice and cheese manufacturing, as well as wine making and brewing, to improve flavor, texture, digestibility, and nutritional value. In terms of enzyme regulations, enzymes used in food can be distinguished into food additives and processing aids. Most food enzymes are considered as processing aids; only a few are used as additives [20]. From a regulatory point of view, distinction between processing aids and additives is very important because the national regulatory context of enzymes differs significantly in different countries. Nevertheless, generally, the processing aids are used during the manufacturing process of foodstuffs, and do not have a technological function demand in the final food. Enzymes used in food processing are typically sold as enzyme preparations, which contain not only the desired enzyme,

but also metabolites from the production strain and several added substances such as stabilizers. All these materials are expected to be safe under the guidance of good manufacturing practice (cGMP).

From the NSPs-enzymes, xylanases are used to break down hemicelluloses, particularly the insoluble arabinoxylans, which tamper with the formation of the gluten network. These NSPs are widely present in cereals. As a result of enzyme action, flour and water distribution is improved and the dough is softer, more flexible and stable, and easier to handle. During the baking process, crumb formation is delayed and the dough is allowed to rise. Besides, the use of xylanases increases the concentration of arabinoxylo oligosaccharides in bread, with a positive impact on human health [29–31]. Furthermore, NSPs-enzymes are used to tackle the turbidity and viscosity of fruit and vegetable juices (mostly due to NSPs including pectins and hemicellulose components) and improve extraction and clarification of fruit and vegetable juices. Pectinases are perhaps the primary type of NSPs-enzymes used in fruit processing, but xylanases and cellulases are used at a lower scale. As briefly mentioned in Chapter 10, Depolymerizating enzymes in human food: bakery, dairy products, and drinks, pectins are NSPs with a backbone of galacturonic acid residues linked by α (1–4) linkage and side chains of diverse sugars. Indeed, the hydrolytic action of pectinases breaks different linkages, resulting in the disintegration of the jellified structure, decreased viscosity of the fruit juice drops, improved pressability, the whole allowing for higher recovery yields and clarified juice [32–35].

11.2.3 NSP-ENZYMES APPLICATION IN AQUACULTURE NUTRITION

Aquaculture is recognized as the fastest growing agribusiness sector and has thus become an important component of the global food supply [36]. However, the increased production of intensively reared fish species necessitates the supply of high-quantity and sustainable feed ingredients in nutritionally balanced formulated diets. NSPs (typically cellulose, arabinoxylan, and mixed-linked-glucans) are major components of plant cell walls and are indigestible to fish. They may cause increased viscosity in the gut, which consequently results in a reduced rate of digestion, nutrient absorption, and reduced feed intake [37]. NSP-enzymes (e.g., cellulase, xylanase, etc.) are capable of disrupting plant cell wall integrity, thereby reducing molecular size characteristics of NSPs. Consequently, this enhances rapid digestion by reducing viscosity in the gut [37,38]. Supplementing fish diet with NSP-enzymes offers potential for better utilization of nutrients from plant ingredients [39]. Nevertheless, the NSPs-enzymes supplementation (Energex (hemicellulases), Bio-Feed (protease) and Alpha galactosidase (α-galactosidase)) did not improve weight gain of rainbow trout fed lupin-based diets [40], as reported previously by Ogunkoya et al. [41] with soybean meal-based diets; but mixed enzymes significantly improved protein efficiency ratio. These authors also suggested that enzyme supplementation studies should be carried out when animals are under restricted feeding regimes as the beneficial effects of enzyme

addition may be hidden by increased feed intake under an ad libitum feeding regime. Supplementation of enzymes including NSPs-enzymes (cellulase and xylanase) increased the growth of Japanese seabass (*Lateolabrax japonicus*) fed with diets of more than 40% plant-protein feedstuffs (soybean meal, rapeseed meal, peanut meal and wheat meal), suggesting that these enzymes were effective in reducing the antinutritional effects of NSPs and improving fish performance [42]. Besides the growth performance, the increase in phosphorus retention and protein utilization suggested that the improved growth was due to increased feed utilization [42]. The fact that protein utilization was improved by NSP-enzymes supplementation provided good support for the hypothesis that a protein-sparing effect and perhaps hydrolysis of protein−carbohydrate complexes occur when exogenous enzymes are added to fish diets. Similarly, supplemental exogenous enzymes including NSP-enzymes such as β-glucanase and xylanase at a dose level significantly improved the specific growth rate, feed efficiency ratio and apparent protein retention of juvenile hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) fed diets with plant-based feedstuffs [43]. These results suggest that NSP-enzymes supplementation can promote the secretion of endogenous enzymes by the fish. In addition, Dalsgaard et al. [44] conducted a feeding trial with rainbow trout in which independent diets with high inclusion levels of three different plant-based feedstuff (soybean, sunflower, and rapeseed meals) meals were supplemented with three different enzymes (β-glucanase, xylanase, and protease) and apparent nutrient digestibility was assessed. Enzyme supplementation had only moderate effects on apparent nutrient digestibility in the sunflower and rapeseed experiments, while β-glucanase and protease improved the apparent digestibility of all nutrients in the soybean meal-based diet. The effect was more pronounced for lipids than for other nutrients. β-Glucanase had a positive effect on energy retention of the fish in the soybean meal experiment, while nitrogen retention and/or production performance were not significantly affected in any of the three experiments [44].

Recently, Yildirim and Turan [45], Ghomi et al. [46], and Zamini et al. [47] found a positive effect of various commercial multienzyme complexes (phytase, xylanase, β-glucanase, β-amylase, cellulase, and pectinase) on the growth performance and feed efficiency of African catfish (*Clarias gariepinus*), great sturgeon (*Huso huso*), and Caspian salmon (*Salmo trutta*), respectively. However, no specified plant-based feedstuffs were used in any of these studies. Moreover, Zhou et al. [15] studied the growth and changes in intestinal microbiota of grass carp fed a duckweed-based diet with an inclusion of NSP-enzyme (cellulase); and their results showed that cellulase supplementation promoted growth and digestive enzyme activities (cellulase, amylase, and protease). Besides, a polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) analysis indicated changes in intestinal microbiota of the fish fed exogenous cellulase in both bacterial species and density. The 16S rRNA gene sequencing identified the emergence of certain bacterial strains including *Bacilli* and *Sphingomonas* that may contribute to the digestion of cellulose. Zhou et al. [15] concluded that the endogenous

cellulase activity of grass carp is far from sufficient to fully digest the ingested fiber, hence exogenous cellulase could be a useful feed additive. Furthermore, Castillo and Gatlin [7] briefly reviewed the beneficial effects of exogenous enzymes including NSPs-enzymes in fish nutrition.

11.3 MECHANISMS OF NSP-ENZYMES IN ANIMAL NUTRITION

To provide insights into underlying mechanisms that relate NSP and NSP-enzymes to gut function and gut health, we followed a review reported by Zijlstra et al. [37]. For instance, Reilly et al. [48] studied the effects of oat-based β-glucans or oat-derived β-glucans in a wheat-based diet with or without a β-glucanase cocktail. Intact β-glucans increased hindgut fermentation and Bifidobacteria and Lactobacilli populations in the colon; the supplementation of β-glucanase reduced these effects. Combined, these results indicate that intact β-glucans stimulate gut health. Smith et al. [49] conducted a study of similar design using barley- and oat-based diets. Oat-based diets had a reduced nutrient digestibility and an increased population of Bifidobacteria and Lactobacilli. Willamil et al. [50] studied maize or wheat-barley-rye diets with or without a β-glucanase and xylanase cocktail. Enzyme increased feed intake and gain for the wheat-based but not the corn-based diet, perhaps due to increased starch digestion in the caecum. Enzyme supplementation decreased Gram-negative bacteria overall, but only increased Lactobacilli, Bifidobacteria, and overall biodiversity for the wheat-based diet. Willamil et al. [51] used the same study design, and indicated that enzyme tended to increase absorptive area and reduced cell proliferation and intraepithelial lymphocytes in the gut. Overall, these studies confirmed that nutrient digestibility differs among cereal grains, and NSP-enzymes can enhance nutrient utilization in wheat-based diets. Both cereal grains and NSP-enzymes could affect components of gut health, including intestinal morphology, bacteria populations, and microbial metabolites in the gut content. Nevertheless, specific mechanisms (up to molecular mechanisms) regarding the interaction between NSPs and NSP-enzymes in plant-based diets need further in-depth studies.

Furthermore, observations on gene expression responses to exogenous enzymes including NSP-enzymes supplementation are important for explaining growth performance responses observed from a physiological perspective. A relevant example is demonstrated in a study with pigs, in which greater starch digestion was found to be associated with greater amino acid absorption [52]. This effect is mediated via glucose absorption because glucose acts as a signaling molecule for amino acid transporters. Observation of such interplay among biochemical pathways may offer insights into the drivers for enhancement of nutrient utilization due to the supplementation of exogenous enzymes [2]. Additionally, because some fish species are known for their inefficient metabolism of glucose

[8,53], it will be important to elucidate how these physiological differences among fish and swine/poultry will affect the responses observed by the addition of exogenous enzymes.

11.4 OPPORTUNITIES AND STRATEGIES FOR THE PRODUCTION OF NSP-ENZYMES

Several researchers have proposed different novel strategies for the production of NSP-enzymes. Novel approaches are based on microbial culture technologies, genetic engineering approaches for strain improvement, and efficient up-scaling technologies. Genomes of several important microorganisms have revealed interesting genes responsible for codifying novel enzymes and organic acids. These genes are the prime interest of current research for superior generation and improved properties of bioproducts [54].

11.4.1 NSP-ENZYME CHARACTERISTICS

There are different strategies to improve the economic feasibility of NSP-enzymes supplementation. In the past, the attention was on producing enzymes that survive longer in the digestive tract by making them less susceptible to proteolysis during transit in the digestive tract. New approaches have aimed to optimize the activity of the NSP-enzymes and designed them to increase their aggressiveness toward the substrate of interest. For instance in aqua-feeds, enzymes including NSP-enzymes used to supplement commercial feeds cannot withstand the rigors of the most aggressive feed processing techniques such as extrusion or pelleting at temperatures in excess of 95°C [7]. According to a recent review by Castillo and Gatlin [7], to avoid high temperature exposure during aqua-feed manufacture, exogenous enzymes including NSP-enzymes can be mixed with oil and spray-coated onto feed pellets after processing. For a more practical approach, a protective coating, usually a lipid, has been used to protect the supplemental enzymes during feed processing. One of the strategies currently being used to enhance enzyme thermostability is to improve coating agents. Characteristics of a desirable coating include its stability during feed processing and its ease of dissolution during digestion. A suitable coating must be strong enough to resist the high temperature and pressure of manufacturing processes, but also must be fragile enough to be digested at lower temperatures. Other approaches may be to genetically manipulate the amino acid structure of the enzyme produced so it is more inherently thermostable or to discover intrinsically thermostable enzymes, e.g., from thermophilic bacteria. The production of much more thermotolerant enzymes may allow dry, powdered enzymes to be used in diets processed at high temperatures [2,55]. Besides their thermolabile nature, exogenous enzymes including NSP-enzymes face other limitations such as pH

specificity and inhibitors in the gastrointestinal tract that could greatly limit the potential benefits of their supplementation. For example, in aquaculture nutrition, it is expected that NSP-enzymes will show different efficiency in different fish species because of the diversity of their digestive systems [7].

11.4.2 NSP-ENZYME COMBINATIONS, SUBSTRATE, AND PRODUCT IDENTIFICATION

Protein is the most costly nutrient in animal diet required for optimum growth performance. Because of the expense of providing sufficient protein to growing animals, numerous studies have been carried out to investigate the possibility of reducing dietary protein and energy using NSP-enzymes in animal feed. Many studies have reported that nitrogen retention and energy utilization are often increased when a cell wall-degrading enzyme (including NSP-enzymes) is added to a plant protein-based animal diet [56–59].

The main antinutrients that may limit the nutritive value of animal feed are the NSPs of plant-based protein sources [7,60,61]. Numerous researchers have reported increased growth and improved feed conversion ratio as a consequence of NSP-enzymes inclusion in plant-based animal diets [62–65]. Effects of exogenous enzymes on growth performance for swine have been variable. Inborr et al. [66] reported that supplementation of barley-based diets with NSP-enzymes significantly increased average daily gain and feed conversion ratio of weaned piglets significantly. Yin et al. [67] showed that a combination of β-glucanases and xylanase improved growth performance and feed gain ratio when piglets were fed with barley-based diets. Lindberg et al. [68] reported that multiple NSP-enzymes (including β-glucanases, xylanases, and cellulase) enhanced the growth performance, especially body mass gain, of piglets when fed with barley-based diets. Thus, the goal in using multiple NSP-enzymes as diet additives is to target different antinutritive compounds in feedstuffs to obtain maximum benefits. The approach in using multiple NSP-enzymes activities may produce greater benefits than a single NSP-enzyme application. Nevertheless, to maximize the efficacy of NSP-enzyme combinations, it is essential to understand how the NSP-enzymes work together to hydrolyze their respective substrates. For example, because some xylanases target soluble and other insoluble arabinoxylans, the most effective combination is achieved when they are used together. Xylanases with only an affinity for insoluble arabinoxylans give soluble hydrolytic products and consequently increase digesta viscosity [69]. Therefore, effective reduction in viscosity is only attainable when the enzyme has an affinity for both the soluble and insoluble NSPs. Studies have shown that mix-linked β-glucan and arabinoxylan are easily hydrolyzed by β-glucanases and xylanases, respectively. Addition of cell-wall-degrading enzymes in vitro increased the release of proteins and nonstarch carbohydrate in barley [70]. Supplementation of exogenous NSP-enzymes to piglet diets can increase the digestibility of barley and pigs' growth [66,71]. This has

been attributed mainly to the breakdown of endosperm cell wall components, resulting in more complete digestion of starch and protein in the small intestine. Moreover, Tahir et al. [59] examined NSP-enzymes such as hemicellulase, cellulase, and pectinase individually and in combination in broilers. They reported that the hydrolysis of hemicelluloses was the rate-limiting step for subsequent hydrolysis of other nutrients that may be trapped in the cell. Therefore, the cellulase and pectinase pair was less effective in improving NSP hydrolysis. Because the results obtained from enzyme combination studies seem to be feedstuff specific, more studies that use individual enzymes, as well as different combinations of enzymes, are needed. Moreover, the specific substrate/enzyme interactions need to be defined properly. A more precise understanding of the presentation of the substrate in feedstuffs will allow development of associative enzymes, which will cooperate to degrade the target substrate(s) [2,55]. In addition, it has previously been demonstrated that a substantial portion of the variation in enzyme efficacy is associated with the inherent intestinal digestibility of starch, protein, and lipid, and so an appreciation for the concentration of undigested fractions of these nutrients in a diet may help to determine the magnitude and consistency of feed enzyme responses. The use of feed ingredient mixtures, generated systematically, with radically different inherent characteristics to explore enzyme effects in a multinutrient space will be instructive and will help to clarify the effects of NSP-enzymes on the nutritional physiology of nonruminant animals [72].

11.4.3 GENETIC ENGINEERING FOR STRAIN IMPROVEMENT

Genetic engineering has provided a platform to overcome serious drawbacks of enzyme productivity and quality such as substrate/product inhibition, or narrow substrate specificity [73]. According to the review reported by Panda et al. [54], the most commonly used genetic engineering technologies for enhanced enzyme production are recombinant DNA technologies, protoplast fusion, and mutation.

11.4.3.1 Recombinant DNA technologies

Genetic improvement of microorganisms has been very useful for commercial production of bioproducts and pharmaceuticals. As reported by Singhania et al. [74] recombinant DNA technology is known to improve the output up to 100-fold, and it is achieved by incorporating a foreign gene of interest into a host where it is subsequently expressed. The host system can either be a prokaryotic or eukaryotic microorganism. Enzymes including NSP-enzymes could be expressed in recombinant microorganisms. Microbial strains are genetically engineered for successful production of different important enzymes. For example, *Fusarium* alkaline protease has been produced by *Acremonium chrysogenum* at 4 g/L. Similarly, in case of Trichoderma *reesei*, higher recombinant enzyme production of 35 g/L was achieved [75].

11.4.3.2 Mutation

Mutations through physical and chemical mutagens are frequently applied for the genetic improvement of microorganisms in terms of environmental adaptability and bioproduct productivity. Fungal strain of *Trichoderma viride* was improved by exposure to UV and microwave radiation followed by EMS (ethylmethane sulfonate) treatment. Higher enzyme recovery, i.e., 13.38 mg/g, was observed for the mutant strain when grown on agricultural waste (wheat bran) [76]. Similarly, spores of *Aspergillus* sp. SU14 were repeatedly treated with different mutagens such as Co60 γ-rays, ultraviolet irradiation, and *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine. One of the mutants (*Aspergillus* sp. SU14-M15) could produce cellulase (one of the NSP-enzymes) at 2.2-fold higher than the wild strain [77].

11.4.3.3 Protoplast fusion

Protoplast fusion is a novel tool for transferring genes for a desired quality and quantity of production. In this technology, two different genetically originated protoplasts from different somatic cells are fused in order to obtain parasexual hybrid protoplasts. Genes representing useful characteristics such as higher bioproduct productivity, improved protein quality, heat and cold resistance can be transferred from one species to another. For instance, intra-strain protoplast fusion was carried out in *T. reesei* strain *PTr2* using lysing enzymes, 0.6 M KCl as osmotic stabilizer, 40% polyethylene glycol with STC (sorbitol, Tris−HCl, $CaCl_2$) buffer. The majority of the fusants showed faster mycelia growth, abundant sporulation, and high levels of extracellular carboxymethyl cellulase (80% of the fusants showed higher enzyme activity and two fusants, *SFTr2* and *SFTr3* were found with twofold increase in enzyme activity) than the nonfusants and wild strains [78].

11.5 CONCLUSIONS AND FUTURE PERSPECTIVES

The overall information may indicate that in animal nutrition, NSP-enzymes improve digestibility of the nutrients and could provide the animals with additional energy, protein, and minerals resulting in environmentally friendly cost-effective, predictable growth performance. Besides the promising results and opportunities found in humans and higher animals, favorable effects were also found in some aquaculture species, which could play an important role in reducing further use of fishmeal in aquatic animal diets and increase the use of plants and their coproducts-based protein feedstuffs, ultimately ensuring sustainability of animal production systems. Indeed, NSP-enzymes do have a future in improving nutrient utilization of plant ingredients and their coproducts-based diets for both humans and animals. In addition, using multiple NSP-enzymes as diet additives can target different antinutritive compounds in feedstuffs to obtain maximum benefits, compared to individual NSP-enzyme. Nevertheless, to maximize the efficacy of NSP-enzyme combinations, it is essential to understand how the NSP-enzymes work together to hydrolyze their respective substrates.

Recently, the application of NSP-enzymes in food production and processing has increased rapidly, which is a representative example of the practical applications of biotechnology. The reasons for such enduring success are related to the specificity, fast action, mild operational conditions required, and biodegradable and biocompatible nature of enzymes. These features allow for chemical modifications of substrates to be performed effectively, and for food processing with preservation of nutrients, while complying with public health-and-safety requirements. Increased insight on catalytic mechanisms and protein configuration at a molecular level allows for a better understanding of the behavior and stability of NSP-enzymes. Thus, actualization of NSP-enzyme applications in industrial processes requires high-performance enzymes with specific characteristics, which will stimulate research to explore new avenues to overcome their weaknesses. Some of the strategies in the field are exploiting novel NSP-enzymes from nature, improving existing catalytic properties, broadening specialized enzymes to serve new functions, optimizing formulation of enzyme preparations, or de novo designing biocatalysts. All these new developments are likely to be pursued in the near future and therefore contribute to improving and expanding the role played by NSP-enzymes in the food, beverage, animal feed, and pharmaceutical industries.

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PART

Proteases

IV

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CHAPTER

Proteases—general aspects 12

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12.1 INTRODUCTION

The term "proteases" defines enzymes that digest proteins, and was invented by S.H. Vines, Professor of Botany at the University of Oxford in 1903 [1,2]. Furthermore, the term "peptidase" was introduced some years later by Petersen and Short [3]. Generally, peptidases are enzymes that cleave peptides into amino acids. Proteases (or proteinases) are enzymes that break down both proteins and peptides. These enzymes start the process of protein catabolism by hydrolysis of the peptide bonds that connect amino acids together.

Almost all organisms require proteolytic enzymes to function. Proteolysis comes along with the release of an amino acid or a peptide from a protein or a larger peptide. It is one of the final stages of posttranslational processing for many proteins, and is unidirectional [1].

Most naturally occurring proteases are initially produced as inactive precursors called zymogens. Zymogen activation involves expression of the protease fused to an activation segment, which prevents proteolytic activity until it is cleaved. The most common activation segments are N-terminal sequences that sterically block the active site [4]. Beyond the role of sterically hindering the active site, activation segments are often important for the folding, stability, and sorting of the precursor protease [5].

According to the Enzyme Commission (EC) classification, proteases belong to group 3 (hydrolases), and subgroup 4 (which hydrolyze peptide bonds) [6].

Generally, peptidases either can act on peptide bonds within the protein molecule (endopeptidases) or can cleave peptide bonds at a free terminus (exopeptidases). The distinction between endo- and exopeptidases does not apply on the protease family level. Endopeptidases can be of any catalytic type. Exopeptidases can be further divided according to whether they act at the N- or C-terminus, and by the number of amino acids released during one cleavage. For example, an exopeptidase that releases one N-terminal amino acid is an aminopeptidase. An exopeptidase that releases a single amino acid from the C-terminus is a carboxypeptidase [1].

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12.2 CLASSIFICATION OF PROTEASES/PEPTIDASES

12.2.1 CLASSIFICATION BY CATALYTIC TYPE

The catalytic type can often help in determining the pH at which the peptidase will operate and the inhibitors that will inactivate it [1]. Currently, proteases are classified into six groups: aspartate, cysteine, glutamate, metallo, serine, and threonine.

This classification is based on characteristic mechanistic catalysis features consistent with each member of a group [4,7]. These six different types can be grouped into protein nucleophiles and water nucleophiles. Protein nucleophiles can be the hydroxyl of a serine or threonine residue, or the thiol of a cysteine, and the catalytic types are known as serine-, threonine-, or cysteine type. A water nucleophile can either be activated by the side of chains of amino acids (aspartates or glutamates) or by a metal ion bound by amino acid side chains (aspartyl-, glutamyl-, or metallopeptidases) [1]. The peptidases of unknown catalytic type are listed under "unknown". Some are grouped into a "mixed" family (Table 12.1).

12.2.2 CATALYTIC MECHANISMS OF PROTEASES

Proteases can act very specifically (limited hydrolysis) and unspecifically (aggressive hydrolysis). The protease specificity is defined by structural properties (amino acid residues) of the active site of the enzyme molecule [8].

Table 12.1 Classification of Proteases by Catalytic Type and by MEROPS (Molecular Structure and Sequence Homology) and Their Usual pH-Optimum [1,7]

Catalytic Type	MEROPS Families *n* (Names From–to)	pH-Optimum[a]
Aspartic (A)	16 (A1–A37)	Acidic
Cysteine (C)	89 (C1–C117)	Acidic
Glutamic (G)	2 (G1–G2)	Acidic
Metallo (M)	72 (M1–M99)	Neutral
Asparagine (N)	10 (N1–N11)	
Mixed (P)	2 (P1–P2)	
Serine (S)	52 (S1–S81)	Neutral to basic
Threonine	6 (T1–T8)	Neutral
Unknown	10 (U32–U74)	
Total	196	

[a]*With exceptions, e.g., at the aspartic peptidases.*

The position of the active site residues (catalytic residues and/or metal ligand) is usually conserved between homologous structures [1].

12.2.2.1 Serine peptidases

In most serine peptidases there is a catalytic triad consisting of a serine, which carries the nucleophilic hydroxyl group, a histidine, which acts as the general base, and an aspartate which is believed to orientate the imidazolium ring of the histidine so that the serine and histidine are close and the hydroxyl group is activated [1].

12.2.2.2 Threonine peptidases

Threonine proteases are characterized by an N-terminal threonine residue within the active site of the enzyme molecule as nucleophile to perform catalysis. There are also N-terminal nucleophile (Ntn-) hydrolases in which the N-terminus is a serine or cysteine. However, many of the Ntn-hydrolases are enzymes other than peptidases, and peptidase activity is restricted to a single processing event to release the propeptide from the precursor [1].

12.2.2.3 Cysteine peptidases

A nucleophile cysteine thiol is involved in the catalytic mechanism of that peptidase.

Seventy-two families of cysteine peptidases have an essential Cys/His dyad in which the cysteine bears the nucleophilic thiol and the histidine acts at a general base. Some third or even fourth residues may be involved in the catalytic process [1].

12.2.2.4 Aspartic peptidases

In the case of aspartic peptidases, the catalysis of the hydrolysis of peptide bonds in a polypeptide chain follows a mechanism in which a water molecule bound by the side chains of aspartic residues at the active center acts as a nucleophile. Aspartic peptidases are all endopeptidases [1].

12.2.2.5 Glutamic peptidases

This category consists of only two families. Peptidases in family G1 use glutamate and glutamine as a nucleophile for catalysis. Peptidases in family G2 are precursors that act autolytically. The catalytic dyad consists of an aspartate and a glutamate, in which the glutamate activates a water molecule [9].

12.2.2.6 Metallopeptidases

The metallopeptidases exist both in the forms of endo- and exopeptidases. The metal ion in a metallopeptidase is usually zinc, but also enzymes with cobalt, manganese, or copper exist [1,10–12]. There are also peptidases with two metal ions.

12.2.3 CLASSIFICATION BY HOMOLOGY

Peptidases of the same catalytic type are not necessarily homologous. Sequences with homologous peptidase units are grouped into a peptidase family according to the MEROPS classification [13] (see Table 12.1). Rawlings [1] gives a brief summary of the classification procedure. A family is built around a peptidase that has been well characterized biochemically, which is termed the "type example." This is often a peptidase for which the sequence, tertiary structure, and specificity is known; e.g., the type example for peptidase family S1 is chymotrypsin. In general, the sequences of peptidase units are determined to be homologous if the expected value in a BlastP [14] or FastA search [15] is less than 0.001.

A family is a set of homologous proteolytic enzymes. The homology is shown by a significant similarity in amino acid sequence either to the type enzyme of the family, or to another protein that has already been shown to be homologous to the type enzyme, and thus a member of the family. The relationship must exist in the peptidase unit at least. A family can contain a single enzyme if no homologous enzymes are known, and a single gene product such as a virus polyprotein can contain more than one proteolytic enzyme each assigned to a different family. Each family is identified by a letter representing the catalytic type of the proteolytic enzymes it contains together with a unique number. Some families are divided into subfamilies because there is evidence of a very ancient divergence within the family, e.g., S1A, S1B [16].

If two peptidases from different families are grouped and have similar tertiary structures (in the peptidase unit), then the two families will be grouped in a clan [1] and further into subclans if there are any characteristics that link some of the families together. An individual peptidase is also given a unique identifier consisting of the family name, a dot and a sequential three digit number. As an example, chymotrypsin A has the MEROPS identifier S01.001. All the available information is listed in the MEROPS database (http://merops.sanger.ac.uk), which is an integrated source of information about peptidases, their substrates, and inhibitors.

12.3 OCCURRENCE OF PROTEASES

It is likely that proteases appeared at the earliest stage of protein evolution as simple destructive enzymes necessary for protein catabolism and the generation of amino acids in primitive organisms [7]. Proteases as such represent the largest single family of enzymes. They occur naturally in all organisms.

Analysis of the human genome has shown that proteases had a proportion of about 2% of the total genome [4,17,18].

Understanding protease biology is complicated, because the potential substrate repertoire is the entire human proteome [19]. Proteases are encoded by more than 550 human genes [20–22]. Beyond nonspecific, degradative functions, e.g., in protein catabolism, proteases act as sharp scissors and catalyze highly specific

reactions of proteolytic processing. Proteases are relevant in the control of multiple biological processes [7].

They are involved in various physiological processes, starting with digestion of food proteins up to complex physiological reactions. They determine the lifetime of other proteins. Proteases play an important role in highly regulated cascades like, e.g., blood coagulation and lysis of the clots, or apoptosis pathways and many other important physiological processes.

They are present in the blood serum (e.g., thrombin) and in the leukocytes (e.g., elastase).

Proteases regulate the fate, localization and activity of many proteins, modulate protein–protein interactions, create new bioactive proteins, and generate, transduce, and amplify molecular signals. Therefore, they influence DNA replication and transcription, cell proliferation and differentiation, tissue morphogenesis and remodeling, heat shock and unfolded protein responses, angiogenesis, neurogenesis, ovulation, fertilization, wound repair, stem cell mobilization, homeostasis, inflammation, etc. [7]. Proteases carry out a vast array of critical functions from intracellular protein recycling, to nutrient digestion, to immune-system amplification [4].

Consistent with these essential roles of proteases in cell behavior and survival and death of all organism, alterations in proteolytic systems underlie multiple pathological conditions such as cancer (tumor invasion), neurodegenerative disorder (e.g., Alzheimer's disease), and inflammatory and cardiovascular diseases (e.g., rheumatoid arthritis) [1,7].

The origin of many proteases is from bacteria. In that context, proteases are also a type of exotoxin, which is a virulence factor in bacteria pathogenesis. Proteases also play important role in many aspects of plant physiology and development [23]. Plant proteases can be further grouped into cytosolic proteases that act in the internal fluid of a plant cell, proteases in the endoplasmic reticulum, and proteases in the lumen of plant vacuoles [24,25]. Proteases also contribute to physiological processes of mitochondria and chloroplasts [26].

Protease activity is a characteristic common to many allergens. Allergen source-derived proteases interact with lung epithelial cells, which are now thought to play vital roles in both innate and adaptive immune responses. Allergen source-derived proteases act not only as allergens, but also as promoters of allergenicity [27].

Protease inhibitors turn off the activity of protease, thus they are no longer needed to defend the organism against various infections. Protease inhibitors are widely distributed in plants and animals. They have a variety of functions, which include preventing digestion of seeds by insects and modifying blood clotting in animals. Synthetic and natural inhibitors suppress two-stage carcinogenesis and breast cancer. Protease inhibitors are unique in that they interfere with cancer development in a variety of ways, including suppression of oxygen radicals, oncogenes, and metastases. Epidemiological evidence supports their prevention of major human cancers in populations that consume foods containing them [28]. However, some protease inhibitors have to be inactivated in food or feed such as, e.g., the trypsin inhibitors found in soybean that have to be inactivated by a heat treatment (toasting process).

12.4 THE DIGESTION OF FOOD PROTEIN

One of the key roles of proteases is the digestion of food protein. This is one of the most important parameters for proteases in human and animal nutrition. The digestion of food protein is more or less basic knowledge, however some main aspects are summarized in the following paragraphs (according to Refs. [29,30]). Protein is a highly diverse substrate. Proteases are very efficient at breaking down the protein bonds of food and feed protein sources.

In order to avoid autolysis by proteolytic activities, proteases are secreted in the form of inactive precursors, which become active only after hydrolytic cleavage of a particular peptide chain. This activation mechanism is, e.g., common for most serine proteases, and helps to prevent autodegradation of the pancreas. In the stomach, the degradation process of protein starts by the denaturation by hydrochloric acid, followed by the hydrolysis by pepsin through reaction of pepsinogen with HCl. The pepsin cleaves most food proteins into polypeptides. There are some exceptions, such as creatine, mucin, protamine, ovomucoid, and other proteins rich in carbohydrates. Preferably, Phe-Leu or Phe-Phe, Phe-Tyr-bonds will be cut by the enzyme.

For example, calves and lambs secrete chymosin (or also called rennin) as inactive proenzyme that can be activated by pepsin or auto-catalytically. The activity of that enzyme leads to the characteristic degradation of casein from milk. Calf rennet, composing of 88%–94% chymosin and 6%–12% pepsin (and lipase), can be extracted from the fourth stomach of the unweaned calf [31].

Synthesized by the pancreas, the serine proteases trypsin and chymotrypsin catalyze the hydrolysis of proteins and polypeptides into peptides. It is important to mention that trypsin is necessary to activate chymotrypsin in the duodenum.

Trypsin is produced as the inactive zymogen trypsinogen in the pancreas. After stimulation by cholecystokinin, it is secreted into the duodenum. Once in the small intestine, the enzyme enteropeptidase activates trypsinogen into trypsin by proteolytic cleavage.

The action of these to enzymes is continued by pancreatic carboxypeptidases and aminopeptidases originating from the lumen. The further degradation into individual amino acids is performed by dipeptidases from the intestinal brush border.

12.5 TECHNICAL ASPECTS

The commercial use of proteases is well established in many technical processes. A broad range of different proteases with adapted technical properties have to be synthesized on a large scale for these various industrial applications.

Enzyme production can be increased by strain improvement. This is usually done by mutating the microorganism that produces the enzyme by techniques

such as classical mutagenesis, which involves exposing the microbe to physical mutagens such as X-rays, g-rays, UV rays, etc., and chemical mutagens such as NTG, EMS, etc. [6].

As the enzyme products often need to be adapted to the technical application, protease engineering can improve the physical characteristics and the production yield of the enzyme.

Current protease products rely on naturally evolved cleavage specificity, although other properties such as solubility and overall stability have been effectively engineered [4].

The application of proteases for commercial use often requires them to maintain high activity in non-physiological conditions such as high temperatures and pH, intensive calcium chelating agents, and detergents [4]. Li et al. [4] discuss in their review on commercial proteases the enzyme subtilisin, an important biocatalyst used in detergent formulations, as a model case study for improvement of protease functions by protein engineering.

High temperatures during the technical processes, including proteases can lead to protein unfolding [32] and covalent mechanisms. Therefore, thermostability enhancement is of extreme importance, as for many other enzymes such as, e.g., phytases or xylanases, except for liquid applications after processing (e.g., in feed applications). Introducing non-natural intramolecular disulfide bonds to stabilize protein structure is one of the well-known strategies to increase the stability of proteins. Mutagenesis and chemical amino acid modification of the proteases molecule are feasible strategies to engineer oxidation-labile residues in proteases for improved oxidation-resistant properties. Metal binding sites that can function to both stabilize and activate protein engineering by directed evolution provide a promising approach to recover lost stability and catalytic activity of proteases in organic solvent. Altering protease substrate specificity by protease engineering is key for the wider application of commercial proteases. High-throughput screening-systems help, e.g., in *Escherichia coli* or yeast-based systems, to expedite the development of protease engineering.

Besides applications of microbial proteases in human and animal nutrition, which will be discussed in detail in the following chapters, proteases are widely used in the detergent and leather industry. Alkaline proteases have been used to remove hair from hides, pancreatic protease extracts for use in detergent, and leather processing [4,6].

The predominant use of proteases in human therapeutics has been in treating cardiovascular diseases; they are also emerging as useful agents in the treatment of sepsis, digestive disorders, inflammation, cystic fibrosis, retinal disorders, psoriasis, and other diseases. Upregulation of proteolysis is associated commonly with different types of cancer and is linked to tumor metastasis, invasion, and growth. Deregulated proteolysis is also a feature of various inflammatory, and other diseases [19]. That is also a broad field of application for protease inhibitors as therapeutics.

12.6 GENERAL NUTRITIONAL ASPECTS

As proteases have key functions in various physiological processes, it is therefore important that external commercial proteases supplemented to food or feed are compatible with the endogenous enzymes that are already synthesized naturally.

The enzyme has to be stable and active in the gut without negative impact through proteolytic activity on inner organs, mucous membranes, and cells. It should mainly work on the food or feed substrate at the pH range that occurs naturally at the physiological stage of the passage through the gut.

Generally, the supplemented proteases could improve protein digestion through the following hypothesis:

- further degradation of feed/food protein substrate through the supplemented proteases delivers additional amino acids (higher digestibility) in addition to the endogenous proteases (additional effect or synergy);
- the exogenous protease initiates the first step of breaking down food protein molecules that will be further digested by the endogenous proteases and leads to higher efficacy in protein digestion as time (passage rate) can be a limiting factor in the passage through the gut;
- the exogenous protease acts on poorly digestible protein substrate that can only be degraded by the endogenous enzymes to a limited extend (e.g., keratin);
- the supplementation of exogenous proteases could have an indirect energy-sparing effect as less endogenous proteases have to be synthesized to digest the feed or food protein.

It has already been mentioned that proteases in particular bear a risk for allergies [27]. It is extremely important to avoid any inhalation of dust while directly handling the protease enzyme preparations at any stage of the process (worker safety). Encapsulation or granulation of fine dusting dry enzyme preparations or use of liquid formulations effectively reduce the risk of dust. Workers have to carefully follow the safety instructions. The allergenic potential is more a problem directly at the enzyme production, where the pure enzymes or concentrates are used within various processes, and less on feed/food production sites and/or at consumer level, where formulated enzyme preparations are used.

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CHAPTER

Proteases—human food

13

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13.1 INTRODUCTION

Proteases are essential for the digestion of food protein and peptides in humans. They assist the hydrolysis of large polypeptides into smaller peptides and amino acids, thus facilitating their absorption by the intestinal cells. The extracellular enzymes play a major role in nutrition due to their depolymerizing activity. The microbial enzymes and the mammalian extracellular enzymes such as those secreted by the pancreas are primarily involved in keeping the cells alive by providing them with the necessary amino acid pool as nutrition [1].

13.2 PROTEASES IN FOOD PROCESSING

Generally, proteases serve in the production of value-added food ingredients. Enzymes that utilize food-grade proteins as substrates have gained recent interest among food scientists. Proteases alter the native conformation of proteins. The utilization of enzymes for structuring foods is an ecologically and economically viable alternative to the utilization of chemical cross-linking and depolymerization agents [2].

Proteases can be generated from plants (e.g., papain, bromelain, keratinases, and ficin). The most familiar proteases of animal origin are gastric pepsins and pancreatic elastase, trypsin, chymotrypsin. and carboxipeptidases A and B [1,3,4]). However, microbial sources are preferred for the production of proteases due to technical and economic advantages [5].

Exogenous proteases are a powerful tool to improve the functional properties of food proteins. They can be used to improve, e.g., gel strength, fat-binding, water-binding, emulsification foaming, or viscous-elastic properties. Furthermore, applications of proteases are in place to improve flavor or palatability in food ingredients. They improve food processing by reducing viscosity. The use of proteases can result in improved drying characteristics, etc. Proteinases from various origins have been used for such purposes. Controlled proteolysis leads generally to more soluble products with improved emulsifying properties but relatively poor foaming properties [6].

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Proteases are used in a wide range of foods and food-processing applications. In the dairy industry, they are used for milk coagulation, cheese ripening, and flavor development. Another application is in the field of meat tenderization. In the baking industry, there are applications for proteases in gluten development and dough preparations. With respect to plant protein processing, proteases are used to improve functionality and processing, e.g., for generation of bioactive peptides. Proteases are useful for obtaining flavor compounds. In fish and seafood processing, proteases are used for the production of fishmeal and for enhanced oil recovery. In aquaculture, proteases can be found in animal protein processing with an impact on improved digestibility, reduced allergenicity, or improved flavor.

13.3 HISTORICAL USE OF PROTEASES

Food processing through proteases is historically a well-established approach [7].

The dairy food sector is a traditional user of enzymes [8]. The production of cheese predates recorded history. Cheesemaking was already known in Europe at the earliest level of Hellenic myth. It originated through the transportation of milk in bladders made of ruminants' stomachs due to their inherent supply of rennet. There is no conclusive evidence indicating where cheesemaking originated [9]. Coming to modern days, in the late 19th century, a Danish company reported the use of rennet (a mixture of chymosin and pepsin) for cheesemaking [7]. Probably that standardized rennet preparation was the first commercial enzyme preparation of any type. It was defined as an extract of ruminant abomasum, ideally mainly containing chymosin [8].

Proteases have been used from ancient times to prepare soy sauce and other soy products [1]. Papain from the leaves and unripe fruit of the pawpaw (*Carica papaya*) have been used to tenderize meats [10].

In the middle of the 19th century, Northrop and Stanley developed a complex procedure for isolating pepsin. Their precipitation technique has since then been used to crystallize many enzymes. A few years later, for the first time a protease was produced by fermentation of *Bacillus licheniformis*. In this way, large-scale production of enzymes became possible, thus facilitating the industrial application of enzymes [8]. The first commercial enzyme (trypsin) was prepared in Germany in 1914 isolated from animals and used in detergents [11]. Later, proteases were traditionally used in breadmaking to treat bucky dough [12].

13.4 PROTEASES IN THE DAIRY INDUSTRY

The major application of proteases in the dairy industry is in the manufacture of cheese. Selection of the most suitable enzymes is crucial for obtaining high-quality cheese with respect to texture and flavor, storage life, or simply the economic yield.

Milk clotting is the first step in the fabrication of cheese. The milk-coagulating enzymes fall into three main categories, animal rennets, microbial milk coagulants, and genetically engineered chymosin. Both animal and microbial milk-coagulating proteases belong to a class of acid aspartate proteases [1].

Rennet obtained from the fourth stomach (abomasum) of unweaned calves contains acid proteases, mainly chymosin and pepsin. Chymosin clots milk by removing a highly charged peptide fragment from κ-casein on the surface of micellar casein [13]. The destabilized casein micelles aggregate and form the structure of the milk clot that is then acidified by lactic cultures to make cheese curd [14]. Chymosin is preferred for this purpose due to its high specificity for casein, which is responsible for its excellent performance in cheesemaking. A world shortage of calf rennet due to the increased demand for cheese production intensified the search for alternative microbial milk coagulants [1].

Microbial coagulants are produced by the production organisms *Rhizomucor* or *Cryphonectria*. Enzyme production technology with yeasts molds and fungi as the primary source has been successfully established. Over half of the total milk-coagulating enzymes used in regions such as the United Kingdom and United States are microbial in origin, mostly from genetically modified (GM) food yeast and mold containing copies of the calf gene for the production of chymosin. The other main type of microbial milk coagulant is made from non-GM mold, *Rhizomucor miehei* [13].

The microbial enzymes exhibited two major drawbacks, i.e., the presence of high levels of nonspecific and heat-stable proteases, which led to the development of bitterness in cheese after storage, and a poor yield. Extensive research in this area has resulted in the production of enzymes that are completely inactivated at normal pasteurization temperatures and contain very low levels of nonspecific proteases [1].

Whey is a by-product of cheese manufacture. It contains lactose, proteins, minerals, and lactic acid. The insoluble heat-denatured whey protein is solubilized by treatment with immobilized trypsin [1].

Cheese ripening is a slow and partially uncontrolled process [15]. Proteases are used alone or in combination with other enzymes in order to improve the maturation of cheese by several months [13]. However, it is difficult to obtain homogenous distribution of the enzyme products when added to the cheese milk. The ripening proteases continue removing soluble peptides from casein resulting in losses of cheese yield into whey. Chees curds can be too soft. Carry-over of ripening enzymes to whey should be avoided.

The principal plant coagulant in use only for artisanal specialty cheeses is extracted from the cardoon flowers of *Cynara cardunculus* [16]. Recent results suggest that this type of coagulant may be applicable for accelerated cheese ripening [13,17,18].

A protease from *Pseudomonas fluorescens* R098 has been reported to hydrolyze the peptides found in cheese, which are responsible for the bitter taste, and thus finds application as a debittering agent [19,20].

The use of protein hydrolysis to lower the allergenicity of baby food formulations has been common practice for more than 60 years [21]. The technology enables production of mother's milk substitutes that are suitable both for the group of babies suffering from cows' milk allergy and those that are at risk of developing it. β-Lactoglobulin is present in cows' milk at relatively high concentration (9.8% of the milk protein) but is absent in human milk. Of the population allergic to milk proteins, 60%–80% are allergic to β-lactoglobulin, 60% to casein, 50% to α-lactalbumin, and 50% to serum albumin. Special proteases are used to cleave milk proteins into small peptides and amino acids [22].

13.5 PROTEASES IN THE BAKING INDUSTRY

In the baking industry, enzymes naturally occurring in wheat and flours or enzymes supplemented to flours at the mill or added at the bakery to suit the formula of the dough and those present in the yeast for leavening bread play an important role. However, the natural proteases of wheat are inactive on gluten and do not appear to play a role in the preparation of doughs and breads [15].

Wheat flour is a major component of baking processes. It contains the insoluble protein, gluten, which determines the properties of the bakery doughs. Endo- and exoproteinases from *Aspergillus oryzae* have been used to modify wheat gluten by limited proteolysis. The proteases are used during the fermentation stage to permit contact with the flour proteins, which are reduced in chain length. Enzymatic treatment of the dough facilitates its handling and machining, and permits the production of a wider range of products. The addition of proteases reduces the mixing time and lowers energy input. It results in increased loaf volumes. Bacterial proteases are used to improve the extensibility and strength of the dough [1,15].

In addition, proteases have largely replaced bisulfite, which was previously used to control consistency through reduction of gluten protein disulfide bonds, while proteolysis breaks down peptide bonds [23].

The enzyme treatment has an impact on the final bread quality. Proteases have additional functional effects, such as, e.g., improved pan flow in bun and roll production, improvement of grain and crumb texture, improved water absorption, improved color, and improved flavor [24–26].

However, infections of wheat can result in negative impacts on wheat protein quality and therefore on dough and bread quality by fungal protease degradation activities at the infection process on the grains [25,27,28].

The use of microbial proteases in baking requires strict control of the bulk fermentation and conditions of the dough to avoid weakening or increased stickiness of the dough. In particular, bacillus proteases and papain should be dosed very carefully to avoid problems with dough collapse, lower bread volume, and a more open crumb structure. In Europe, application of proteases in bread making is

limited. Particularly because the flour qualities can be weaker than in the United States or Canada, overdosing of protease is a risk. Protease hydrolysis can increase stickiness of the dough [25].

13.6 PROTEASES IN MEAT PROCESSING

Aging of meat permits the natural proteolytic enzymes of meat tissues (the cathepsins) to function autolytically and produce a tendering effect by causing proteolytic changes in the muscle and connective tissue proteins [15].

The use of exogenous proteases to improve meat tenderness has attracted much interest recently, with a view to consistent production of tender meat and added value to lower-grade meat cuts [29].

Various exogenous proteases capable of digesting connective tissue and muscle proteins have been chosen for the purpose of meat tenderization [30]. Papain from papaya latex, bromelain from pineapple stem, and ficin from fig tree latex are used on commercial scales [31]. Furthermore, proteases have been used for bone cleaning and flavor formation in the meat industry [32]. These enzymes have broad substrate specificity and they hydrolyze both myofibrillar proteins and connective tissue [33,34]. Bromealin appears to be more effective in attacking tendons than papain [15]. The common plant proteases do not hydrolyze native collagen, but they act upon the gelatine, the heat-denatured form of collagen [32] that is generated during cooking [30]. Attempts to tenderize collagen-rich connective tissue with tenderizing proteases inevitably led to extensive hydrolysis of noncollagen proteins and resulted in meat that was too soft. Currently, food-grade collagenase preparations are not commercially available although the need is evident [30]. Actinidin from kiwi fruit and zingibain found in ginger rhizomes as well as microbial enzyme preparations have been of recent interest due to controlled meat tenderization and other advantages [29]. Actinidin tenderizes meat and it has been also found to hydrolyze collagen to a certain degree [35,36]. An actinidin protease preparation was found to be effective at hydrolyzing beef myofibril proteins and a zingibain protease preparation to be effective at hydrolyzing connective tissue proteins [37]. A cucumicin-like serine protease extracted from Kachri fruit, *Cucumis trigonus Roxburghii*, a fruit that had been traditionally used for meat tenderization in the Indian subcontinent, could be used for meat tenderization [38,39].

Proteases can be also applied for production of protein hydrolysates from different meat byproducts [30].

13.7 PROTEASES IN FISH PROCESSING

For the fishery industry, proteases are used as processing aids for many products. These include recovery of pigment and flavoring compounds, production of fish protein hydrolysates, viscosity reduction, skin removal, and roe processing. About

one-third of the dry matter in crustacean shell waste is protein. Carotenoids used for pigmentation can be recovered from crustacean wastes for reuse in feed of farmed fish and shellfish. Application of trypsin resulted in higher recovery compared to pepsin and papain [40].

Fish sauce is a traditionally fermented fish product mainly consumed in Southeast Asia. Traditional fermentation takes from 6 months up to 3 years for complete hydrolysis and flavor development [41]. Addition of exogenous proteases (e.g., plant proteases such as bromelain or trypsin/chymotrypsin preparations) to the fermentation can accelerate the process. However, flavor characteristics in the final product are inferior compared to the traditional fermentation [40].

Seafood flavors are in high demand for use in products such as artificial crab and fish sausage. Proteolytic enzymes can aid the extraction of flavor compounds from shells and other materials [40].

Deskinning requires the removal of fish skin without causing damage to the flesh. In order to avoid risk of damaging the flesh and high amounts of waste due to rough mechanical procedures, enzymatic applications have been implemented [42]. Preparations of proteases and carbohydrases are effective in deskinning of fish and peeling of shrim shells [40].

With respect to the production of fish protein hydolysates (FPH), the enzymatic hydrolysis is technically and economically attractive compared to chemical hydrolysis. Processing requires addition of protease preparations from different sources as plant proteases, microbial proteases, crude enzyme preparations and endogenous enzymes from fish [42,43]. FPH products have interesting functional properties for food applications, e.g., emulsification and foaming ability, gelling activity, protein solubility, oil-binding capability, and water holding capacity [42,44–46]. FPH has a bitter taste. Enzymatic debittering with exopeptidases and removal of free amino acids seems to be promising [47].

Proteases are also involved in the ripening of salted fish [42]. The activity of enzymes from the digestive tract (chymotrypsin and trypsin) is most significant in the ripening process, although muscle proteases (cathepsin) play a role [48–50].

Proteases can be employed to process inedible fish and scrap fish to produce oil, meal, and fish solubles. The type of proteases and the action of the enzymes need to be properly controlled to avoid development of bitter off-flavors. Fishmeal treated with Aspergillus proteases eliminates much of the off-odor and fishy taste making the product acceptable for human consumption [15].

13.8 MANUFACTURE OF SOY PRODUCTS

Soybeans serve as a rich source of food, due to their high content of good-quality protein. The alkaline and neutral proteases of fungal origin play an important role in the processing of soy sauce. Proteolytic modification of soy proteins helps to improve their functional properties. Treatment of soy proteins with alcalase at pH

8 results in soluble hydrolysates with high solubility, good protein yield, and low bitterness. The hydrolysate is used in protein-fortified soft drinks and in the formulation of dietetic feeds [1].

13.9 PROTEASES IN PROCESSING OF PROTEIN HYDROLYSATES

Protein hydrolysates have several applications, e.g., as constituents of dietetic and health products, in infant formulae and clinical nutrition supplements, and as flavoring agents [1].

The market for hydrolyzed proteins ranges from relatively low-value products used for instance in pet food to high-value nutritional products, such as special peptides in enteral nutrition, baby food formulas or bioactive peptides. Proteases have been used for a long time for the production of low allergenic mother's milk substitutes for reduction of the allergenicity of the proteins. Production of highly soluble proteins/peptides for protein fortification plays an important role. The properties of the hydrolyzed proteins are adjusted by the selection of the protease, pretreatment of raw material, the parameters of hydrolysis, as well as the downstream processing of the hydrolysate. Hydrolysis of proteins to smaller peptides enables modifications of the protein properties. Due to differences in specificity of the enzymes the resulting peptides also widely differ [22].

Intact proteins are tasteless in purified form [51]. The bitter taste of protein hydrolysates is a major barrier to their use in food and healthcare products. When proteins are hydrolyzed, there are two main contributions to the taste of the peptides. One is the release of flavor components "hidden" in the protein structure, which are released when the protein is hydrolyzed. More important is the formation of small peptides with a relative high content of hydrophobic amino acids which tend to create bitterness [51].

The intensity of the bitterness is proportional to the number of hydrophobic amino acids in the hydrolysate. The presence of a proline residue in the center of the peptide also contributes to the bitterness. The peptidases that can cleave hydrophobic amino acids and proline are valuable in debittering protein hydrolysates. A careful combination of an endo-protease for the primary hydrolysis and an aminopeptidase for the secondary hydrolysis is required for the production of a functional hydrolysate with reduced bitterness [1].

13.10 USE OF PROTEASES IN BEER BREWING AND BEER STABILIZATION

The two most important steps in brewing are mashing (production of fermentable sugars) and fermentation (alcohol production by yeasts). Various enzymes, among them proteases, arising from the malt (barley) are active during

the mashing process [15]. While about 95% of starch from malt is solubilized by the end of mashing, only about 30%–40% of the malt protein is solubilized. The principal groups of enzymes involved in the breakdown of malt proteins are endoproteases and exopeptidases [52].

Protein and decomposition products from proteolysis (free amino acids, peptides, and degraded proteins) are major components of all beers. Protein plays a key role in foam formation and foam stabilization of a beer. It has a positive influence on the flavor of a beer and it is necessary for adequate yeast nutrition. Amino acids and di–tri peptides play a key role in the metabolism of yeast, thus having an indirect influence on the flavors developed during fermentation. Protein also plays a role in both the colloidal stabilization and destabilization of beer. It will interact with grain-derived polyphenols, which leads to haze formation in the final beer. Proteolysis will reduce haze problems that could be caused by proteins and can also influence the foam stability of beer. The function of proteolytic enzymes during mashing is to hydrolyze the large-chain protein molecules, to make starch molecules more accessible to enzymatic attack, and to produce sufficient levels of amino acids and di–tri peptides for an optimal fermentation. Most of the proteolysis will already have occurred during malting [52].

Storing of beer for prolonged periods at preferably $<0°C$ to prevent or slow down beer haze development is a costly and time-consuming solution. The current trend is to remove the haze-forming components (proteins and polyphenols). Papain is a proteolytic enzyme extracted from pawpaws, the fruit of the papaya tree. It is applied to prevent colloidal instability problems imparted by protein and therefore extend the shelf-life of beer. A mixture of cysteine proteinases with broad specificity can be used for that purpose too. It can be added during transfer between fermentation and maturation or directly into the maturation tank itself. If the beer is pasteurized at temperatures $>70°C$, the enzyme will be inactivated [52].

13.11 SYNTHESIS OF ASPARTAME

Although proteases generally act as hydrolases, they can catalyze the reverse reaction under certain kinetically controlled conditions. Aspartame is a noncaloric artificial sweetener, which is 200 times sweeter than sucrose, approved for use in the food industry. The dipeptide is composed of L-aspartic acid and the methylester of L-phenylalanine. An immobilized preparation of thermolysine from *Bacillus thermoproteolyticus* is used for the enzymatic synthesis of aspartame by enzyme-catalyzed condensation (reverse synthesis) in organic solvent [1,15,53].

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CHAPTER

Proteases—animal feed

14

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14.1 PROTEASES IN ANIMAL FEED

Digestion of dietary protein is an important factor in animal nutrition, as protein and amino acids are among the most expensive nutrients. Besides the important role of endogenous proteases in the whole metabolism of animals, there are two main applications for exogenous proteases in animal nutrition: (1) during processing of food or feed ingredients, e.g., protein hydrolysates, or (2) direct application of exogenous proteases as supplements to feed or during processing of final feed formulations.

Precise knowledge on the digestibility of amino acids in feed ingredients and the requirement of the animal is important for formulation of diets containing protease. Feeding dietary protein and amino acids at a level that fulfills nutritional requirements is essential for optimal performance of the animal. However, if the dietary protein level is too high, there is a risk for problems with gut health and in poultry for wet litter. Another important aspect is the environmental impact of high-nitrogen excretions through animal manure. The use of exogenous proteases in feed can be an option to reduce dietary protein levels maintaining high performance levels.

14.2 USE OF PROTEASES IN PROCESSING OF PROTEIN HYDROLYSATES FOR USE IN ANIMAL FEED

The use of protein hydrolysates has a long tradition in animal nutrition. They can be of animal or plant origin. Different feed applications of hydrolysates from fish protein, animal co- or by-products such as tissues or blood, whey from milk, and vegetable products from soybean, wheat, etc., exist, especially in diets for young and rapidly growing animals.

A selection of protease enzymes that are used for the production of protein hydrolysates is listed in Table 14.1. The enzyme products can be from animal, plant, or microbial origin. They can be applied as single enzymes or enzyme mixtures.

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Table 14.1 Examples for Proteases Used for Enzymatic Hydrolysis of Protein

Catalytic Type of Enzyme	Name of Enzyme
Aspartatic (A)	Chymosin, pepsin A
Cysteine (C)	Bromelain, cathepsin B, ficin, papain
Metallo (M)	Alanine carboxypeptidase, aminopeptidase, aminopeptidase Y, bacillolysin, carboxypeptidases A and B, carboxypeptidase S, dipeptidase 1, dipeptidyl peptidase III, glycine carboxypeptidase, membrane dipeptidase, prolyl dipeptidase, thermolysin
Serine (S)	Chymotrypsin, dipeptidyl peptidase IV, subtilisin, trypsin

Source: *Adapted from Hou Y, Wu Z, Dai Z, Wang G, Wu G. Protein hydrolysates in animal nutrition: industrial production, bioactive peptides, and functional significance. J Anim Sci Biotechnol 2017;8 (24):13. doi: 10.1186/s40104-017-0153-9.*

Protein hydrolysates can be obtained by chemical, microbial, or enzymatic hydrolysis. Enzymatic hydrolysis of proteins is often the method of choice in processing animal by-products or plant-source feedstuffs. Enzymatic hydrolysis is mild, specific, and the enzymes used during the process can be easily deactivated by heat treatment after the hydrolysis process. However, costs and enzyme inhibitors in the protein substrate can be disadvantageous in certain processes [1] compared to especially chemical hydrolysis. For example, considerable amounts of fish-processing coproducts are generated every year with high costs for waste disposal. Enzymatic hydrolysis processes have been demonstrated advantages on a lab scale due to mild reaction conditions, superior product quality and functionality, but are still not in use on an industrial scale at the moment due to high costs compared to chemical hydrolysis [2].

Enzymatic hydrolysis takes place under mild conditions (e.g., pH 6−8 and 30−60°C) and minimizes side reactions. The peptide bonds of proteins can be broken down by many different kinds of proteases (exo- and endopeptidases alone or in combination, respectively). The choice of enzymes depends on the protein source and the degree of hydrolysis. The resulting smaller or larger peptides from that process can be used, e.g., for feeding purposes. However, the peptides can have both nutritional and physiological or regulatory functions in the animals. Some peptides of plant or animal sources also have antimicrobial, antioxidant, antihypertensive, and immunomodulatory activities. Peptides that can serve for biological functions beyond their nutritional value are defined as bioactive peptides. Fractionation of protein hydrolysates is optionally performed to isolate specific peptides or remove undesired peptides [1].

Peptides generated from the hydrolysis of plant and animal proteins are included in the diets for feeding pigs, poultry, fish, and companion animals. The outcomes are positive and cost-effective for the improvement of intestinal health, growth, and production performance. Vegetable protein hydrolysates are of growing importance [1,3].

Protein, in particular, plays a critical role in the growth and development of young animals [3]. In piglets, the use of enzymatic digestion techniques with blended abattoir by-products such as poultry tissues, swine blood, and enzymatically hydrolyzed feathers, improved the feeding value to make them comparable to high-quality ingredients such as fishmeal, spray-dried animal blood cells, and spray-dried plasma protein, providing an additional formulation alternative [4].

Plant protein hydrolysates can partly or even to a large extent replace fishmeal in aquaculture. That point is of extreme importance due to the worldwide shortage of fishmeal. Hydrolyzed soy protein products can replace skim milk powder for rearing veal calves [1,5].

Hydrolysis of some proteins (e.g., tryptic hydrolysates from soybean) can generate bitterness [6] and can require additional treatments for reduction of bitter taste [1].

Techniques to improve savory characteristics through application of the Maillard reaction [7] and by generating specific flavor enhancers with the use of enzymatic processed protein hydrolysates can provide cost-effective solutions for the pet-food industry. In addition, protein hydrolysates deliver peptides in the range of 800−1500 Da, small enough to avoid allergenic reactions of companion animals [8].

Use of protein hydrolysates from by-products at the slaughterhouse or at fish-processing facilities and from plant ingredients that would otherwise not be used in animal feed, are important under the environmental aspects of effective recycling of nutrients and sustainable use of resources. However, local regulations sometimes exclude the use of certain animal by-products for different reasons.

Substrates can differ widely and for, e.g., different fish-processing substrates it has been demonstrated that chemical composition and palatability (here in dogs) are affected the substrate composition, respectively [9].

Pedersen et al. [10] demonstrated efficacy of four different keratinases in hydrolyzing chicken feathers.

Nchienzia et al. [11] showed the feasibility of hydrolyzing poultry by-products that went through a rendering process using different brands of commercial proteases. The authors concluded that findings from this research are important in the preparation of palatants, in which relatively inexpensive hydrolyzed poultry meal could be used to improve the flavor of companion animal food products.

Coll et al. [12] could show with fluorescence-labeled protease from *Bacillus licheniformis*, that treatment with meat and bonemeal to increase solubility and inactivate prions is technically possible. However, soft tissue particles showed greater resistance to enzyme infusion compared to bones.

14.3 APPLICATION OF PROTEASES IN ANIMAL FEED

Feed protein digestion is accomplished by an endogenous proteolytic enzyme system in the digestive tract, involving pepsin, trypsin, chymotrypsin, elastase, as well as aminopeptidases and carboxypeptidases [13].

The animals do not digest and absorb all of the proteins and amino acids present in the diet. The undigested component is a substrate for increased microbial fermentation mainly in the hindgut in monogastric animals [14] which can lead to digestive disorders.

Differences determined between ileal and excreta digestibilities in broiler chickens demonstrated that amino acid metabolism by hindgut flora may be substantial [13,15].

Additional supplementation of feed with exogenous proteases can further improve protein digestibility of ingredients through enhanced protein solubilization and hydrolysis of dietary proteins and reduce substrate for undesirable hindgut fermentations.

Caine et al. [16] demonstrated in vitro the effects of a pretreatment of soybean meal (SBM) with a *Bacillus subtilis* subtilisin-protease on soluble crude protein (it was significantly increased) and on soybean trypsin inhibitors (that were decreased in some cases).

Exogenous feed protease visibly degraded protein storage vacuoles from whole soybeans in vitro. Protein degradation could be visualized as holes in the protein storage vacuoles [17].

Whether the methods of determining protein and amino acid digestibility are also the best methods to determine the effectiveness of exogenous proteases is still not clear [18]. The findings on protease application in feed still remain, to a certain extent, inconclusive. Positive effects of proteases on protein and amino acid digestibility could be demonstrated in a large number of experiments in various animal species, whereas a certain number of trials did not show any beneficial effects on these parameters. These results may depend on specific trial conditions such as the test protease, trial duration, level of protease supplementation, age of animals, composition of basal diets, or test parameters and methods. The interaction of endogenous protease with exogenous protease supplemented to the feed and its regulation in the metabolism could also be a factor.

In general, activities from endogenous proteases are carefully regulated because their activity in the wrong location can lead to digestion of the animal's own tissues and/or may activate inflammatory pathways [19]. The pancreas is able to adapt its secretion of enzymes in response to the composition of diet. This was observed for the first time by Pavlov [20] and was later widely confirmed in several studies using more sophisticated techniques [21–24]. In an animal adapted to a specific nutrient-enriched diet, the high level of this nutrient in the digestive tract is very rapidly followed by increased corresponding enzyme levels in the pancreas [23,25]. These adaptive measures are entirely intuitive and suggest that the process of digestion is rather carefully regulated to ensure that overproduction of digestive juices is avoided. Exogenous proteases could bridge a gap between the composition of the feed and the digestive enzyme complement of the animal. The supplementation of feed with exogenous proteases could additionally spare maintenance energy through reduced secretion of endogenous enzymes, improving performance not necessarily by increasing digestibility [19].

Further research activities on optimal conditions for evaluation of proteases in animal nutrition could help to establish a basal set-up for experiments in various animal species for efficacy studies, comparisons among proteases, and transfer of results into practical feed-formulation advice. Recent publications on exogenous proteases with focus on gastrointestinal health, intestinal morphology or under challenge conditions showed interesting effects of supplementation with the enzyme.

14.3.1 POULTRY

Protein and amino acid nutrition in poultry has been one of the most prominent areas in poultry research in recent decades [18].

In the literature, until recent years, many studies been reported evaluating combinations of proteases with carbohydrases and other enzymes, demonstrating positive effects on growth performance, energy- or protein- utilization mainly in broiler chickens and turkeys, and in some other poultry species. Under practical conditions, enzyme admixtures or supplementation of enzymes in various combinations, e.g., a phytase, a xylanase, and a protease, are justified, depending on the composition of the basal diets and the formulation targets. It has to be mentioned that summarizing effects of single enzymes should follow an asymptotic curve and therefore cannot be 100% additive. However, as it is not possible to distinguish between the effects of single enzymes, these publications on enzyme mixtures are not further presented or discussed in this chapter.

First experimental results showing beneficial effects of two fungal proteases alone or in combination on weight gain and feed conversion ratio (FCR) in broilers fed protein-restricted diets were published a long time ago by Kasbaoui and Guillaume [26], Vogt and Harnisch [27], Lewis and Hiller [28]. Supplementation with protease resulted in almost the same growth performance as that obtained with standard diets. Direct effects on *N*-balance were not observed in the studies performed by Vogt and Harnisch [27] and Lewis and Hiller [28]. These early enzyme products were never launched for practical use for various reasons [13].

The effects of proteases on the nutritive value of SBM were evaluated in different studies. Marsman [29] evaluated the effects of treatment of SBM with protease and carbohydrase, respectively, and in combination. Enzyme treatment improved apparent ileal digestibility (AID) of crude protein (CP) and nonstarch-polysaccharides compared with no enzyme treatment (85.2% vs 83.7% and 20.6% vs 14.5%, respectively); however, enzyme treatments did not result in better growth performance of the chicks.

Ghazi et al. [30] evaluated the effects of pretreatment of SBM with a *Bacillus* protease (P1) and an *Aspergillus* protease (P2). Treatment with *Aspergillus* proteases improved chick performance; from 7 to 28 days of age, chicks fed on treated SBM had greater feed intakes and gained more weight than chicks fed on untreated SBM. Both proteases P1 and P2 significantly reduced chick serum antisoya antibodies, while protease P2 treatment increased apparent ileal nitrogen (N) digestibility and apparent N retention across the whole digestive tract.

Two tube-feeding experiments confirmed that, of the treated SBMs used in experiment 1, only protease P2 treatment improved apparent N digestibility and true metabolizable energy (TME). Ghazi et al. [31] observed in further experiments with tube-fed cockerels or in broiler chickens improvements of TME and true nitrogen digestibility for two *Aspergillus niger* proteases (P2 and P3) and only little effect for the *Bacillus* protease (P1). It was possible to demonstrate similar effects of protease supplementation by simple addition of the enzyme to the diets compared to pretreatment of SBM.

Thacker [32] compared wheat and corn as energy sources and effects of supplementation with protease in broiler chickens. Enzyme treatment had no significant effects on body weight (BW) gain. Feed conversion was improved for birds fed protease. Birds fed wheat-based diets had significantly higher digestibility coefficients for dry matter (DM) and energy than birds fed corn-based diets. Nitrogen retention was unaffected by choice of cereal grain. There were no significant effects on DM digestibility, energy digestibility, or nitrogen retention due to enzyme supplementation.

A keratinase from *B. licheniformis* improved BW and FCR at 21 days of age and BW (in experiment 1) or overall FCR (in experiment 2) in broilers fed either protein reduced or high protein basal diets [33].

Wang et al. [34] performed a broiler trial, where diets were formulated at low (95%), medium (100%) or high (105%) crude protein and amino acids level relative to industry standards. Supplementation with the keratinase from *B. licheniformis* resulted in improvements in BW and FCR. The improvements were greatest at low protein and amino acid levels. Breast-meat yield improvements were greatest at medium and high dietary protein levels.

In another experiment, the effects of keratinase were evaluated in broilers fed diets with inclusion of the enzyme subsequently pelleted at 85°C. The authors observed a 55-g increase in BW gain and 0.17 improvement in FCR per bird after supplementation with the enzyme [35].

Yu et al. [36] tested a protease derived from a *Bacillus* strain in two experiments that were repeated in two different seasons in broiler chickens and in an in vitro experiment. Protease supplementation increased feed intake for all diets in the cool season, but significantly decreased feed intake in the low-protein diet and increased in basal diet during the hot season. BW gain and feed conversion were improved in the treatment supplemented with protease. The enzyme supplementation did not improve in vivo digestibility of the DM and protein. SBM hydrolysis rate and protein digestibility were increased in the in vitro assay as compared to the respective ingredient without supplementation.

No effect of a protease inclusion was observed on growth performance of turkey hens fed diets from day 0 to day 72 either with no inclusion or with 30% DDGS (wheat distillers dried grains with solubles) [37].

Protease improved the ileal digestibility of a large number of amino acids of wheat DDGS for broilers and turkeys [38]. The experiments were carried out with wheat DDGS as the only source of amino acids.

A serine protease expressed in a genetically modified strain of *B. licheniformis* was tested in several experiments.

Supplementation of a serine protease in broilers fed diets at high or moderate amino acid density over 42 days resulted in improved feed conversion and breast-meat yield with results dependent on dietary amino acid density [39].

Fru Nji et al. [40] observed significant effects of serine protease in vitro on protein solubilization, hydrolysis and digestibility in a gut simulation model. Furthermore, the effects of supplementation with protease on growth performance and nutrient digestibility were tested in two broiler experiments. In both experiments, the addition of the enzyme had significant benefits on the performance of the animals, mainly by increasing the digestibility of crude protein and consequently digestible energy. In feeds with lower nutrient digestibility, the effect of the exogenous enzyme seemed to be more pronounced.

Freitas et al. [41] evaluated the effects of adding the serine protease to corn-, SBM-, and meat and bonemeal-based broiler diets. In the first experiment, protease supplementation had no effect on the BW of broiler chickens. Feed efficiency (FE) was improved in a quadratic manner as protease level was increased. In the second experiment, protease supplementation improved FE as well as digestibilities of fat and crude protein ($P \leq .01$) in broilers, regardless of dietary protein or energy concentration.

Angel et al. [42] evaluated the effects of increasing dosages of serine protease on performance and protein utilization in broiler chickens fed low-protein corn-SBM diets (20.5% CP) compared to a positive control (22.5 % CP). Birds fed low-protein diets supplemented with the protease grew as well as birds fed the positive control diet. Supplementation with protease restored digestibility of crude protein. When benefits in digestibility of amino acids occurred, they were similar at all protease inclusion levels.

Effects of a serine protease on apparent ileal amino acid digestibility and ileal protease activity were evaluated in broilers [43]. The addition of the enzyme showed no significant effects.

Aureli et al. [44] recorded improvements of standardized ileal amino acid digestibility of valine and threonine of wheat DDGS and of glycine and histidine in wheat middlings by supplementation with a serine protease in broiler chickens.

In another experiment, Aureli et al. [45] studied the effects of a serine protease in broilers fed on a diet based on 40% triticale on AID of nitrogen and amino acids, which were improved by inclusion of the enzyme.

Camara et al. [46] studied the effects of inclusion of a serine protease on AID of crude protein and amino acids of SBMs of different origins (USA, Brazil, Argentina) in broilers. Supplementation with the protease increased numerically the AID of amino acids and DM, but the differences were not significant.

Erdaw et al. [47] concluded from their experiment that supplementation with a serine protease may enable the utilization of low levels of raw full-fat SBM in diets for broiler chickens partly replacing commercial (heat treated) SBM.

Araujo et al. [48] studied the effects of exogenous protease in mashed and pelleted diets for broilers. Low-protein basal diets were prepared with reduction of crude protein and amino acids by 4% compared to a positive control. The addition of protease to the low basal diets did not have main effect on performance, carcass yields, and cuts of broilers. Interactions among supplementation with protease and nutritional levels or breast yield were observed.

Viera et al. [49] conducted a study in turkeys from day 1 to 26 days of age fed corn-soy diets with reduced levels of crude protein and amino acids (negative control) and with or without inclusion of a serine protease, compared to a positive control. The authors observed a main effect of protease on FCR. Protein digestibility was improved with supplementation of protease compared to the negative control. Digestibilities of specific amino acids were better for turkeys fed diets supplemented with protease.

The serine protease preparation improved weight gain and feed efficiency in female turkeys during the first 4 weeks of life [50].

Boghun et al. [51] performed an experiment to evaluate amino acid digestibility of SBM and DDGS without and with supplementation of a serine protease. The level of digestibility was much lower in DDGS than in SBM, which confirmed findings from studies in broilers. The results of that study indicated that protease in the young turkey's intestine is not the factor limiting protein digestion as differences between supplemented and nonsupplemented DDGS were not significant.

Barrekataina et al. [52] evaluated effectiveness of a protease on broiler chickens fed sorghum distillers dried grains with solubles (sDDGS). Supplementation with the protease could improve the digestibility of DM and crude protein for the diet containing the highest levels of sDDGS. The authors concluded that supplementation with protease could alleviate the adverse effects of sDDGS on performance, in particular FCR, in broilers.

Xu et al. [53] performed a study to evaluate the effects of coated compound proteases (CC proteases) from *A. niger, B. subtilis*, and *B. licheniformis* on performance, nutrient utilization, gut morphology and carcass traits of broilers fed corn-soya- or sorghum-soya-based diets. Average daily gain, apparent metabolizable energy and DM, gross energy and nitrogen retention were increased by supplementation with enzymes. Gut morphology parameters were improved in the starter phase. Breast muscle weight and pH24 value were improved and breast muscle drip lost was reduced in treatments supplemented with enzyme.

Carvalho et al. [54] did not observe effects for protease supplementation comparing vegetable diets based on corn-SBM with diets based on meat and bonemeal, feathers and bloodmeal and viscera and bonemeal in broilers.

Law et al. [55] concluded that beneficial effects of exogenous protease supplementation appeared to be dependent on the enzyme source and not dependent on the dietary level of protein. They evaluated two different serine protease products

at two dietary protein levels (16% and 19% crude protein, respectively) observing improvements in digestibility coefficients of energy, crude protein, and the majority of amino acids for one of the enzyme products.

The incidence of food pad lesions is one of the main concerns in the broiler industry with wet litter as one of the main risk factors. Supplementation with a serine protease improved protein digestibility in broiler chickens [56]. The authors concluded that supplementation with protease could contribute to improve intestinal health, resulting in better broiler performance and litter quality.

Olukosi et al. [57] reported another experiment where a serine protease improved growth performance of broiler chickens reared on used litter. It was concluded that the enzyme might have enhanced nutrient utilization to a greater extent in these birds, partially compensating for the depressed feed intake observed in birds fed diets without supplementation with the enzyme.

The impact of the serine protease with or without its matrix values on growth performance traits, feed digestibility, serum parameters, selected immune parameters and bacterial counts, carcass traits, as well as duodenal morphology was studied in broiler chickens [58]. Supplementation of protease on top and to basal diets low in metabolizable energy (ME) surpassed all groups in weight gain, feed consumption, FCR, and in dressing percentage. Significant improvement of crude protein digestibility was noted, respectively. Both treatments resulted in significant reduction of total ileal clostridia species counts. In addition, supplementation of protease on top showed an increase in villus height and villus to crypt ratios compared to other experimental groups.

Dietary supplementation with a protease reduced the negative impact of a coccidiosis infection on BW gain of broilers, although the coccidial lesions and oocyst excretion remained unaffected [59].

14.3.2 SWINE

The effects of supplementation with exogenous proteases have been studied in piglets and growing fattening pigs in order to increase digestibilities of dietary protein/amino acids or to use alternative feed ingredients. However, the number of publications is still lower, compared to publications in poultry. The findings are inconclusive and the reasons for this are still unclear. There are experiments showing beneficial effects of protease inclusion, but several publications showed no effects. Secretion of sufficient amounts of endogenous proteases, especially in the growing/finishing pigs could be partly a possible explanation. However, supplementation with exogenous proteases could complement the digestion of protein and help to lower energy requirements for maintenance. Further research activities to determine optimal conditions for evaluation of proteases would be needed to draw conclusions.

The effect of protease supplementation on the nutritive value of peas for growing and finishing pigs were evaluated in two experiments [60]. In experiment 1,

the addition of protease had no effect on nutrient digestibility or digestible energy content of the diets. In experiment 2, the addition of protease improved FCR in the growing pig.

The effects of serine protease supplemented to a corn-SBM-based diet on AID of nutrients were evaluated in weaned piglets [61]. No effects of protease on AID of nutrients after 14 days were recorded. After 29 days of feeding, AID of crude protein was increased for the treatment supplemented with the enzyme compared to the control. The AID of the indispensable amino acids, Met + Cys, and branched-chain amino acids was increased. In the protease supplemented pigs, the AID of the individual amino acids was increased for Arg, Asp + Asn, Glu + Gln, His, Ile, Lys, Phe, Thr, Tyr, and Val.

Alpine et al. [62] studied effects of protease on growth performance and nutrient digestibility in finisher pigs. The pigs fed diets containing protease enzymes had reduced daily gain and final BW compared to pigs offered diets without protease enzymes (experiment 1). Pigs offered diets supplemented with protease had increased coefficients of AID of N and gross energy (GE) compared to pigs offered diets without protease supplementation. Protease had any effect on total tract digestibilities of GE or N. In conclusion, protease supplementation showed no positive effects on grower−finisher pig performance.

De Barros et al. [63] evaluated the effects of protease addition on the total tract apparent digestibility of piglet diets containing hard endosperm corn. No effects of protease supplementation on digestibility parameters were observed in that study.

Zuo et al. [64] conducted a study to investigate the effect of dietary protease supplementation at three inclusion levels compared to a positive and to a negative control on the growth performance, nutrient digestibility, intestinal morphology, digestive enzymes, and gene expression in weaned piglets. Results indicated that final BW, average daily gain, average daily feed intake, crude protein digestibility, enzyme activities of stomach pepsin, pancreatic amylase and trypsin, plasma total protein, and intestinal villus height were higher for the positive control diet and for supplementations of 200 and 300 mg protease per kg NC diet than for the NC diet.

Two experiments were conducted to evaluate effects of CC protease on apparent total tract digestibility (ATTD) of nitrogen (N) and energy, and AID of amino acids and nutrients in diets for pigs [65]. CC protease supplementation increased digestible and metabolizable N and energy values and the digestibility and retention rate of N in the diet. The ATTD of energy and nutrients were improved in the diet supplemented with CC protease. The CC protease increased the AID of crude protein and some essential AA including arginine, isoleucine and leucine. CC protease improved AID and ATTD of energy and nutrients, but the hindgut digestibility of nutrients was unaffected.

14.3.3 RUMINANTS

Supplementation of ruminant diets with exogenous enzymes could complement the endogenous enzyme system and the enzymes occurring in the rumen. To date,

numerous experiments have been performed with ruminants such as cattle, both dairy and beef, goats, sheep, and less with buffalo. The results seem to be inconsistent. Positive results, e.g., on feed intake, nutrient digestibility, growth performance, other production parameters, manure nutrient excretion, etc., have been obtained. There is evidence that exogenous proteolytic enzyme (EPE) could increase the total tract digestibilities of DM, organic matter (OM), acid detergent fiber (ADF), and neutral detergent fiber (NDF) with larger increases in digestibility of cows though the feeding of proteolytic enzyme [66].

In ruminants, supplementations of EPEs are not as common as in nonruminants because of an abundance of endogenous proteolytic enzymes produced by the microorganisms in the rumen [67].

Recent results on effects of proteolytic enzymes are hereafter mentioned. The effects of EPEs in dairy cows fed low and high forage diets on intake, digestibility, ruminal fermentation, and milk production were determined in a double 4×4 Latin square experiment [68]. Supplementation with proteolytic enzyme (EPE) increased total tract digestibilities of DM, OM, N, ADF, and NDF, with larger increases in digestibility observed for cows fed low forage diets. Ruminal xylanase and endoglucanase activities increased with addition of EPE to the diet, which may have accounted for improvements in fiber digestion. However, feeding EPE unexpectedly decreased feed intake of cows, which offset the benefits of improved feed digestibility. Consequently, milk yield of cows fed high or low forage diets decreased with adding EPE. Nevertheless, dairy efficiency, expressed as milk/DM intake, was highest for the LF + EPE diet. Addition of EPE to the diet increased milk fat and milk lactose percentages, but decreased milk protein percentage of cows fed a low forage diet. For cows fed high forage diets, EPE only increased milk lactose percentage. Efficiency of N use for milk production was decreased for both the high and low forage diets when EPE was added to the diet. Mean ruminal pH was lowered when EPE was added to a low forage diet, likely due to the increased degradation of forage and concentrate, but there was no effect of EPE on rumen pH when cows were fed high forage diets. Profiles of volatile fatty acids (VFA) and microbial yield were not affected by adding EPE to the diets. Adding EPE to a total mixed ration containing alfalfa hay, barley silage, and concentrate improved nutrient digestibility in the total tract, and the response was maximized with a high-concentrate diet. However, improvements in digestibility were offset by decreased feed intake, likely due to increased ruminal acidosis. Lazzarini et al. [69] demonstrated that DM intake decreased as rumen ammonia nitrogen concentration exceeds 15.3 mg/dL in cattle; therefore, the increased rumen ammonia concentration may have negatively affected feed intake [67].

Vera et al. [70] investigated the effects of adding an EPE on the growth performance of beef steers fed growing and finishing diets containing 30% DDGS. The addition of EPE during the growing phase increased DMI but had no effects growth performance Adding EPE during the growing phase decreased NDF digestibility, whereas the digestibility of DM, CP, and ADF were not affected.

There was a tendency for both average daily gain (ADG) ($P = .09$) and final BW ($P = .11$) to increase during the finishing phase without affecting BW change and G:F. As opposed to the growing phase, EPE increased digestibility ($P < .04$) of DM, CP, NDF, and ADF. The authors concluded that the use of protease enzyme products might be more effective in high-concentrate diets such as finishing beef steer diets containing DDGS.

Sucu et al. [72] evaluated the effects of supplementation with alkaline protease on production parameters in dairy cows. There was no treatment effect on milk yield (37.6 kg/day), but cows fed diets supplemented with protease consumed less DM compared to controls, as observed by Eun and Beauchemin [68] and therefore tended to have improved feed efficiency ($P = .06$). Protease decreased blood urea nitrogen compared to the control. However, feeding enzyme-supplemented diets had no effect on milk fat and protein content but tended ($P = .08$) to increase milk lactose concentration and tended ($P = .10$) to decrease milk urea nitrogen levels and somatic cell score. Results indicated that supplementation of diets with protease may enhance production efficiency and improve parameters of nitrogen status.

A number of studies have shown exogenous alkaline and serine-type proteases to have a potential to increase feed digestibility in ruminants [72–74]. This may be due to their abilities to bypass rumen degradation and thus contribute to protein digestion in the small intestine. It has also been suggested that exogenous proteases may work closely with endogenous enzymes by potentially providing endogenous enzymes with greater access to nutrients, particularly in cell wall matrices [75], thus creating a symbiotic relationship between endogenous and exogenous enzymes and enhancing overall feed digestibility [67].

14.3.4 AQUACULTURE

One of the major factors limiting the expansion of aquaculture is the development of nutritionally adequate, cost-effective diets [76]. Dietary proteolytic enzymes in aquaculture have gained attention in recent years, mainly because of the need for better utilization of proteins from existing sources [77].

In particular, alternative protein sources for replacement of fishmeal in feed for carnivorous fish are of growing interest due to major concerns about the limited world fish supply.

Drew et al. [78] studied the effect of adding an alkaline serine endopeptidase to coextruded flax:pea or canola:pea products on nutrient digestibility and growth performance of rainbow trout. The addition of protease to the canola:pea diet resulted in a significant improvement in feed efficiency. However, the addition of protease to the flax:pea diet had no effect on performance. The authors suggested that proteases should be assessed for individual ingredients.

Dalsgaard et al. [79] investigated whether supplementing diets with high inclusion levels of plant-based protein with serine protease could improve the apparent nutrient digestibility in juvenile rainbow trout. Digestibilities of three diets with

high inclusion levels of either dehulled, solvent extracted SBM, sunflower meal, or rapeseed meal were studied in subsequent trials, with and without supplementation of enzyme, respectively. Enzyme supplementation had only moderate effect on apparent nutrient digestibility in the sunflower and rapeseed experiments, while supplementation with protease improved the apparent digestibility of all dietary nutrients in the soybean experiment. The effect was more pronounced for lipids than for other nutrients.

Yigit et al. [80] evaluated effects of the serine protease on growth performance and nutrient digestibility of rainbow trout fed diets based on fishmeal and dehulled SBM. Addition of protease could not increase growth and nutrient digestibility in trout in the present study.

14.3.5 COMPANION ANIMALS

The most common use for proteases in the pet-food industry is in the production process of digest, a hydrolyzed concentrate that is used as a flavor in dry pet food to improve its palatability. Digest is produced using proteases for hydrolysis of clean animal tissues (as, e.g., meat or meat by-products), thus liquefying the raw material and creating a good flavor for the pets.

14.4 ENVIRONMENTAL ASPECTS

Nitrogen is considered as a major environmental pollutant. Animal manure is a source of gaseous emissions of NH_3 and N_2O. N_2O emissions contribute to the emission of greenhouse gases. Depending on the livestock density in the region, both N_2O and ammonia emissions lead to increased nutrient enrichment and eutrophication and acidification potential, respectively. More than 90% of the NH_3 emissions in Europe come from livestock production. Adaptation of feed formulation by reducing dietary protein has a direct impact on the reduction of N excretion.

Oxenboll et al. [81] evaluated the environmental consequences of decreased dietary protein content and decreased nitrogen excretion in a life cycle assessment (LCA), which considered all steps in broiler production from production of feed ingredients to the broilers leaving the chicken house, including the use of manure. Significant benefits were obtained for all impacts considered. Most important were the benefits related to reduced emissions of ammonia, which help reduce both health risks and environmental impacts, such as acidification and eutrophication. The largest effects were obtained when protease was used as a tool to allow for lower diet protein content. However, even when used in a diet with normal protein levels, significant benefits were observed.

Leinonen and Williams [82] performed a calculation to quantify the effects of the use of a commercial serine protease in broiler feed on the environmental

impacts of (1) broiler production chains (at the farm gate) and (2) broiler feed production chains (at the feed mill gate). This was done by using an LCA modeling approach with data from trials using both standard soya-based broiler diets and reduced-protein diets with added protease. The use of protease in the broiler diets reduced the environmental impacts of both feed production and broiler production. The results for the feed production chain showed that there was a reduction in all environmental impact categories when protease was used in the diets. The biggest reduction occurred in the category of global warming potential, mainly as a result of decreased carbon dioxide emissions from land use changes related to soya production. In the results for the broiler production chain, there were relatively bigger reductions in eutrophication potential and especially in acidification potential, mainly as a result of reduced feed protein content and subsequent nitrogen emissions from housing and manure management. The reduction was mainly through reduced NH_3 emissions that will be beneficial with respect to animal health aspects.

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PART

Other Enzymes

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CHAPTER

Enzymes as therapeutic agents

15

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15.1 INTRODUCTION

Enzymes have found a considerable role in clinical laboratories in the diagnosis as well as in the treatment of a number of diseases. The assay of enzyme level in the extracellular body fluids is an important aid to clinical diagnostics and management of diseases. Enzyme assay can make significant contributions to the diagnosis of diseases because a minute change in enzyme concentration can be easily measured. Measurement of the changes in enzyme levels, therefore, offer a greater degree of organ and disease differentiation as compared to other possible clinicochemical parameters such as albumin or gamma globulin.

The best known uses of enzymes in analysis are as soluble enzyme "kits," especially useful in clinical biochemistry for measuring blood glucose using glucose oxidase and catalase, and serum cholesterol levels using cholesterol oxidase, which are derived from a number of organisms such as *Mycobacteria, Nocardia, Streptomyces*, etc. The other enzyme used in serum cholesterol assay, cholesterol esterase, is produced by *Pseudomonas fluorescens* and *Candida rugosa*. More recently, the use of enzyme electrodes in which enzymes are bound to a transducer making use of potentiometric or amperometric detection have become widespread [1].

One of the most exciting and significant therapeutic application of enzymes is in the field of medicine and pharmaceuticals. Already, pharmaceuticals account for a half of the total enzyme industry and this application has a high growth potential in the future [2]. In contrast to the industrial use of enzymes, therapeutically useful enzymes are required in relatively tiny amounts but at a very high degree of purity and (generally) specificity. The favored kinetic properties of these enzymes are low K_m and high V_{max} in order to be maximally efficient even at very low enzyme and substrate concentrations. Thus the sources of such enzymes are chosen with care to avoid any possibility of unwanted contamination by incompatible material and to enable ready purification. Therapeutic enzyme preparations are usually marketed as lyophilized pure preparations with

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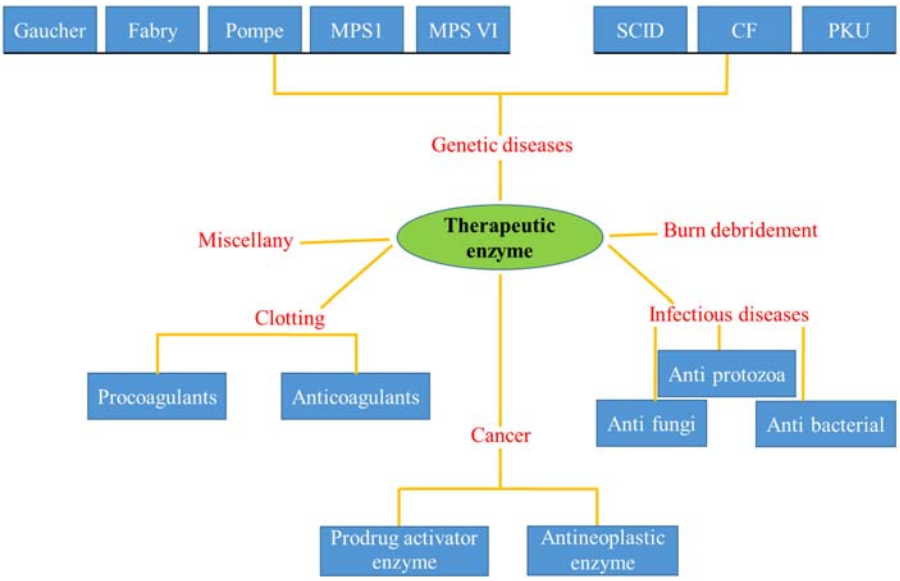

FIGURE 15.1

Application of therapeutic enzymes in different disorders and diseases [4–8].

biocompatible buffering salts and mannitol diluent. The costs of these enzymes are high, but do not exceed or are comparable with those of therapeutic agents or treatments [3]. As an example, urokinase (UK) is derived from human urine and used to dissolve blood clots. One of the major applications of therapeutic enzymes is in the treatment of cancer and various other diseases, as shown in Fig. 15.1.

15.2 ENZYME SOURCES

Therapeutic enzymes are widely distributed in animals, plants, and microbes. Nevertheless, the attributes of the enzymes' origin determines the convenience, cost and recovery process. Broadly speaking, several enzymes analogous to those of plants and animals can be obtained from microorganisms as well. There is a disposition to employ the enzymes from microbial sources for commercial purposes because of economic feasibility, since they can be obtained abundantly by this approach [9]. The microbial enzyme industry offers rapid and robust growth of microbial sources, and thereby enables one to produce enzymes in large quantities. Better strains can be obtained through recombinant DNA (rDNA) techniques by the manipulation of microbes at the genetic level, chiefly to better the quality or characteristics of the enzyme and also to obtain higher yields [10].

15.3 ENZYME PRODUCTION

The enzyme industry flourished in the 19th up until the mid-20th century, when it was established that enzymes can be obtained from microbes. Traditionally, enzymes used to be extracted from animal and plant sources, which resulted in a reduced level of accessibility, swollen cost, and inferior development of the enzyme industry. With the help of genetic engineering, desirable proteins are largely brought forth to satisfy the needs of the enzyme industry. Therefore, to the highest degree biopharmaceuticals made nowadays are genetically modified products [11]. In rDNA technology, cloning of cDNA for the desired protein takes place, then the insertion of cloned cDNA into expression vectors, transformation of *E*scherichia *coli* with the expression vectors, its overexpression and finally purification of the expressed proteins [9]. The yield, quality, production time, and ease of extraction are crucial parameters for appropriate expression systems for recombinant enzyme manufacturing [12]. Several enzyme expression systems have been established. These include cell cultures of bacteria, molds, mammals, yeasts, plants or insects, or via transgenic animals and plants. So far, 400 human proteins have been produced by rDNA technology for therapeutic use [13]. Various fermentation methods are employed to produce enzymes. Microbial cultures are grown on large-scale fermenters for enzyme production under optimized growth conditions [14,15]. These include solid-state fermentation, submerged fermentation and immobilization, etc. These different methods of fermentation are utilized to produce therapeutic enzymes on commercial scale rather than liquid cultures in huge bioreactors [16]. Various production techniques and downstream processing of large-scale production of microbial therapeutic enzymes have also been reported [13].

15.4 THERAPEUTIC APPLICATIONS

Therapeutic enzymes have a broad variety of specific uses: as oncolytics, anticoagulants or thrombolytics, as replacements for metabolic deficiencies, digestive aids, metabolic storage disorders, etc. In addition, there is a growing group of miscellaneous enzymes of diverse function (Table 15.1).

15.5 ONCOLYTIC ENZYMES

15.5.1 ASPARAGINASE

Asparaginase is a tetrameric enzyme that catalyses the hydrolysis of the amino acid, asparagine (Asn). It may be purified from a wide variety of microorganisms (yeast, fungi, bacteria such as *E. coli*). Asn is required for normal

Table 15.1 Some Therapeutically Important Enzymes [2]

Enzyme	Source	Therapeutic Application
Asparaginase (amidase)	*E. coli*, guinca pig serum	Cytotoxic agents
Bromelain (protease)	*Ananas comosus*	Inflammation, oedema
Chymotrypsin (protease)	Bovine pancreas	Inflammation, oedema, ophthalmology and upper respiratory tract diseases
Collagenase	*Clostridium histolyticum*	Cleaning necrotic tissue
Deoxyribonuclease (DNA hydrolysis)	Bovine pancrease	Reduces viscosity of pulmonary secretions (liquefies blood clots)
Dextranase (dextran hydrolysis)	*Penicillium funiculosum*	Dental plaque restriction
Diastase (starch hydrolysis)	Malt	Amylaceous dyspepsia
Galactosidase (lactose hydrolysis)	*Aspergillus niger*	Inherited β-galactosidase deficiency
Hyaluronidase	Animal testes	Increase absorption rate, increase effectiveness of local anesthetics
(mucopolysaccharide hydrolysis)	Egg white	Antimicrobial activity (eye therapy)
Lysozyme	Animal pancreas	Pancreatitis
Pancreatin	*Carica papaya*	Dyspepsia and gastritis
Papain (protease)	*Bacillus cereus*	Penicillin allergy
Penicillinase	Plasminogen	Thrombotic disorders anticoagulation
Plasmin (protease)	Streptococci	Depolymerization of DNA in purulent exudates
Streptodornase (DNA-ase)	Streptococci	Thromboemolic diseases
Streptokinase (protease)	Animal pancreas	Cleaning necrotic tissue
Trypsin (protease)	Recombinant DNA tech.	Thromboemolic diseases
Tissue plasminogen activator	Human plasma	Stops bleeding
Thrombin	Human urine	Thromboemolic diseases
Urokinase (protease)		

metabolic activity. Most human cells are capable of synthesizing Asn, but certain malignant cells do not. This can be used in the destruction of malignant cells. Sources of clinically used asparaginase include: (1) *E. coli*: two isozymes of which only one is effective; and (2) *Erwinia chrysanthemi.* This enzyme is important in the treatment of childhood leukemia. The side effects include severe allergic reactions, nausea, vomiting, fever, compromised kidney and liver function. Allergic reaction is greatly reduced by coupling the asparaginase with polyethylene glycol (PEG) [17]. Asparaginase has also been produced by a recombinant *Pichia pastoris* strain harboring *S. cerevisiae* ASP3 gene ([18]).

An important characteristic of the process of oncogenesis is proliferation. Enzymes influencing this process have also been studied and targeted for

production. It has been shown that the removal of chondroitin sulfate proteoglycans by chondroitinase A, C and, to a lesser extent, by chondroitinase B inhibits, tumor growth, neovascularisation and metastasis as reported by Su et al. [19] and Blain et al. [20].

Antibody-directed enzyme prodrug therapy (ADEPT) illustrates further applications of enzymes as therapeutic agents in cancer. In ADEPT, a monoclonal antibody carries an enzyme specifically to cancer cells, where the enzyme activates a prodrug, destroying these cells, but not the normal cells, as reported by Xu and McLeod [21] and Jung [22]. This approach is being utilized to discover and develop a class of cancer therapeutics based on tumor-targeted enzymes that activate prodrugs.

15.5.2 OTHER ONCOLYTIC ENZYMES

Diphtheria toxin (an oncolytic enzyme still in the experimental stage), catalyzes transfer of the adenosine diphosphate ribose (ADP-ribose) moiety of nicotinamide adenine dinucleotide (NAD) to elongation factor 2. This enzyme halts protein synthesis. The protein synthesis in tumor cells is 100−10,000 times more sensitive to this toxin than the analogous process in normal cells.

Enzymes that degrade macromolecules include neuraminidase, ribonuclease, and a diverse group of proteases. Neuraminidase removes sialic acid residues from the surface of (neoplastic) cells, thereby altering their immunogenicity and rendering them sensitive to immune response. In 2000, the Food and Drug Administration (FDA) approved the Orphan Drug application of Wobe-Mugos as an adjunct therapy for multiple myeloma. Wobe-Mugos (vitamins + proteolytic enzymes) has been used successfully in Europe in conjunction with chemotherapy since 1977.

15.6 ENZYMES AS DEBRIDING AGENTS

Debriding agents effectively clean open wounds by removal of foreign matter and any surrounding dead tissue. Trypsin, papain, and collagenase (all proteolytic enzymes) have often been used. Trypsin from mammalian pancreas hydrolyzes peptide bonds involving arginine and lysine. Papain from the leaves and the unripe fruit of the papaya tree hydrolyze peptide bonds involving basic amino acids (e.g., lysine, arginine, histidine). Collagenase is from culture extracts of various animal cells or normally from various *Clostridium* species (pathogenic).

15.7 ENZYMES AS ANTIINFLAMMATORY AGENTS

Administration of some enzymes is shown to be effective in the reduction of various inflammatory responses. Chymotrypsin: chymotrypsinogen (the zymogen form produced in the pancreas) is converted to the active form in the small

intestine. Bromelains: plant proteases purified from the stem or the fruit of pineapple. Their antiinflammatory action is not known in detail. Probably their ability to degrade protein-based inflammatory mediators plays a role in their action.

15.8 ENZYMES AS THROMBOLYTICS

Thrombolytic diseases are today a major cause of morbidity and mortality. Thrombolytic/fibrinolytic agents are plasminogen activators that convert the zymogen plasminogen to the active enzyme plasmin, which degrades fibrin. Fibrinolytic enzymes have apparent significance in thrombosis therapy in man. Therefore, great attention has been directed towards a search for thrombolytic agents of various origins. Streptokinase (SK) was first isolated in 1933, entered clinical use in mid-1940s, and was used to treat peripheral arterial occlusive (PAO) disease in 1974 by Dr. Charles Dotter [23,24]. The fibrinolytic potential of human urine was first described by Macfarlane and Pilling in 1947 [24]. The active molecule was extracted, isolated and named as urokinase (UK) in 1952. Its precursor was discovered in urine in 1979 and was first licensed for its use in France in 1972 [23,24]. Staphylokinase (SAK) was obtained from *Staphylococcus aureus*. In 1980, recombinant SAK was produced and its thrombolytic property was reevaluated in humans. However, the discovery of streptokinase and urokinase ushered in the use of these enzymes as thrombolytic agents to degrade the fibrin network with an issue of systemic hemorrhage. In 1982, Genentech Corporation announced the successful production of recombinant tissue plasminogen activator "alteplase" (second-generation plasminogen activator), partially controlled this problem and got US FDA. approval for the treatment of myocardial infarction in 1987 [24]. "Tenecteplase" got US FDA approval in June 2000 [24]. The third-generation molecules, majorly *t*-PA variants, showed desirable properties of improved stability, safety and efficacy with enhanced fibrin specificity. Plasmin variants are produced as direct fibrinolytic agents as a futuristic approach with targeted delivery of these drugs using liposome technology. The novel molecules from microbial, plant, and animal origin present the future of direct thrombolytics due to their safety and ease of administration.

15.9 REPLACEMENTS FOR METABOLIC DEFICIENCIES

15.9.1 ENZYMES AS DIGESTIVE AIDS

Most digestive aid preparations are based on depolymerases responsible for the breakdown of polysaccharides, proteins, and lipids. Such preparations may include a single enzyme or multiple enzymes. All amylases are glycoside hydrolases and hydrolyze α(1-4) glycosidic bonds. Amylase from *B. subtilis* or species of *Aspergillus* have various industrial applications. Oral amylase administration is

used to aid digestion. *Lactase* is an enzyme that hydrolyzes lactose down into galactose and glucose. In many geographical regions, several adults have greatly reduced lactase activity.

There are various proteolytic enzymes, e.g., papain, pepsin, etc. The main function of these protease enzymes is to aid digestion of various foods. For example, Pancreatin is a preparation extracted from pancreas containing various enzymes. İt is used in deficiencies related to secretion of pancreatic enzymes (e.g., chronic pancreatitis, pancriatic carcinomas, cyctic fibrosis). Since one problem associated with oral administration of enzymes is gastric inactivation, coadministration of inhibitors of gastric acid secretion is performed. Enteric coated tablet or capsules and use of microbial proteases, amylases, and lipases are also pursued.

15.10 SUPEROXIDE DISMUTASE

This is an important enzyme in all aerobic organisms.

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Two forms are found in eukaryotes: cytoplasmic (zinc and copper) and mitocondrial (manganese). Isolates from bovine liver and erythrocytes clinically used as antiinflammatory agent (injection into patients with osteoarthritis of the knee).

15.11 ORAL AND INHALABLE ENZYME THERAPIES

In contrast to the treatments mentioned so far, there are some diseases that do not require intravenous injection of an enzyme of human origin. Updating the age-old application of enzymes as digestive aids, several diseases have yielded to oral enzyme formulations. Congenital sucrase-isomaltase deficiency (CSID), e.g., is treatable with sacrosidase—a β-fructofuranosidefructohydrolase from *Saccharomyces cerevisiae* that can be taken orally, as reported by Treem et al. [25].

Phenylketonuria (PKU) is another genetic disorder requiring strict compliance with a specialized diet. PKU is caused by low or nonexistent phenylalanine hydroxylase activity, which catalyzes the conversion of phenylalanine to tyrosine. An oral treatment, phenylase, is being developed based on the use of recombinant yeast phenylalanine ammonia lyase (PAL). PAL has been shown by Sarkissian et al. [26] to degrade phenylalanine in the gastrointestinal tract.

Inhalable enzyme formulations have found application in the treatment of cystic fibrosis (CF). Pulmozyme (Dornase α), a DNase, received one of the fastest approvals by the FDA under the Orphan drug status. DNAse I produced by expression of cDNA in CHO cell lines (Pulmozyme) has been approved for

medical use. Dornase α liquefies accumulated mucus in the lung. The use of Dornase α in CF patients can also diminish pulmonary tissue destruction by lowering the level of matrix metalloproteinases in the broncho-alvelolar lavage fluids [27].

15.12 ENZYME-REPLACEMENT THERAPY (ERT)

Enzymes as drugs have two important features that distinguish them from all other types of drugs. First, enzymes often bind and act on their targets with great affinity and specificity. Second, enzymes are catalytic and can convert multiple target molecules to the desired products. These two features make enzymes specific and potent drugs that can accomplish therapeutic biochemistry in the body that other small molecules cannot. These characteristics have resulted in the development of many enzyme drugs for a wide range of disorders.

In 1987, the first recombinant enzyme drug, Activase (altepase: recombinant human tissue plasminogen activator), was approved by the FDA. This "clot-buster" enzyme is used for the treatment of heart attacks caused by the blockage of a coronary artery by a clot. In 1990, Adagen, a form of bovine adenosine deaminase (ADA) treated with polyethylene glycol (PEG), was approved for use in treating patients affected with a type of severe combined immunodeficiency disease (SCID), which is caused by a chronic deficiency of ADA.

Adagen, used for the treatment of SCID, represents the first successful application of an enzyme therapy for an inherited disease [28]. As reported by Hershfield [29], the success of the treatment depends on the modification of bovine adenosine deaminase (ADA) with PEG, which enhances the half-life of the enzyme and reduces the possibility of immunological reactions due to the bovine origin of the drug.

Ceredase (alglucerase injection) used for the treatment of Gaucher disease, a lysosomal storage disease (LSD), was the first enzyme-replacement therapy in which an exogenous enzyme was targeted to its correct compartment within the body [30].

15.13 ENZYMES AS NERVE AGENT SCAVENGERS

Currently, fielded treatments for nerve agent intoxication promote survival, but do not afford complete protection against either nerve agent-induced motor and cognitive deficits or neuronal pathology. The use of human plasma-derived butyrylcholinesterase (HuBuChE) to neutralize the toxic effects of nerve agents in vivo has been shown to both aid survival and protect against decreased cognitive function after nerve agent exposure. Recent work has focused on paraoxonase-1 (PON1), a naturally occurring human serum enzyme (catalytic scavengers) with

the capacity to catalyze the hydrolysis of nerve agents, albeit too slowly to afford dramatic protection [31]. It may be possible to engineer a mutant form of PON1 with enhanced activity and stereospecificity (stoichiometric scavengers) for some of the most toxic nerve agent isoforms.

15.14 TOPICAL ENZYME THERAPY FOR SKIN DISEASES

DNA damage has an important role in the development of skin cancer and precancerous skin lesions. The topical administration of bacterial DNA repair enzyme (T4 endonuclease V) in a liposomal delivery vehicle to sun-damaged skin of patients with xeroderma pigmentosum lowered the rate of development of two forms of these lesions with no adverse effects during a year of treatment [32].

In the past, a large number of proteolytic enzymes of plant and bacterial origin have been studied as replacements for the mechanical debridement (removal of dead skin) of burns. Unfortunately, the results have been variable due, perhaps, to the poor quality of the enzymes used [33,34]. Several products of higher quality and purity, some owing to their recombinant origin, are now under clinical trials. Debrase gel dressing, comprising a mixture of enzymes extracted from pineapple, received clearance in 2002 by the US FDA for a Phase II clinical trial for the treatment of partial and full-thickness burns. Vibrilase (recombinant vibriolysin), a proteolytic enzyme from the marine microorganism *Vibrio proteolyticus*, has been shown to have efficacy against denatured proteins such as those found in burned skin [35].

15.15 ENZYMES IN INFECTIOUS DISEASES

Lysozyme has been used as a naturally occurring antibacterial agent in many foods and consumer products because of its ability to break carbohydrate chains in the cell walls of bacteria. Lysozyme has also been shown to possess activity against HIV, as has RNase A and urinary RNase U, which selectively degrade viral RNA as reported by Lee-Huang et al. [36], opening exciting possibilities for the treatment of HIV infection. Other naturally occurring antimicrobial agents are chitinases. As an element of the cell wall of various pathogenic organisms, including fungi, protozoa and helminths, chitin is a good target for antimicrobials [37].

15.16 FUTURE PROSPECTS

The prospects of industrial and pharmaceutical uses of enzymes have increased greatly in the 21st century. More biopharmaceuticals have entered the drug discovery and development pipelines in the recent times. Enzymes have already

been used as clinical test reagents and further development in this field can be expected. Development in the field of clinical application of enzymes is also seen. Various industries including enzyme manufacturers are carrying out enzyme research currently to find new and improved methods for using enzymes, to improve yields of industrial microbial enzymes, and find new enzymes for industrial and medical purposes. There is a great and growing market for therapeutic enzymes. Occurrence of many diseases, and outbreak of unknown ones, have increased the demand for enzymes as therapeutic agents. Presently, therapeutic enzymes are available as pills, capsules, powders, and food supplements. Medically important enzymes produced by microorganisms are used as anticoagulants, oncolytics, thrombolytics, fibrinolytics, mucolytics, antiinflammatories, antimicrobials, and digestive aids. Diseases which are resurging after acquiring resistance to antibodies can also be treated using enzymes. Combinations of enzymes and drugs also have the ability to induce synergistic effects and can treat various diseases by counteracting their side effects. Hence, it can be concluded that there is indeed the need for research in the near future in these biomolecules which will later on prove relevant and beneficial to mankind.

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CHAPTER

Enzymes as direct decontaminating agents—mycotoxins

16

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16.1 INTRODUCTION

16.1.1 ENZYMES AS DECONTAMINATING AGENTS

Enzymes are biocatalysts that increase the rate of a reaction and accelerate the conversion of substrates into products that occur many millions of times faster by lowering the activation energy of the reaction. The active site (region) of the enzyme is directly involved in the catalytic process [1]. An enzyme may have one or more groups that are prerequisite for catalytic activity associated with the active sites through either covalent or noncovalent bonds. Being catalytic, the enzymes are immensely effective and can detoxify many times their own weight of agent in seconds or minutes. The biocatalytic nature and detoxification abilities of enzymes make them efficient decontamination agents [2].

The aim of decontamination technology progress efforts is to develop systems that are rapid and effective in detoxifying chemical and biological agents, are environmentally safe, that do not impact the operation effectiveness of the machinery being decontaminated, and minimize the procedural impact on operations [3]. Unlike most chemical catalysts, different types of enzymes with different specificities can be mixed together in a single formulation. Because enzymes function excellently at near neutral pH, there are minor, if any, compatibility or corrosion concerns as long as the material being decontaminated can tolerate water. In a water-based system, there will not be any flammability concerns. Similar to enzymes in commercial laundry detergents, an enzyme-based decontaminant, being biodegradable, will pose little or no health or environmental danger and leave no hazardous products that would need to be dealt with [4]. Potentially, an enzyme-based detoxifying agent will be favorable enough to be used directly on the skin of personnel and casualties. Another major advantage is

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that an enzyme-based decontaminant might be provided as a dry granulated powder that is added to whatever water-based spray or foam system that the user has available [5].

An appropriate blend of enzymes and other natural products provides considerable advantages over other decontaminants. A number of key advantages over reactive chemistry-based decontaminants are offered by enzymatic approaches. All enzymes are biodegradable proteins produced by living organisms that act as natural catalysts, accelerating the specific biochemical reaction for which they were designed [6].

16.1.2 ENZYME CATEGORIES BASED ON DETOXIFICATION NATURE

All the enzymes are categorized into six classes: (1) the oxidoreductases, (2) the transferases, (3) the hydrolases, (4) the lyases, (5) the isomerases, and (6) the ligases (synthetases). Oxidoreductases catalyze the transfer of electrons and protons from a donor to an acceptor. Transferases catalyze the transfer of a functional group from a donor to an acceptor. Hydrolases promote the cleavage of C−C, C−O, C−N, and other bonds. Lyases catalyze the cleavage of C−C, C−O, C−N, and other bonds by elimination, leaving double bonds, or catalyze the addition of groups across double bonds. Isomerases facilitate geometric or structural rearrangements or isomerizations. Ligases catalyze the joining of two molecules [7].

16.1.2.1 Oxidoreductases

Oxidoreductases mediate the detoxification of toxic organic compounds through oxidative coupling. These enzymes catalyze the biochemical reactions by cleaving chemical bonds and by assisting the transfer of electrons from a reduced organic substrate (donor) to another chemical compound (acceptor). In such oxidation−reduction reactions, the contaminants are finally oxidized to harmless compounds. Oxidoreductases aid in decolorizing azo dyes [8], and through polymerization, copolymerization with other substrates, or binding to humic substances, they can also detoxify toxic xenobiotics [9].

16.1.2.2 Oxygenases

Oxygenases belong to the group of oxidoreductase enzymes. They participate in oxidation of reduced substrates by transferring oxygen from molecular oxygen (O_2) utilizing flavin adenine dinucleotide (FAD)/nicotinamide-adenine dinucleotide, reduced (NADH)/nicotine adenine disphosphonucleotide, reduced (NADPH) as a cosubstrate. On the basis of number of oxygen atoms used for oxygenation, these enzymes were further classified as the monooxygenases and dioxygenases. The monooxygenases consist of a versatile superfamily of enzymes that facilitate oxidative reactions of substrates ranging from alkanes to complex endogenous molecules such as steroids and fatty acids. The ammonification, biotransformation, biodegradation, dehalogenation, denitrification, desulfurization, and hydroxylation

of various aromatic and aliphatic compounds are catalyzed by monooxygenases. Dioxygenases are multicomponent enzyme systems that introduce molecular oxygen into their substrate. Aromatic hydrocarbon dioxygenases belong to a huge family of Rieske nonheme iron oxygenases. These dioxygenases catalyze the oxygenation of broad range of substrates, enantio-specifically. Dioxygenases primarily oxidize aromatic compounds. Laccases are another class of oxidases containing copper as a cofactor and catalyze a wide range of ortho- and paradiphenols, aminophenols, polyphenols, polyamines, aryldiamines, and lignins, as well as some inorganic ions [10–12]. Laccases, besides oxidizing phenolic and methoxyphenolic acids, also decarboxylate them and attack their methoxy groups (demethylation). Laccases are a fascinating group of ubiquitous, oxidoreductase enzymes among the biological agents, that show great potential for biotechnological and bioremediation applications [13].

16.1.2.3 Peroxidases

The oxidative transformation of different organic and inorganic compounds at the expenditure of peroxide, usually hydrogen peroxide (H_2O_2) is the principle biological reaction catalyzed by peroxidases. Peroxidases oxidize phenolic compounds to extremely reactive free radical species, which instinctively generate insoluble oligo- or polymeric derivatives. These insoluble derivatives can be removed from solutions by simple techniques such as filtration and sedimentation [14]. As the lignin peroxidase (LiP), manganese-dependent peroxidase (MnP), and versatile peroxidase (VP) processes demonstrate high potential to degrade toxic substances in nature, they are the most studied among all other peroxidases.

LiPs are heme proteins. LiP degrades lignin and other phenolic compounds in the presence of a cosubstrate (H_2O_2) and a mediator (veratryl alcohol (VA)). In the reaction, the cosubstrate H_2O_2 gets reduced to H_2O by accepting an electron from LiP (which itself gets oxidized). By gaining an electron from VA, the LiP (oxidized) returns to its native reduced state, and VA is converted to veratryl aldehyde. Veratryl aldehyde then again reduces back to VA by accepting an electron from the substrate. This results in the oxidation of halogenated phenolic compounds, polycyclic aromatic compounds, and other aromatic compounds, followed by a series of nonenzymatic reactions [15,16].

Manganese peroxidases catalyze the multistep oxidation reaction which converts Mn^{2+} to the oxidant Mn^{3+}. In the presence of H_2O_2 (cosubstrate), Mn^{2+} triggers the production of MnP and serves as a substrate for MnP. The Mn^{3+}, produced by MnP, serves as a mediator to oxidize a variety of phenolic compounds.

VP enzymes are capable of oxidizing Mn^{2+}, methoxybenzenes, and phenolic aromatic substrates similar to that of MnP, LiP, and horseradish peroxidases. VPs, which combine typical properties of both manganese and LiPs, couple with a wide substrate range [17–19]. Even though this class of peroxidases has huge affinity towards manganese and dyes, they are also effective in oxidizing VA and 2,6-dimethoxyphenol (DMP) in a manganese-independent reaction [19]. Manganese peroxidases are capable of oxidizing phenolic substrates only in the

presence of manganese, but the VPs are able to oxidize such substrates both in the presence and absence of manganese.

16.1.2.4 Transaminases

Transaminases are a class of enzymes that catalyze the transfer of an amino group from an amine to an acceptor, creating a keto- and amino acids with a single chirality. Pyridoxal phosphate (PLP: the biologically active form of vitamin B6), aids as cofactor to this group of enzymes. Normally, the PLP binds covalently to the lysine amino acid at the active site by an internal aldimine (Schiff's base). The mechanism of action of the reaction catalyzed by transaminases is divided into two half-reactions. During the first half of the reaction, the donor substrate donates its amino group to the PMP (cofactor), producing a keto acid and enzyme-bound pyridoxamine-phosphate and in the second half of the reaction, PMP transfer an amino group to an acceptor (keto acid), resulting in an amino acid and reconstituting the PLP internal aldimine [20]. For example, the liver contains peculiar transaminases to transfer an amino group from glutamic acid to α-keto acid that is related to most of the other amino acids. Other transaminases catalyze reactions in which an amino group is transferred from glutamic acid to other compounds, e.g., to aldehydes to form amines. Transamination is one of the crucial mechanisms for the production of necessary amino acids in protein metabolism [21].

16.1.2.5 Hydrolases

Hydrolytic enzymes destroy notable chemical bonds in toxic molecules, resulting in the reduction of their toxicity. Biodegradation of oil spills, organophosphate, and carbamate insecticides can be effectively performed by this mechanism. Hydrolases also catalyze a number of other related reactions such as condensations and alcoholysis. The prime advantages of this class of enzymes are ready availability, lack of cofactor stereo selectivity, and toleration of the addition of water-miscible solvents. Hydrolases fall under group 3 of the enzyme classes, and may further be classified according to the type of bond hydrolyzed [22]. Amylases, cellulases, proteases, lipases, DNases, pullulanases, and xylanases are some of the subclasses of hydrolases.

Cellulase enzymes are capable of catalyzing the degradation of crystalline cellulose to glucose. Since the early 1990s, cellulases have been used in the manufacture of detergents and employed in the paper and pulp industry, for the removal of ink during recycling of paper. Cellulases are also used in the brewing industry increasing the liberation of juice from fruit pulp, and for the production of ethanol from cellulosic biomass [23].

Proteases belong to a group of enzymes that catalyze the hydrolysis of peptide bonds in an aqueous environment. Proteases have ample of applications in the food, leather, detergent, and pharmaceutical industries [24,25]. Proteases are further classified as endopeptidases and exopeptidases based on the type of peptide chain catalyzed. Endopeptidases are further divided depending on the active site

position, such as aspartic endopeptidases, cysteine peptidase, metallopeptidases, and serine endopeptidase. The exopeptidases exert their effect only near the terminal amino or carboxylic position of the peptide chain. The proteases that react at free amino and carboxyl terminals are known as aminopeptidase and carboxypeptidase, respectively.

Lipases are a unique class of enzymes that catalyze the hydrolysis of triacylglycerols to glycerol and free-fatty acids. Lipolytic reactions occur at the lipid–water interface, where lipolytic substrates usually form equilibrium between monomeric, micellar, and emulsified states. These enzymes are able to catalyze a broad range of reactions such as aminolysis, hydrolysis, esterification, interesterification, and alcoholysis [26]. Lipase activity was found to be the most effective indicator for testing the degradation of hydrocarbons in soil [27,28]. Besides its diagnostic application in bioremediation, lipase has many other potential uses in food, chemical, detergent manufacturing, cosmetic, and paper-making industries, but its production cost has limited its industrial application [29].

16.1.2.6 Lyases

Lyases are enzymes that cleave C–C, C–O, C–N, and other bonds by elimination to form multiple bonds or rings. Lyases utilize an extraordinary range of prosthetic groups which include thiamine pyrophosphate [30,31], iron sulfur clusters [32], NAD(P)(H), pyridoxal 5b-phosphate [33–35], biotin [36] and back-bone dehydroalanine [37], or 4-methylidene-imidazole-5-one residues [38]. Hydroxynitrile lyases, aldolases, ketolases, and a certain type of thiamine-diphosphate-dependent lyases play important roles in industrial biotransformations.

16.1.2.7 Isomerases

Isomerases belong to the EC 5 primary class of enymes that catalyze the reactions involving a structural rearrangement of a molecule. Alanine racemase, e.g., catalyzes the conversion of L-alanine into its isomeric (mirror-image) form, D-alanine. An isomerase known as mutarotase catalyzes the conversion of α-D-glucose into β-D-glucose. They are subdivided into three hierarchical levels: six subclasses, 17 subsubclasses, and 231serial numbers. These serial numbers are related with almost 300 biochemical reactions; e.g., EC 5.1.1.9 illustrates the racemization of arginine, lysine, or ornithine, and it is therefore associated with three different reactions [39]. Isomerases are used in many applications. In metabolic engineering, xylose isomerase (EC 5.3.1.5) has been traditionally used to convert glucose to fructose in the syrup industry. D-Tagatose is isomerized from galactose by L-arabinose isomerase. This class of enzymes has potential applications both in pharmaceutical and agrofood industries [40].

16.1.2.8 Ligases

Ligases are enzymes that are capable of catalyzing the reaction of joining two large molecules by establishing a new chemical bond, generally with concomitant hydrolysis of a small chemical group on one of the bulky molecules or simply linking of two compounds together (e.g., enzymes that catalyze joining of C–O,

C−S, C−N, etc.). They are classified under EC 6 primary class of enzymes and further divided into six subclasses known as ligases establishing carbon−oxygen bonds, carbon−sulfur bonds, carbon−nitrogen bonds, carbon−carbon bonds, phosphoric−ester bonds, and nitrogen−metal bonds [7]. The usual names of ligase enzymes generally include the word "ligase," including DNA ligase, an enzyme often used in molecular biology laboratories to join DNA fragments. Other general names for ligases include synthetases, as they catalyze the reaction of synthesizing new molecules and they require energy from nucleoside triphosphates for catalyzing the synthetic reaction.

16.2 ENZYMES FOR DECONTAMINATION OF MYCOTOXINS

16.2.1 MYCOTOXINS INTRODUCTION

Mycotoxins are secondary metabolites produced by fungi causing adverse effects to humans and other animals. Several hundred mycotoxins are currently documented, with aflatoxin (AF), ochratoxin, fumonisin, deoxynivalenol, and zearalenone (Fig. 16.1) being the most toxic and consequently the best studied. AF and ochratoxin are known as Nature's most toxic compounds as they are extremely toxic at

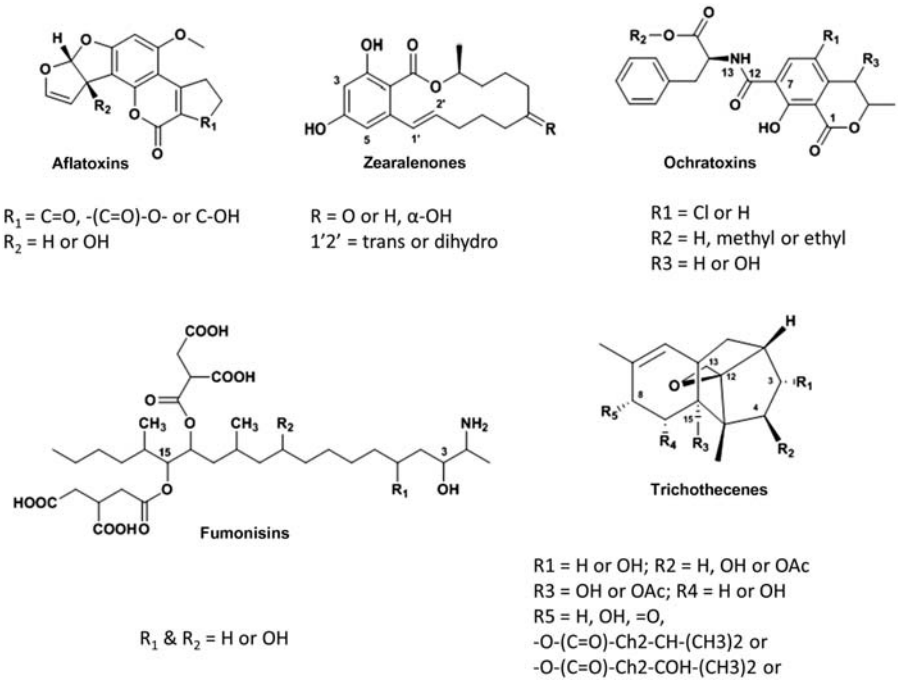

FIGURE 16.1

Schematic representation of mycotoxins.

the parts-per-billion level. They often occur in a broad range of agricultural commodities [41,42] and thus cause a serious threat to humans and animal health. Since the 1970s, in situ enzymatic detoxification and decontamination of mycotoxins has been used as a strategy for the elimination of mycotoxins from grain, feed, and food. In the 1980s and 1990s, extensive research on the biological decontamination of trichothecenes was carried out in military facilities and by extramural military-funded research [43,44].

Biodegradation of AFs by microorganisms or enzyme preparations is an attractive means of food detoxification because it is potentially efficient, highly target-specific, environmentally friendly, and likely to preserve the quality of the food product [45]. Mycotoxin formation appears to be unavoidable under certain adverse conditions, even though the primary goal is the prevention of mycotoxin contamination. Mycotoxin formation can be prevented by the chemical treatment of grains, but it is not desirable. In addition, both physical and chemical decontamination methods have been employed (such as for ergot physically) with varying success in the past, primarily for feed [46].

Mycotoxins can be partially removed by dry cleaning of the grain and in the milling process. During the milling process mycotoxin levels may be increased in bran and reduced in flour. Heat treatment, typically applied in food technology does not have a significant effect on the mycotoxin levels, as the majority of mycotoxins are heat-stable [47]. Several efforts have been made in different countries to find an economically acceptable way of destruction of mycotoxins into nontoxic products using different chemicals such as alkali and oxidative agents. Even though such treatment reduces or completely removes the mycotoxins, these chemicals may also cause losses of some of the essential nutrients and such treatment is too drastic for, e.g., grain destined for food uses. Recent studies show that many physical adsorbents are commercially available preparations as animal feed additives. Nevertheless, many of these adsorbents can bind to only a small group of toxins while showing very little or no binding to others [48]. Biological decontamination and biodegradation of mycotoxins with microorganisms or enzymes have been used more recently [49,50]. Many species of fungi and bacteria degrade mycotoxins enzymatically [51–53]. There are no significant losses in nutritive value and palatability of decontaminated food and feed when mycotoxins are degraded enzymatically without using any harmful chemicals.

To reduce mycotoxins in food and feed commodities, several technologically diverse decontamination strategies are used, which are based on physical, chemical, or biochemical principles. Some physical processes aim to remove highly contaminated fractions from bulk material [54–56] through sorting [57], milling [58,59], dehulling [60,61], cleaning [62], heating, irradiation, or combinational approaches [63]. Physical removal strategies include the use of inorganic or organic mycotoxin binders [64]. Even though these adsorbing binders have some good features, some may have adverse nutritional effects due to their binding capacity of vitamins and minerals [65] or reducing the efficacy pharmacokinetics of antibiotics [66].

Conversion of mycotoxins via chemical reactions involves remediation strategies using chemical methods. Some of these chemical methods include ammoniation, alkaline hydrolysis, peroxidation, ozonation, and the use of bisulfites, which are effective on one or more mycotoxins, but a detailed insight into the toxicity of eventual end products or the impact on palatability and nutritive quality is questionable [67].

The most frequently used approaches for biodegradation of mycotoxins includes the microorganisms that are able to degrade the given mycotoxin and treatment of food or feed in an appropriate fermentation process [48]. Thus the microbes and enzymes are preferred to be considered for use as detoxifying or decontaminating agents. Therefore this type of decontamination method could be a useful approach for partially overcoming the problem of some mycotoxins [47].

The use of enzymes or engineered microorganisms as processing aids in the food sector would also prove to be beneficial, if these methods were allowed by legislation. As the major food crops in the world are contaminated with mycotoxins, safer ways to decontaminate these foods are essential. The biological decontamination of mycotoxins involves their degradation or conversion into less toxic molecules by enzymes and selected microorganisms. It has been established that enzymes produced from some microorganisms could modify the structure of mycotoxins and/or proteins that can conjugate these compounds, making them less active as pathogenic agents [68]. However, only a few microorganisms have shown the capacity to degrade mycotoxins. Toxicity can be eliminated from absorbable mycotoxins through an adsorption process; however, for the non-adsorbable mycotoxins, such as zearalenone, ochratoxins, and trichothecenes, biotransformation is crucial. The toxic structure of mycotoxins is converted into nontoxic, harmless metabolites by biotransformation, which is enabled by enzyme-producing microorganisms.

16.2.2 TYPES OF MYCOTOXINS AND THEIR DECONTAMINATION PROCESS

16.2.2.1 Aflatoxins

Biodegradation of AFs using enzymes is one of the well-known approaches for the decontamination of AFs in foods and feeds. The major drawback of using microorganisms for detoxification is avoided, as it may change the flavor or reduce the nutritional value, and thereby the acceptability of the product is limited [45]. Enzymes responsible for the degradation of AFs have been identified as laccases, peroxidases, oxidases, and reductases [69,70].

An immobilized enzyme, named AF-detoxifizyme (ADTZ) from *Armillariella tabescens* (E-20), was reported to show complete detoxification by opening the difuran ring of aflatoxin B1 (AFB1) that led to its subsequent hydrolysis. The AF oxidase was also reported to have acted on versicolorin A, 3,4-dihydro-2H-pyran, and furan ring, suggesting that the 8,9-unsaturated carbon−carbon bond of AFB1

is the reactive site for AF oxidase [71]. The extracellular enzyme from *Pleurotus ostreatus* showed AF-degradation activity [72] and suggested that the enzymes cleaved the lactone ring of the AF molecule, converting it to a nontoxic form. An F420H2-dependent reductase was identified from *Mycobacteria smegmatis* that catalyzes AF degradation by reducing an α,β-unsaturated ester and subsequently, destabilized the lactone ring [73]. The AF degradation mechanism of labeled myxobacteria AF degradation enzyme (MADE) from *M*yxococcus *fulvus* was identified [74]. An MnP from *Phanerochaete sordida* YK-624 catalyzes AFB1 detoxification with oxidation by the MnP and then hydrolyzes to AFB1-8,9-dihydrodiol. A multienzyme isolated from *A. tabescens* showed degradation: AFB1 was first transformed to AFB1-epoxide, followed by hydrolysis of the epoxide to give the dihydrodiol. Then, the difuran ring opened in the subsequent hydrolysis step. Enzymes isolated from *P. ostreatus* and *A. tabescens* also showed AF-degradation activity.

16.2.2.2 Ochratoxin

Ochratoxin A (OTA) is a major mycotoxin present in food and agricultural products causing damage to humans and animals. It is produced by several fungi, such *Aspergillus* and *Pencillium* species. Further, OTA has several toxicological effects such as teratogenic, hepatotoxic, nephrotoxic, carcinogenic, and immunosuppressive. [75]. Numerous microorganisms are capable of degrading, adsorbing, and detoxifying OTA. Numerous decontamination procedures of OTA in food and feeds have been suggested. By heating under reflux of hydrochloric acid [76] or by hydrolysis with carboxypeptidase A [77], OTA which is a stable compound can be converted into ochratoxin-α (OTα) and L-b-phenylalanine. Chemicals such as ammonia or hydrogen peroxide are used to inactivate OTA [78,79], or dichloromethane is used to extract it [80]. Nevertheless, the use of such approaches may lead to substantial losses in nutritive value and palatability of decontaminated products. Biodegradation with enzymes is considered to be a better solution for decontamination.

Enzymatic degradation of OTA is the cleavage of the amide bond into nontoxic phenylalanine and OTα moiety [81,82]. Proteolytic enzymes may possess significant OTA degrading activity and among them carboxypeptidases could be considered to be mostly effective. These enzymes cleave amide bonds at the carboxy-terminal end of a peptide, and carboxypeptidase A shows some preference for aromatic amino acids like phenylalanine as the terminal amino acid [77]. Further, traditionally carboxypeptidase A was the first enzyme demonstrated to be most effective in degradation of OTA [83], and it is also used as a standard for the activity of OTA-degrading enzymes [84]. Other proteolytic enzymes such as chymotrypsin exhibit lesser OTA-degrading activity, while trypsin shows no activity [77]. To some extent, protease A and pancreatin (with protease, lipase and amylase activity) have also been effective in degradation of OTA [84]. Relevant OTA degrading activity was also observed from lipase of *A. niger*, and a crude lipase (Amano) has been identified from the same species

which substantially hydrolyzes OTA into OTα [85]. In vitro hydrolysis of OTA by carboxypeptidase A and in lesser amounts by α-chymotrypsin was also reported [77].

16.2.2.3 Fumonisin

The product of the decontamination of fumonisins in food is determined by the method used and the leftover toxicity of the treated sample. The best decontamination procedure should be easy to use, economical, and should not lead to the formation of compounds that are still toxic, or may reverse to reform the parent mycotoxin, or alter the nutritional and palatability properties of the grain or grain products [86]. Decontamination of the fumonisin contents in foods is performed using physical, chemical, and biological methods. Most of the reported physical and chemical methods to reduce fumonisin B1 concentrations in food have been incompetent or slightly difficult to implement into the production process [87]. Hence, some efforts have been initiated to discover enzymes or organisms that are able to metabolize fumonisins, therefore providing a biotechnological solution to the problem.

Enzymes and genes from microorganisms such as black yeast *Exophiala spinifera* and from bacterium ATCC 55552 are efficient in fumonisin degradation into less toxic products and these may further enhance the prospects for enzymatic detoxicification of fumonisins in food and other products. All the well-known fumonisin catabolism pathways start with the hydrolytic release of the two tricarballylic acid side chains, and in the second step 2-amino groups are removed from the core chain by diverse enzymatic mechanisms. Further, recombinant carboxylesterase was able to catalyze the deesterification of fumonisin B1 to a less toxic hydrolyzed fumonisin B1. In the next step, hydrolyzed fumonisin B1 was deaminated by the heterologously expressed aminotransferase in the presence of pyruvate and pyridoxal phosphate. Microbial enzymes and genes for catabolism of fumonisins and their potential for fumonisin detoxification in transgenic plants have also been developed [88]. Another application for fumonisin detoxification in the gastrointestinal tract of animals can be considered in the processing of maize. To be able to evaluate the requirements, and the potential of a new enzyme-based fumonisin detoxification technology, an overview of the state of the art of fumonisin elimination and the known chemical reactions of fumonisins in processing or decontamination is also given.

The effect of the recombinant enzymes on fumonisin B1and hydrolyzed fumonisin B1 was determined in earlier studies. Bacterial enzymes, including one that catalyzes a new reaction in fumonisin catabolism, were also identified [89]. The applications of such enzymes in food or feed processing, or as an additive animal feed for the gastrointestinal tract, seem achievable. The same reaction product, 2-keto-hydrolyzed fumonisin B1, is produced by the bacterial strains via a different enzymatic mechanism, catalyzed by an aminotransferase [89]; recently, it has been registered and commercialized, under the trademark FUMzyme, a fumonisin

esterase from *Komagataella pastoris* aimed at the detoxification of fumonisin from swine and poultry diets [90].

A promising application of genes from fumonisin catabolism pathways for generation of transgenic plants and fumonisin detoxification in plants has been considered [88].

16.2.2.4 Deoxynivalenol

Enzymatic detoxification of deoxynivalenol (DON) in feed additives is a promising approach for the decontamination of feed and food. Toxicity of trichothecenes is due to its 12,13-epoxide ring, and a reductive deepoxidation triggered by specific enzymes (deepoxidase) entails a significant loss of toxicity. *Eubacterium* BBSH 797 was isolated to eliminate the toxic effects of trichothecenes (DON) and the enzymes produced by this organism in the gastrointestinal tract (epoxidases) play an important role by cleaving the toxic epoxy ring and resulting in deepoxy trichothecenes. The destruction of the epoxide ring in DON may also be associated with the action of oxidative enzymes that would catalyze the opening of the epoxide ring [91,92]. DON can also be converted into an acetylated derivative by trichothecene 3-*O*-acetyltransferases. These enzymes are encoded by the genes TRI101 or TRI201 and are produced by fungi in the genus Fusarium [92,93,94]. Attachment of an acetyl group to the C-3 hydroxyl moiety of the trichothecene molecule is involved in the enzymatic modification [95], forming the derivative 3-acetyl deoxynivalenol (3ADON) [96]. The product of mycotoxin-degrading enzyme, with esterase, epoxidase, and peptidase activity, could block epoxide formation in DON metabolism and esterase transformed ZON into a nontoxic metabolite in pigs [97].

Enzymes from *Agrobacterium-Rhizobium* strain E3-39 can detoxify DON by converting DON into 3-keto-DON under aerobic conditions [97]. An enzymatic detoxification pathway leading to 3-oxo-DON and 3-epi-DON was discovered in soil bacteria. The Tri101 gene from *Fusarium sporotrichioides* was used; *Fusarium graminearum* enzyme which retains higher activity towards DON would apparently be a better choice. Other bacterial enzymes catalyzing epimerization, oxidation, and less likely deepoxidation of DON may include this list in future.

Enzymes responsible for the acetylation of DON can be used in genetically engineered crops, and can thus be regarded as an example of pathogen-derived resistance, which has been mainly applied to resistance to plant viruses [98]. Isolation and identification of trichothecene-3-*O*-acetyltransferases from seven *Fusarium* species for decontamination of DON has been described earlier by Khatibi et al. [94]. The DON-catabolic genes and enzymes may provide new approaches to decontaminate DON in DON-degrading microbes.

16.2.2.5 Zearalenone

Zearalenone (ZON) is a secondary fungal metabolite, produced predominantly by *F. graminearum* and *Fusarium culmorum*. ZON acts as a strong estrogenic

compound and binds to estrogenic receptors, has genotoxic and hepatotoxic properties, and may contribute to the increasing occurrence of cancer [99]. ZON is an estrogenic mycotoxin which is converted to a nonestrogenic product by a detoxifying enzyme of *Clonostachys rosea*. It is most often found in corn, but has been also reported in other cereals such as wheat, barley, oat, sorghum, and rice. Laccases are multicopper enzymes which catalyze diphenols and are generally found in plants and fungi, and in some bacteria and insects. They also oxidize polyphenols, aromatic diamines, methoxy-substituted phenols and a variety of other compounds [100]. Laccases of fungi have great potential for industrial and biotechnological applications. In the food industry, the potential applications of laccases are related to bioremediation, detoxification and for decontamination [12]. Takahashi-Ando identified and characterized a lactonohydolase enzyme in fungus *C. rosea* which converts ZON to a less estrogenic compound [101]. Enzymic detoxification methods could offer a practical and efficient method of ZON decontamination. *A. niger* strain FS10 can also be used to eradicate ZON contamination, thereby alleviating hazards to human and animal health.

16.3 CONCLUSION

We have reviewed different formulations of enzyme-based decontaminants and their mechanisms of action against mycotoxins from the literature in the present chapter. In summary, enzymes can be redesigned, through the use of molecular biology and protein engineering tools, which are more effective against nonnatural substrates, that have broader specificity to a class of compounds, or they may have narrow specificity to a single chemical in a class, to the other extreme. In addition a final product of enzymes is able to inactivate a variety of classes of compounds or a single identified material. Further, enzymes are safe, easy to use, environmentally benign, and effective in low-volume doses. Moreover, compared to traditional chemical approaches, their manufacturing, shipping, storage and shelf-life offer additional advantages. Advancements in enzyme technology like enzyme immobilization and synthesis of enzyme nanoparticles can aid in developing the robust decontamination, detoxification, and protective agents against the xenobiotic pollutants. The economic production of potent enzymatic decontaminants will be beneficial in the commercial sector.

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CHAPTER

17

Enzymatic decontamination of antimicrobials, phenols, heavy metals, pesticides, polycyclic aromatic hydrocarbons, dyes, and animal waste

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17.1 INTRODUCTION

Waste material was traditionally disposed of by accumulation in more or less defined areas. That is still the case in large underdeveloped regions of the world. In industrialized countries, this is no longer possible due to a lack of available land. The most frequently used technologies for waste disposal are high-temperature incineration and chemical decomposition. However, such techniques have several inconveniences. Instead, strong efforts are currently focused on innovative, environmentally friendly alternatives such as bioremediation. Bioremediation can be defined as the enzymatic transformation and/or degradation of hazardous compounds by bacteria, fungi, algae, and plants, or by extracellular enzymes. Bioremediation depends on the enzymatic capacity to convert pollutants into innocuous products.

Laccases (Lacs) are blue multicopper oxidases, which catalyze the monoelectronic oxidation of a broad spectrum of substrates, e.g., ortho- and para-diphenols, polyphenols, aminophenols, and aromatic or aliphatic amines, coupled with a full, four-electron reduction of O_2 to H_2O. Lacs decolorize and detoxify the industrial effluents and help in waste-water treatment. They act on both phenolic and non-phenolic lignin-related compounds as well as highly recalcitrant environmental pollutants, and they can be effectively used in paper and pulp industries, textile industries, xenobiotic degradation, and bioremediation, and act as biosensors.

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The Lacs can oxidase highly recalcitrant compounds such as environmental pollutants, pesticides, and antimicrobials, and could therefore be used in the detoxification of industrial effluents, mostly from the paper and pulp, textile, and petrochemical industries. Moreover, their potential extension to the nanobiotechnology field is receiving increased interest [1].

A rapid industrialization and extensive use of pesticides in agriculture have led to an increased contamination of soil, water, and air which is a serious environmental problem of today. Polychlorinated biphenyls (PCBs), benzene, toluene, ethyl benzene, xylene, polycyclic aromatic hydrocarbons (PAHs), pentachlorophenol (PCP), 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (DDT), and trinitrotoluene are the substances which are known for their carcinogenic as well as mutagenic effects and are persistent in the environment. Fungi degrade a wide variety of hazardous chemicals, and thus they have attracted particular interest from researchers [2]. The fungus *Trametes versicolor* is used for the bioremediation of atrazine in soil with low moisture and organic contents that are normally found in semiarid and Mediterranean-like ecosystems [3]. Udayasoorian and Prabu [4] obtained lacs from *T. versicolor* and *Pleurotus ostreatus* for the degradation of PCBs as well as phenol, and found that increased chlorination decreased degradation rate. The same authors concluded that 3-hydroxy biphenyl was more resistant to Lac degradation than 2- or 4-hydroxy analogs [4]. After 5 days of incubation, when glucose and fructose were used as a cosubstrate, 71% of *p*-hydroxy benzoic acid and 56% of protocatechuic acid were degraded [5].

Lacs have a wide range of substrate specificity which opens possibilities to find applications within the pulp industry and for waste-water remediation. Lacs have also been used in combined pretreatment of biomass hydrolyzates to remove enzymatic and fermentation inhibitors. *T. versicolor* crude culture filtrates (CCFs) remove phenolic inhibitors by 76% and 94% from the dilute acid hydrolysates of rice straw and poplar, respectively, and xylo-oligosaccharide concentrations by 64%. *T. versicolor* CCFs can be proposed as a low-cost technology for decreasing enzymatic and fermentation inhibitors [6]. The bioremediation process of industrial waste can be made more efficient using ligninolytic Lacs. They catalyze the monoelectronic oxidation of a substrate in a process that requires expenditure of molecular oxygen. These enzymes contribute to the cross-linking of monomers involved in the degradation of a wide range industrial pollutants. In recent years, Lacs have gained applications in pulp and paper, textile, and food industries [1,7,8].

Removal of excess polyphenolic compounds which has low biodegradability constitutes a challenge in waste-water treatment [9]. Large amounts of polyphenol are present in the beer factory waste-water which is dark brown in color and can be degraded by the white-rot fungus *Coriolopsis gallica* [10]. Lac from *Trametes* spp. bioremediates the distillery waste-water generated from the sugarcane molasses fermentation with a high content of organic matter [11] as well as olive mill waste-water. Since Lacs catalyze the electron transfer reactions

without additional cofactors, they can also be used as biosensors to detect various phenolic compounds, oxygen, and azide. Recently, Lac has been used as a biocatalyst for the synthesis of organic substance as well as in the design of a biofuel cell. Low-cost Lac production is becoming an important factor determining increased use for the treatment of many types of chemical wastes as well as pollutants.

The phytoremediation of soils contaminated with organic pollutants offers a low-cost method for the removal of such pollutants. To enhance the environmental decontamination functions of plants, the Lac of *Coriolus versicolor* was introduced into tobacco plants [12]. The positive results should stimulate further efforts to develop plant-based technologies for the removal of environmental pollutants from contaminated environments.

Aquatic hyphomycete *Clavariopsis aquatica* degrade water contaminants with endocrine activity like xenoestrogen nonylphenol (t-NP). Lac preparations derived from *C. aquatica* cultures also converted t-NP leading to the formation of products with higher molecular masses than that of the parent compound. The role of fungi occurring in aquatic ecosystems in the degradation of such contaminants is emphasized. Two different mechanisms employed by fungi isolated from aquatic environments to initiate t-NP degradation are suggested: (1) hydroxylation of individual t-NP isomers at their branched nonyl chains and further breakdown of the alkyl chains of certain isomers, and (2) attack of t-NP by extracellular Lac, the latter leading to oxidative coupling of primary radical products to compounds with higher molecular masses [13].

Bioremediation of waste-water represents an important treatment methodology, especially when examined against the backdrop of ever-stricter legislation that is evolving in order to regulate effluent release into the environment. Crucial questions surrounding the treatment of effluents include: efficiency of the process, economic feasibility, legal requirements, and the mechanisms involved in the remediation process. The last requires special attention [14].

Obviously, one important factor determining efficiency is temperature. It has been found that degradation of polysaccharides, proteins, lipids, and DNA at 4°C, 16°C, and 28°C by 174 microbial cultures originating from the deep water of the South Pacific Gyre show clear temperature dependence. Thus, the amylase, chitinase, cellulase, lipase, lecithinase, caseinase, gelatinase, and DNase found in these sample cultures were mostly (85.6%) cold-adapted enzymes at 4°C [15]. Such enzymes are produced from isolates distributed primarily in *Alteromonas* and *Pseudoalteromonas* genera of the *Gammaproteobacteria*. *Pseudoalteromonas* degraded nine types of macromolecules but not cellulose. *Alteromonas* secreted eight enzymes except for cellulase and chitinase. Interestingly, the enzymatic activities of *Gammaproteobacteria* isolates at 4°C were higher than those observed at 16°C or 28°C [15]. These cold-active enzymes may have huge potential for industrial applications, including bioremediation in cold environments.

17.2 ANTIMICROBIALS

Antimicrobials have been extensively and effectively used in human- and veterinary-medicine. They are often excreted unchanged as active compounds. Several actors play an important role in the urgent need for reducing the release of antimicrobials into the environment. The main source of antibiotic contamination is waste-water from antibiotic manufacturers, large-scale animal feeding operations, households, and hospitals. How to eliminate the antibiotic residues in various wastes is critical to minimizing potential environmental contamination and the ecological health risk caused by antibiotic pollution.

Antibiotics can challenge untargeted microbial populations and they must therefore be considered as important pollutants. Residues from human environments and from farms may contain antibiotics and spread antibiotic resistance genes that can contaminate the environment. The clearest consequence of antibiotic release in natural environments is the selection of resistant bacteria [16].

About 100,000 tons of antibiotics are used annually worldwide. More strains of pathogens have become antibiotic resistant, and some have become resistant to many antibiotics and chemotherapeutic agents, i.e., multidrug resistant. Common multidrug-resistant organisms are usually bacteria. Two examples are Vancomycin-Resistant *Enterococci* and Methicillin-Resistant *Staphylococcus aureus* (MRSA). MRSA is resistant to several antibiotics as well as to disinfectants [17]. MRSA organisms become a main threat for patients in several hospitals.

Due to their antimicrobial and persistence features, antibiotics generally cannot be efficiently removed by traditional methods applied in waste-water treatment plants, which include both physiochemical methods, e.g., coagulation, flocculation, sedimentation, and filtering, and biological processes, e.g., activated sludge and anaerobic digestion. In contrast, newly developed physicochemical techniques including oxidation and advanced oxidation processes (AOPs), such as chlorination, ozonation, fenton and photo-fenton reactions, photolysis, and photocatalysis, can overcome the disadvantages of the biological methods. The use of these techniques has shown success in the remediation of antibiotics contamination in waste-water.

The new analytical protocols for antibiotic residue and their metabolites determination and quantification are great tools for surveillance of antibiotic contamination at spatial and temporal levels. Elucidating degradation pathways and identification of intermediate products that might be used as biomarkers for environmental monitoring is highly warranted.

In addition to detecting antibiotic residue in the environment, the identification and quantification of antibiotic resistance genes are also important areas that need to be developed. Because of the availability of omic- and metaomics technologies and the low cost of next-generation sequencing techniques, it is possible to investigate the abundance and diversity of bacteria resisting antibiotics and antibiotic resistance genes in those bacteria, and to compare the distribution of such bacteria

and genes at local, regional, and global scales. In addition modern bioinformatics tools might allow a better understanding of the transfer and evolution of antibiotic resistance genes in the environment.

The removal of β-lactams, sulfonamides, fluoroquinolones, tetracyclines, macrolides, and trimethoprim by biodegradation and adsorption are the major ways bacteria use to develop resistance to antibiotics. Kumar and Cabana [18] showed that complete removal of pharmaceuticals such as acetaminophen, diclofenac, mefenamic acid, atenolol and epoxy carbamazepine and partial removal of fenofibrate, diazepam, trimethoprim, and ketoprofen by Lac was achieved within 12 hours of incubation, whereas efficient removal of indometacin required the presence of mediator. In addition, Cerrena Lac cross-linked enzyme aggregates (M-CLEAs) were most effective in degradation of tetracycline and oxytetracycline, followed by ampicillin, sulfamethoxazole, and erythromycin.

Immobilized Lac (from *T. versicolor*) in combination with the mediator syringaldehyde (SYR) were tested in removing a mixture of 38 antibiotics. Lac alone did not reduce the load of antibiotics significantly, but with the addition of SYR as much as 32 were degraded significantly. The addition of SYR resulted in a time-dependent increase in toxicity. If the toxicity issue can be resolved, enzymatic treatment may be a valuable addition to existing water-treatment technologies [19].

Sulfadimethoxine (SDM) is commonly used in animal feeding and is released into the environment. Released antimicrobials affect terrestrial and aquatic organisms. Incubation with Lac, horseradish peroxidase (HRP), and lignin peroxidase from sandy soil led to significant reduction in the fraction of extractable SDM, indicating the formation of bound residues. These finding can help to the development of remediation methods to mitigate the release of sulfonamides from soil to water [20].

Antimicrobials have been widely spread in the environment a fact that creates threats both to the ecological system and to human health. Intrinsic reaction kinetics of the Lac-catalyzed removal of two emerging antimicrobials, chlorophene (CP) and dichlorophen (DCP), show that their removal follows second-order reaction kinetics, first-order with respect to both the enzyme and the substrate concentration. It was also found that fulvic acid could suppress the transformation of CP and DCP by reversing the oxidation reactions through its action as a scavenger of the free radical intermediates produced from reactions between Lac and the substrates. Several toxicity evaluation tests using *Scenedesmus obliquus* confirmed that the toxicity of CP and DCP is effectively eliminated during the reaction processes [21].

Fluoroquinolone-based antimicrobials in natural waters represent a significant emerging environmental problem. A novel alkaline bacterial Lac, SilA, from *Streptomyces ipomoeae* is able to degrade the antimicrobials, Ciprofloxacin (Cip) and Norfloxacin (Nor) under alkaline conditions in the presence of natural mediators by more than 90% of both fluoroquinolones. The SilA-acetosyringone system is able to reduce the toxicity of Cip and Nor by 90% and 70%, respectively [22].

When soil adsorption is introduced into the Lac-oxidation system to assist the simultaneous removal of 14 sorts of sulfonamide, tetracycline, and quinolone antibiotics differing in structures and chemical properties, the complementary effects facilitate the simultaneous removal. At pH 6 and 25°C, the removal rates of each antibiotic exceed 70% in 15 minutes and are close to 100% in 180 minutes, demonstrating the potential for practical utilization [23].

Sulfonamides are removed mainly by Lac-stimulated oxidation and quinolones mainly by soil adsorption. Tetracyclines are removed by both treatments in the coupled system, but Lac oxidation dominates.

17.3 OTHER DRUGS

Seven genes were cloned from a *Yersinia enterocolitica* strain and inserted in a pET28 vector for subsequent expression in *Escherichia coli* BL21 (DE3). The preparation showed Lac activity when oxidized with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and guaiacol [24]. The protein has an optimum pH of 9 and is stable at 70°C. The Lac biotransforms diclofenac and aspirin [24].

Lactobacillus plantarum J16 (CECT 8944) produces a Lac showing degrading activities of biogenic amines (Bas). The new Lac cloned and overexpressed in *E. coli* has properties of blue multicopper oxidases and typical activity toward Lac substrates ABTS and 2,6-dimethoxyphenol. Recombinant *L. plantarum* Lac oxidizes mainly tyramine and has the potential to eliminate toxic compounds present in fermented food and beverages [25].

The endophytic fungus *Phomopsis liquidambari* can effectively degrade the xenobiotic sinapic acid (SA), one of the most representative methoxy phenolic pollutants in industrial waste-water. SA is first transformed to 2,6-dimethoxy-4-vinylphenol, further degraded via 4-hydroxy-3,5-dimethoxybenzaldehyde, syringic acid, gallic acid, and citric acid involving catalysis by phenolic acid decarboxylase, Lac, and gallic acid dioxygenase [26].

The white-rot fungi *T. versicolor* and *Ganoderma lucidum*, individually or combined, totally remove diclofenac, gemfibrozil, ibuprofen, progesterone, and ranitidine. Lower removals are achieved for 4-acetamidoantipyrin, clofibric acid, atenolol, caffeine, carbamazepine, hydrochlorothiazide, sulfamethoxazole, and sulpiride. It appears that both extracellular and intracellular oxidation mechanisms contribute to the removal of pharmaceuticals [27].

17.4 PHENOLS

Oxidation of the classical Lac substrate ABTS by the laccase of *Tetracystis aeria* is widespread in chlorophycean algae. For example, species of the *Moewusinia*,

including *Chlamydomonas moewusii* and *T. aeria*, excrete putative "true" Lacs. Phenolic substrates are oxidized by these enzymes optimally at neutral to alkaline pH. The *Tetracystis laccase* efficiently transforms other compounds such as bisphenol A, 17α-ethinylestradiol, nonylphenol and triclosan in the presence of ABTS as redox mediator, while anthracene, veratrylalcohol, and adlerol remain unchanged. Possible, natural functions of the enzymes, such as the synthesis of complex polymers or detoxification processes, may assist the survival of the algae in adverse environments. In contaminated surface waters, Lac-producing green algae might contribute to the environmental breakdown of phenolic pollutants [28].

Putative Lac (CotA) from *Bacillus pumilus* MK001 cloned and expressed in *E. coli was* found to be thermostable exhibiting a half-life of 60 minutes at 80°C and show potential binding affinities with ferulic acid, caffeic acid, and vanillin [29].

Phenols are known inhibitors for cellulase and fermentative microorganisms in biorefining processes. The addition of Lac removes the phenolic compounds and subsequently reduces the lag phase of the fermentative microorganism. However, the application of Lac diminishes glucose release during the enzymatic hydrolysis. The proportion of lignin and the composition of phenols are key players in the inhibition of cellulase when the enzymatic hydrolysis is combined with Lac detoxification [30].

One interesting application in environmental biotechnology is the immobilization of Lac to eliminate phenolic contaminants via oxidation. Fumed silica nanoparticles have an interesting potential as support material for Lac immobilization via sorption-assisted immobilization in the perspective of applications such as the elimination of micro-pollutants in aqueous phases. Immobilizing Lac from a *Leviathan* genus, *Coriolopsis polygonal*, *Cerrena unicolor*, *P. ostreatus*, and *T. versicolor* onto fumed silica nanoparticles, separately or in combination, produces an increased activity over a pH range between 3 and 7. The different Lacs differ in their pH optima and substrate affinity. Exploiting their differences allowed the formulation of a tailor-made nanobiocatalyst able to oxidize a broader substrate range than the dissolved or separately immobilized enzymes. The nanobiocatalyst has the potential for biochemical oxidation in the elimination of multiple target pollutants [31]. It has been confirmed that nonvocational nanobiocatalysts obtained by Lac immobilization onto silica nanoparticles possessed a broad substrate spectrum regarding the degradation of recalcitrant pollutants, such as phenolic EDCs (bisphenol A) [32].This underlines the potential of fumed silica nanoparticles/laccase composites for advanced biological waste-water treatment.

Lacs are able to catalyze one-electron oxidation of phenolic compounds into radical intermediates that can subsequently couple to each other via covalent bonds. These reactions are believed to play an important role in the humification process and the transformation of contaminants containing phenolic functionalities in the environment. A reaction model was developed by integration of metal-HA binding equilibriums and kinetic equations, predicting the transformation rate of triclosan in the presence of HA and divalent metal ions including Ca^{2+}, Mg^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+}, Ba^{2+}, and Zn^{2+} [33].

Hydroxylated polybrominated diphenyl ethers (OH-PBDEs) have been frequently found in the marine biosphere as emerging organic contaminants. The production of OH-PBDEs is likely a result of the coupling of bromophenoxy radicals, generated from the Lac-catalyzed oxidation of 2,4-DBP or 2,4,6-TBP. The transformation of bromophenols by Lac is pH-dependent, and is also influenced by enzymatic activity. In view of the abundance of 2,4-DBP and 2,4,6-TBP and the phylogenetic distribution of Lac in the environment, Lac-catalyzed conversion of bromophenols may be potentially an important route for the natural biosynthesis of OH-PBDEs [34].

Phanerochaete chrysosporium belongs to a group of lignin-degrading fungi that secrete various oxidoreductive enzymes, including lignin peroxidase (LiP) and manganese peroxidase (MnP). However, the production of Lacs in this fungus has not been completely demonstrated and remains controversial. The coexpression of the *LacIIIb* gene from *T. versicolor* and the *vpl2* gene from *Pleurotus eryngii*, and also the endogenous genes *mnp1* and *lipH8* improved the cooverexpression of peroxidases and laccases up to fivefold as compared with wild-type species. Transformant strains have a broad spectrum in phenolic/nonphenolic biotransformation and a high percentage in synthetic dye decolorization in comparison with the parental strain and are an easy and efficient coexpression of Lacs and peroxidases in suitable basidiomycete species [35].

Recently, Lac has been applied to nanobiotechnology, which is an increasing research field, and catalyzes electron transfer reactions without additional cofactors [36].

Carbon nanoparticles are promising candidates for enzyme immobilization. Compared to free Lac, the immobilized enzymes have significantly reduced reaction rates. Diffusional limitation induced by the aggregation of carbon nanoparticles cannot be ignored because it can lead to increased reaction times, low efficiency, and high economic costs. Furthermore, this problem is exacerbated when low concentrations of environmental contaminants are present [37].

Bisphenol A (BPA) is an endocrine-disrupting chemical that is ubiquitous in the environment because of its broad industrial use. Extracellular Lac of the most widely cultivated mushroom in the world (i.e., white-rot fungus, *P. ostreatus*) efficiently degraded BPA. The exposure to BPA has no harmful effects on this edible fungus [38].

The removal of BPA by Lac in a continuous enzymatic membrane reactor assessed in synthetic and real biologically treated waste-waters in a reactor configuration based on a stirred tank reactor coupled to a ceramic membrane, showed almost complete removal of BPA. Polymerization and degradation are probable mechanisms of BPA transformation by Lac [39].

Organisms from the phylum *Basidiomycota* have enormous bioremediation potential by their phenol oxidases in the degradation of phenolics. Lac and tyrosinase are mainly in *T. versicolor* and *Agaricus bisporus*, respectively. New promising wild-type producers of enzymes have emerged and a number of recombinant strains were also constructed, based mainly on yeasts or *Aspergillus* strains

as hosts. The constructs allow applications for the degradation of phenols, polyphenols, cresols, alkylphenols, naphthols, bisphenols, and halogenated (bis)phenols. Biological and physicochemical methods could be combined to make the processes fit for industrial use [40].

Plant peroxidases have strong potential utility for decontamination of phenol-polluted waste-water. Large-scale use of these enzymes for phenol depollution requires cheap, abundant, and easily accessible peroxidase-containing material. Potato pulp, a waste product of the starch industry, contains large amounts of active peroxidases, and potato pulp enzymes maintain their activity at pH 4–8 and are stable over a wide temperature range. Phenol removal efficiency of potato pulp is over 95% [41].

Immobilized Lac on nanoporous silica beads degrade more than 90% of 2,4-dinitrophenol in a short time (12 hours). The immobilization process improves the activity and sustainability of Lac for degradation of the pollutant. Temperatures above 50°C reduce the enzyme activity to about 60%. However, pH and the mediator concentration could not affect the enzyme activity. The degradation kinetic is in accordance with a Michaelis–Menten equation [42].

Aqueous exudates of ryegrass (*Lolium perenne*) can degrade BPA both in the absence and presence of natural organic matter (NOM). In exudates with the addition of NOM, the degradation process is longer than without NOM. Peroxidase and Lac activities in exudates suggested a significant involvement of these enzymes in BPA degradation [43].

Organofluorine compounds have become important building blocks for a broad range of advanced materials, polymers, agrochemicals, and pharmaceuticals. The concept for the introduction of the trifluoromethyl group into unprotected phenols by employing a biocatalyst (Lac), tBuOOH, and either the Langlois' reagent or Baran's zinc sulfinate was achieved. The method relies on the recombination of two radical species, namely the phenol radical cation generated directly by the Lac and the CF3-radical. The Lac-catalyzed trifluoromethylation proceeds under mild conditions and degrades trifluoromethyl-substituted phenols that were unavailable by classical methods [44].

Lac-producing freshwater ascomycete *Phoma* sp. strain UHH 5-1-03 has potential for practical micropollutant removal. Bisphenol A (BPA), carbamazepine (CBZ), 17α-ethinylestradiol (EE2), diclofenac (DF), sulfamethoxazole (SMX), technical nonylphenol (t-NP), and triclosan (TCS) are substrates with the rank order: EE2 ≫ BPA > TCS > t-NP > DF > SMX > CBZ. The obtained metabolites indicate hydroxylation, cyclization, and decarboxylation reactions, as well as oxidative coupling typical for Lac reactions. The observations strongly suggest that the extracellular Lac of *Phoma* sp. largely contributes to fungal biotransformation [45].

Increased attention has been given to nanobiocatalysis. Lac reversibly immobilized onto Cu(II)- and Mn(II)-chelated magnetic microspheres was successful in removing BPA from water. In comparison with free Lac, the thermal and storage stabilities of immobilized Lac are significantly improved. More than 85% of BPA was removed under optimum conditions [46].

However, using enzymes in solution for water treatment has limitations of nonreusability, short enzyme lifetimes, and high cost of single use. Chen et al. [47] developed a new type of biocatalyst by immobilizing fungal Lac on the surface of yeast cells using synthetic biology techniques. The biocatalyst is referred to as surface display Lac (SDL) and can be re-used with high stability as it retained 74% of initial activity after eight repeated batch reactions. SDL effectiveness and proof of concept in treating contaminants of emerging concern were demonstrated with bisphenol A and sulfamethoxazole.

17.5 HEAVY METALS

Sources of heavy metals in the environment include geogenic, industrial, agricultural, pharmaceutical, domestic effluents, and atmospheric sources. Most essential heavy metals exert biochemical and physiological functions in plants and animals. They are constituents of several key enzymes playing important roles in various oxidation−reduction reactions. Copper for example serves as an essential cofactor for several oxidative stress-related enzymes including catalase, superoxide dismutase, peroxidase, cytochrome C oxidases, ferroxidases, monoamine oxidase, and dopamine β-monooxygenase. Heavy metals also affect cellular components such as cell membrane, mitochondrial, lysosome, endoplasmic reticulum, nuclei, and some enzymes involved in metabolism, detoxification, and damage repair.

However, environments contaminated by heavy metals and trace elements need to be cleared. Several techniques are available to remove such pollution: chemical precipitation, oxidation or reduction, filtration, ion-exchange, reverse osmosis, membrane technology, evaporation, and electrochemical treatment. But most of these techniques are expensive and often ineffective [48].

Biological methods like biosorption and/or bioaccumulation for removal of heavy metals may be an attractive alternative to physicochemical methods, and the use of microorganisms and plants for remediation purposes is thus a possible solution. However, accumulation of heavy metals in the soil causes considerable modification of the microbial community, despite their vital importance for the growth of microorganisms at relatively low concentrations.

Because microorganisms have developed various strategies for their survival in heavy-metal-polluted habitats, these organisms are known to develop and adopt different detoxifying mechanisms such as biosorption, bioaccumulation, biotransformation, and biomineralization, which can be exploited for bioremediation. Microorganisms' uptake of heavy metals can be active (bioaccumulation) and/or passive (adsorption). The microbial cell walls offer many functional groups that can bind heavy metal ions, and these include carboxylate, hydroxyl, amino, and phosphate groups. Fungi of the genera *Penicillium*, *Aspergillus*, and *Rhizopus* are some of the potential microbial agents for the removal of heavy metals from aqueous solutions together with endophytic bacteria from the copper-tolerant species of *Elsholtzia apliendens* and *Commelina communis*.

The long-term effects of biochar on PTEs (potential toxic elements) immobilization depends on biochar's own property and its aging process in soil. These results suggest that it is important to select the right kind of biochar, particularly the lignin content, to stably decrease the bioavailability of PTEs (Cd and Cu) in contaminated soils [49].

Genomics is a new tool for bioremediation which makes it possible to analyze the microorganism on the basis of not only biochemical parameters, but also at the transcriptome level. This provides an overview of the genes related to the sensitivity of microbes toward toxic metals in the soil.

However, bioremediation technology has some limitations. Several microorganisms cannot break toxic metals into harmless metabolites, and these have inhibitory effects on microbial activity. Engineering of outer membrane proteins of potential interesting bacteria improving metal binding abilities is one way to enhance their capacity for biotransformation of toxic metals. Public concerns regarding genetically engineered microorganisms should be taken into account, and proof of nontoxicity to the environment is mandatory before practical use.

17.6 PESTICIDES

There are hundreds of pesticides used worldwide. Some of them, like chlordecone, have been withdrawn in most countries whilst others, like dichlorodiphenyltrichloroethane (DDT), are banned in some regions of the world and are under strong pressure for banishment in almost all others. DDT is, for instance, still used in tropical regions in the fight against malaria. The World Health Organization established a new classification of pesticides in 2009 [50]. The classification has the following five classes of pesticides: extremely hazardous (class Ia—hexachlorobenzene, calcium cyanide, mercuric chloride, chlordecone, parathion, lindane, etc.), highly hazardous (class Ib—acrolein, azinphos-ethyl, coumaphos, dichlorvos, edifenphos, etc.), moderately hazardous (class II—acifluorfen, azamethiphos, bilanafos, chlordane copper sulfate, etc.), slightly hazardous (class III—benazolin, biphenyl, chloridazon, malathion, metoxuron, etc.), and active ingredients unlikely to present acute hazard in normal use (bromacil, dimethylphthalate, flucarbazone-sodium, mepronil, norflurazon, etc.).

Microorganisms, have been used to transform pesticides. It is known that part of the soil biota can quickly develop the ability to degrade certain pesticides, when they are continuously applied to the soil. In general, microorganisms that have been identified as able to degrade pesticide have been isolated from a wide variety of sites contaminated with some pesticide. Microbial processes that eliminate organic environmental contamination are important. Progress in the biotechnology of biodegradation relies on the underlying sciences of environmental microbiology and analytical geochemistry. General biodegradation and aromatic-hydrocarbon biodegradation are based on the characterization of microorganisms

from pure-culture isolates, laboratory enrichment cultures, and in contaminated field sites. *Pseudomonas* is the most efficient bacterial genus for the degradation of toxic compounds. The most frequent efficacious fungus species are *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus terreus*, *Absidia* and *Rhizopus microsporus* var *corymberifera microsporis*. For example, *Botrytis cinerea* can eliminate the linuron and metroburon herbicides almost completely, while another 31 isolated species can also eliminate metroburon. *Trichoderma viride* has the ability to degrade endosulfan and methyl parathion.

The oxidizing capacity of Lacs on highly recalcitrant environmental pollutants, like pesticides, makes them very interesting agents of environmental protection and remediation. In surface waters, phototransformation can substantially contribute to degradation of pesticides. In "direct" phototransformation, photons are absorbed by the contaminant, while in "indirect" phototransformation, reactive species are formed through photon absorption by other substances. Even though their effects are lowered, transformation products may remain problematic. Some transformations, such as oxidation, leave active moiety intact. The issue is specifically addressed in major regulatory frameworks. In Europe, for instance, "non-relevant" metabolites are distinguished from metabolites that are "relevant for groundwater resources" or even "ecotoxicologically relevant."

Optimized Lac from *T. versicolor* has a high capacity for degrading pesticides such as chlorpyrifos, chlorothalonil, pyrimethanil, atrazine, and isoproturon. The optimized enzyme is stable at a pH range from 5 to 7 and temperature from 25°C to 30°C. The best mediators are violuric acid for pyrimethanil and isoproturon, vanillin for chlorpyrifos, and acetosyringone and HBT for chlorothalonil and atrazine, respectively. Pesticides were degraded by up to 90%−100% [51].

MnP completely degrades the widely used pesticide glyphosate whilst Lacs only do it in the presence of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), $MnSO_4$ and Tween 80. Lignin peroxidase and HRP have no degradation capacity on glyphosate. MnP, together with $MnSO_4$ and Tween 80, degrade glyphosate in its commercial formulation Roundup Bio. The same enzyme mixture can degrade 22 other pesticides, with degradation percentages ranging between 20% and 100%. These results highlight the potential of ligninolytic enzymes to degrade pesticides [52].

Despite their agricultural benefits, pesticides are often considered as a serious threat to the environment because of their persistent nature and the anomalies they create. The use of biological resources to degrade or remove pesticides from the environment has emerged as a powerful tool for their in situ degradation and remediation. Fungi are among such bioresources that have been widely characterized and applied for biodegradation and bioremediation of pesticides. Fungal isolates appear as an effective tool to degrade different pesticides including lindane, methamidophos, endosulfan, chlorpyrifos, atrazine, cypermethrin, dieldrin, methyl parathion, heptachlor, etc.

Fungal strains exhibit biochemical processes including hydroxylation, demethylation, dechlorination, dioxygenation, esterification, dehydrochlorination,

oxidation, etc., during biodegradation. Biodegradation of pesticides is mediated by enzymes including Lac, hydrolase, peroxidase, esterase, dehydrogenase, MnP, lignin peroxidase, etc. [53]. Lindane-degrading capabilities of *G. lucidum* GL-2 strain make it a potential candidate for managing lindane bioremediation at contaminated sites [54].

Microbial degradation plays important roles in the removal of various pesticides in soil. Several carbofuran- and chlorpyrifos-degrading bacteria have been isolated from natural environments, and their hydrolysis strongly reduces toxicity providing a useful enzymatic detoxification method. So far, several recombinant strains have been constructed for degradation of different classes of pesticides. Among them, a recombinant strain capable of simultaneously degrading methyl parathion and carbofuran was constructed by random insertion of a methyl parathion hydrolase (*mph*) gene into the chromosome of a carbofuran degrading *Sphingomonas* sp.

Lac in the presence of the mediator 2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) is effective in the degradation of the persistent herbicide dymron. They are able to degrade over 90% of dymron within 24 hours, while the half-life of dymron is 50 days in soil, suggesting that oxidation of dymron results into compounds with a single aromatic ring. Degradation occurs at acidic pH and at a broad range of temperatures. The Lac-mediator system can be suggested for the treatment of soils and rainwater contaminated with herbicides [55].

In the environment, *Actinobacteria* play important ecological roles including recycling of substances, degradation of complex polymers, and production of bioactive molecules. Their biotechnological potential was demonstrated by their ability to remove organic and inorganic pollutants. Strategies such as bioaugmentation, biostimulation, cell immobilization, production of biosurfactants, design of defined mixed cultures, and the use of plant−microbe systems were developed to enhance the capabilities of *Actinobacteria* in bioremediation [56].

Organochlorines are persistent pesticides that are still present in the environment despite not being used any longer. These chemicals pose a threat to human health, particularly during periods of increased sensitivity, such as gestation, young mental development, and in the induction of dysplasia. Chlordecone is an organochlorine pesticide that had been principally used to control the banana root borer in Central and South America and in the Caribbean. Some soils in current and previous banana fields and some waterways are permanently polluted. Chlordecone is an endocrine disruptor with estrogen-like and progestin-like characteristics both in vitro and in vivo, as well as a potential carcinogen that has been shown to cause hepatic tumors in laboratory rats and mice. The carcinogenic and hormonal properties of chlordecone and its long biologic half-life raise concerns regarding the long-term effects, such as cancer. Long-term chlordecone exposure in adulthood favors the development of prostate cancer, with a particularly high incidence of this disease observed in the French West Indies [57]. Lacs are effectively agents for the degradation of a large set of pesticides. So far, there

is no information on the sensitivity of the chlordecone to hydrolysis by these enzymes so that specific investigation of its degradation by such enzymes is highly recommended.

The white-rot fungus *Irpex lacteus* produces ligninolytic enzymes: Lac, lignin peroxidase, and MnP. It is an efficient degrader of PAHs such as phenanthrene, anthracene, fluoranthene, and pyrene, PCBs, and PCP. The role of MnP in PAHs degradation by *I. lacteus* includes cleavage of the aromatic ring [58].

Silica nanostructures, on account of their sorbent properties, porous nature, and increased surface area, allow effective extraction of pesticides. They can be modified (with ionic liquids, silanes or amines), coated with molecularly imprinted polymers, or magnetized to improve the extraction. Degradative enzymes (organophosphate hydrolases, carboxyesterases, and Lacs) and bacterial cells that produce recombinant enzymes have been immobilized on silica nanostructures for mediating pesticide degradation. After immobilization, these systems show increased stability and improved degradation capacity [59].

Contaminants from agricultural herbicides have very slow degradation rates in the aquatic environment. Despite the more rapid degradation observed for most herbicides in the presence of light and sediments, the half-lives remained at more than 100 days for the photosystem II herbicides. The degradation of herbicides is 2- to 10-fold more rapid in the presence of a diurnal light cycle and coastal sediments. The effects of light and sediments on herbicide persistence were likely due to their influence on microbial community composition and its ability to utilize the herbicides as a carbon source [60].

It has been shown that the amount of lindane degraded by a subtropical white-rot fungus DSPM95 was directly proportional to the initial lindane concentration in the medium [61]. Lac and MnP were produced in both stationary batch and in immobilized cultures. The highest percent degradation of the pesticide by DSPM95 was 81%.

The yeast *Candida* sp. VITJzN04 isolated from sugarcane fields is able to utilize lindane as the sole carbon source for growth, and furthermore efficiently degraded 600 mg/L of lindane within 6 days in mineral medium under the optimal conditions (pH 7; temperature 30°C, and inoculum dosage 0.06 g/L). Enzymes such as dechlorinase, dehalogenase, dichlorohydroquinone reductive dechlorinase, lignin peroxidase, and MnP appeared to be involved in the degradation process of lindane by *Candida* sp. VITJzN04 [62].

Metabolite formation from radical-based oxidation of triclosan (TCS) was compared using an ascomycete (*Phoma* sp. UHH 5-1-03) and a basidiomycete (*T. versicolor*) laccase, laccase-redox mediator systems, and electrochemical oxidation (EC). Lac oxidation predominantly yielded TCS di- and trimers, but notably also caused TCS ether bond cleavage. The latter was more prominent during EC-catalyzed TCS oxidation, which generally resulted in a broader and more divergent product spectrum. By contrast, only quantitative but not qualitative differences in TCS metabolite formation were observed for the two Lacs.

These observations establish 2,6-dimethoxy-1,4-benzoquinone as a potential cause of toxicity effects of the presumable natural laccase redox mediator syringaldehyde laccase systems [63].

Manganese peroxidase, MnP-Tra-48424, from *Trametes* spp. decolorizes dyes (indigo, anthraquinone, azo and triphenylmethane) and degrades different PAHs (fluorene, fluoranthene, pyrene, phenanthrene, anthracene) and also PAHs in mixtures. The combination of MnP-Tra-48424 and Lac-Tra-48424 improves the decolorization rate [64].

Peniophora incarnata KUC8836, a white-rot fungus, produces lignolytic enzymes managing the hazardous effects of PAHs. Tween 80 is a viable cosubstrate for *P. incarnata*, as it enhances the ability of *P. incarnata* to manage effective biodegradation of PAHs. The genes encoding for laccase and manganese-dependent peroxidase are involved in the production of lignolytic enzymes from *P. incarnata* KUC8836 [65].

By a semirational engineering approach, an efficient and thermostable organophosphate hydrolase has been designed, starting from a lactonase scaffold (SsoPox from *Sulfolobus solfataricus*). The triple mutant C258L/I261F/W263A retains its inherent stability and has an enhanced hydrolytic activity on paraoxon up to 300-fold. The mutant is efficacious in cleaning pesticide-contaminated surfaces, using formulations of different solvents (methanol or ethanol) or detergents (SDS or a commercial soap) [66].

As mentioned, a strain of *Candida* sp. VITJzN04 is able to utilize lindane as sole carbon source for growth. The strain efficiently degrades lindane in mineral medium under the optimal conditions (pH 7; temperature 30°C). Addition of H_2O_2 in the medium enhance lindane degradation by 32%. The enzymes dechlorinase, dehalogenase, dichlorohydroquinone reductive dechlorinase, Lac, lignin peroxidase, and MnP are present during lindane degradation [67].

The thermotolerant white-rot fungal strain RYNF13 of *Trametes polyzona* completely degrade phenanthrene as well as 90% of fluorene and 52% of pyrene, and is superior to most white-rot fungi. RYNF13 secrete different ligninolytic enzymes (MnP, Lac and lignin peroxidase), during PAH biodegradation at 30°C. At temperatures from 30°C to 37°C and 42°C, only MnP and Lac are detectable. RYNF13 is a potential fungus for PAH bioremediation, especially in a tropical environment where the temperature can be higher than 40°C [68].

Numerous approaches, including chemical, physical, and biological decontamination, have been considered for developing decontamination methods against organophosphates (OPs). Efforts have been dedicated during the past decades to the development of efficient OP degrading biocatalysts. Among these, the promising biocatalyst SsoPox isolated from the archaeon *S. solfataricus* is emphasized in the light of recently positive effects and the hyperthermostable enzyme appears to be particularly attractive for external decontamination purposes with regard to both its catalytic and stability properties [69].

Recombinant CotA Lac from *Bacillus subtilis* oxidizes PAHs with high Lac activity and copper independence, indicating that CotA is a better candidate for

the remediation of PAHs than Lac CueO from *E. coli* [70]. HRP appears more advantageous than *T. versicolor* lac in removing triclosan from an aqueous matrix considering the reaction parameters pH, temperature, catalytic efficiency and enzyme concentration [71].

Trametes maxima—Paecilomyces carneus monoculture and coculture extracts are able to degrade atrazine at 100% in a clay-loam soil microcosm contaminated at field application rate (5 μg/g).The best degradation rate is achieved by the *T. maxima* monoculture extract in an acid soil (pH 4.86) [72].

17.7 DYES

Environmental pollution is an increasing problem due to the indiscriminate and frequently deliberate release of hazardous, harmful substances.

Research efforts have been directed at developing new, low-cost, low-technology, ecofriendly treatments capable of reducing and even eliminating pollution in the atmosphere, the hydrosphere, and soil environments.

Effluents of several industries contain important quantities of synthetic organic dyes. The discharge of these colored compounds into the environment causes considerable nonaesthetic pollution and serious health-risks. Applications of typical methods such as electrocoagulation, electrochemical reduction, EC, and indirect electrooxidation with active chlorine species are reported. Among biological agents, enzymes, both intracellular and extracellular, have a great potentiality to effectively transform and detoxify polluting substances and are being explored as biochemical means of waste-water treatment. They can selectively degrade a target pollutant without affecting the other components in the effluent. Therefore, enzymatic treatment is suitable for effluents that contain relatively large amounts of the recalcitrant target pollutants in comparison to others. They can also operate under mild reaction conditions, especially temperature and pH. In this respect, enzymes outperform the regular catalysts (transition elements like Cu, Ni, etc.). From the environmental perspective, enzymes are more acceptable due to their biodegradability. Considering that colorants such as azo dyes can be degraded physicochemically by oxidation (i.e., AOPs), a majority of the enzymes that are being investigated for their dye degradation potential belong to the enzyme class oxidoreductases. These enzymes are involved in electron transfer reactions. Hence, at the end of the reaction the enzyme is regenerated and is available for the next catalytic cycle. Some of the oxidative enzymes such as the peroxidases require hydrogen peroxide (H_2O_2) or alkyl peroxide (R_2O_2) to act as the electron acceptor. Others, such as Lacs, utilize molecular oxygen for this purpose. Peroxidases such as HRP (E.C. 1.11.1.7), MnP (E.C. 1.11.1.13), and lignin peroxidase (E.C. 1.11.1.14) are ferric-ion-containing heme proteins and require peroxides such as H_2O_2 to function. Lignin peroxidase and MnP are obtained from fungi. There are various plant sources of peroxidases, such as horseradish,

soyabean, radish, beetroot, and peanut. Most of these peroxidases have been tested to determine their potential to treat synthetic and actual waste-waters. Lacs are mainly obtained from lignin-degrading fungi such as *T. versicolor* and *Trametes villosa*, as well as fungi like *Fusarium solani* and *Cladosporium cladosporioides*. The azo groups in the azo dyes are converted to amines by reductive cleavage, a reaction catalyzed by azo reductase. Azo dyes undergo reductive splitting relatively easily under anaerobic conditions. The anaerobic reduction of certain azo dyes, however, yields aromatic amines that are potentially carcinogenic. Peroxidases and Lacs show a wide substrate range, especially with regard to phenols and amines. This suggests that these oxidative enzymes may not have specific substrate binding sites. The ability of these enzymes to act on different pollutants is affected by the molecular structure of the substrate, the temperature and pH of the treatment, as well as the presence of intermediates. Lacs may require mediators like 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) to act as an intermediate substrate for the enzyme.

Azo dyes account for >70% of the global industrial demand (~9 million tons). Owing to their genotoxic/carcinogenic potential, the annual disposal of ~4,500,000 tons of dyes and/or degraded products is an environmental and socioeconomic concern. Phytoremediation has emerged as a green, passive, solar-energy-driven, and cost effective approach for environmental clean-up when compared to physicochemical and even other biological methods. Plant-based treatment of dyes is relatively new and hitherto has remained an almost unexplored area of research. Use of macrophytes such as *Phragmites australis* and *Rheum rhabarbarum* have shown efficient removal of Acid Orange 7 and sulfonated anthraquinones, respectively. Plants namely *Aster amellus*, *Portulaca grandiflora*, *Zinnia angustifolia*, *Petunia grandiflora*, *Glandularia pulchella*, and many ferns and aquatic plants have also been advocated for their dye-degradation potential [73].

Also, plants' oxidoreductases such as lignin peroxidase, Lac, tyrosinase, azo reductase, veratryl alcohol oxidase, riboflavin reductase, and dichlorophenolindophenol reductase are known as key biodegrading enzymes which break the complex structures of dyes.

Conventional waste-water treatment plants cannot degrade the majority of these pollutants and thus powerful methods for the decontamination of dyes waste-waters should be developed. In comparison to physicochemical methods, microbe-mediated dye degradation is considered to be low-input, cost-effective, and environmentally safe. The majority of studies on microbial dye-degradation focus on decolorization with least attention toward detoxification. In the absence of ecosystem-based studies, the results of laboratory-based studies on dye degradation, metabolite production and their genotoxic impact on model organisms are used to predict the possible fate and consequences of azo dyes/metabolites in the environment. These studies are fundamental for better knowledge on dye-microbe-environment interactions and their applications to combat dye-induced environmental toxicity, and will provide directions for undertaking environmentally sound microbial dye detoxification.

Both oxidative and reductive pathways are involved in the metabolism of azo dyes [74]. The majority of azo dyes undergo reduction catalyzed by enzymes of the intestinal microorganisms and/or hepatic enzymes including microsomal and soluble enzymes. The oxygen sensitivity of reduction discriminates because liver is aerobic, whereas the microorganisms of the lower bowel exist in an anaerobic environment. Many of the azo dyes in common use today have highly charged substitutes such as sulfonate. These resist enzymatic attack and for the most part are poorly absorbed from the intestinal tract, providing poor access to the liver, the major site of the mixed-function oxidase system. Lipophilic dyes which are often carcinogenic, readily access oxidative enzymes and are activated by both mixed-function oxidase and conjugating systems. Reduction of the carcinogenic dyes usually leads to loss of carcinogenic activity. By contrast, most of the highly charged water-soluble dyes become mutagenic only after reduction. Even then, most of the fully reduced amines require oxidative metabolic activation. An outstanding example is the potent human bladder carcinogen benzidine, which derives from the reduction of several azo dyes. Many problems regarding mutagenic and carcinogenic activation remain to be solved. It seems that both oxidative and reductive pathways yield toxic products. This is critical in the treatment of waste from chemical plants where there is a great need for soil bacteria that catalyze reduction aerobically. Secondary pathways and metabolites are also of great concern. For example, azoreduction of carcinogenic dyes removes carcinogenic activity although oxidative metabolism of the primary amines generates mutagenic products. Such dilemmas must be dealt with when considering metabolism/toxicity relationships for azo dyes.

White-rot fungi produce various isoforms of extracellular oxidases including Lac, Mn peroxidase and lignin peroxidase, which are involved in the degradation of lignin as well as in that of various xenobiotic compounds and particularly dyes [75].

Salvinia molesta, an aquatic fern has the potential to degrade azo dye Rubine GFL up to 97%. *S. molesta* has lignin peroxidase, veratryl alcohol oxidase, Lac, tyrosinase, catalase, DCIP reductase, and superoxide dismutase activities. After treatment, toxicity analysis on seeds of *Triticum aestivum* and *Phaseolus mungo* reveal the decreased toxicity of dye metabolites. Anatomical tracing of dye in the stem of *S. molesta* confirms the presence of dye in tissues and subsequent removal after 48 hours of treatment [76].

Chemical industries are moving from traditional chemical-based concepts to a catalytic alternatives. Biocatalysis has numerous benefits within biotechnological and environmental applications. These processes are more energy efficient than conventional methodologies under moderate processing, generating no or negligible secondary waste pollution. Solid-phase biocatalysts can be economically tailored on a large scale. Immobilization of lignin-modifying enzymes including lignin peroxidase, MnP, and Lac of white-rot fungi are nowadays potentially interesting tools for efficient biocatalysis [77].

Waste-water from textile and leather industries, particularly in developing countries requires treatment in an environmentally safe manner. Many studies

have shown that a variety of cultured bacteria have efficient enzymatic systems that can carry out complete mineralization of dye chemicals and their metabolites (aromatic compounds) over a wide range of environmental conditions. Complete mineralization of azo dyes generally involves a two-step process requiring initial anaerobic treatment for decolorization, followed by an oxidative process that results in degradation of the toxic intermediates that are formed during the first step. Molecular studies have revealed that the first reductive process can be carried out by two classes of enzymes involving flavin-dependent and flavin-free azoreductases under anaerobic or low oxygen conditions and the second step is carried out by oxidative enzymes that are broad-specificity peroxidases, such as Lacs and tyrosinases [78].

The potential of the enzymes—peroxidases, MnPs, lignin peroxidases, Lacs, microperoxidase-11, polyphenol oxidases, and azoreductases—has been exploited in the decolorization and degradation of dyes. Some of the recalcitrant dyes are not degraded/decolorized in the presence of such enzymes. The addition of certain redox mediators such as 1-hydroxybenzotriazole, veratryl alcohol, violuric acid, and 2-methoxy-phenothiazone enhance the range of substrates and efficiency of degradation. Soluble enzymes cannot be exploited on a large scale due to limitations such as stability and reusability. Treatment of waste-water on a large scale is also possible using reactors containing immobilized enzymes [79].

The *Enterococcus faecalis* YZ 66 strain has the ability to decolorize various industrial dyes; in some of which, it showed complete decolorization and degradation of toxic, sulfonated recalcitrant diazo dye Direct Red 81 at pH 7 and at 40°C. Metabolites produced by degradation are nontoxic [80].

Some plant enzymes constitute potential tools for detoxification and decolorization of textile dye. In vitro grown *Petunia grandiflora* and *Gaillardia grandiflora* plantlets show 76% of dye giving 94% of decolorization of simulated dye mixtures. Root cells have induced activities of veratryl alcohol oxidase, laccase, tyrosinase, riboflavin reductase, and lignin peroxidase.

Metabolites formed after the degradation of dyes reveal the reduced cytogenotoxicity on *Allium cepa* roots cells when compared with untreated dye mixture solution and phytotoxicity [81].

A gene coding for a multicopper oxidase (BopA) was identified through the screening of a metagenomic library constructed from waste-water treatment activated sludge. The recombinant BopA produced in *E. coli* exhibit oxidation activity toward 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) in the presence of copper, suggesting that BopA is actually a Lac.

The *bopA* gene is of bacterial origin, possibly within the phylum *Deinococcus-Thermus*. Purified BopA is active over a pH range from 6 to 11. At pH 7.5 it has maximum activity with bilirubin as its substrate. Its activity remains intact even after a heat treatment at 90°C for 60 minutes. BopA is a thermostable-bilirubin oxidase with much higher thermostability than that previously reported for Lacs [82].

E. faecalis YZ 66 strain is able to decolorize several industrial dyes. It completely decolorizes and degrades toxic, sulfonated recalcitrant diazo dye, Direct Red 81. The optimum pH and temperature for decolorization is 7.0 and 40°C, respectively. The degradation of Direct Red 81 produces nontoxic metabolites [80].

A lignin-degrading bacterial strain decolorizing Azure-B dye identified as lignin peroxidase (LiP) producing strain LD-5 isolated from pulp and paper mill effluent is suggested to be *Serratia liquefaciens*. The strain LD-5 reduce pollution parameters (color 72%, lignin 58%, chemical oxygen demand (COD) 85%, and phenol 95%) of real effluent together with toxicity reduction of treated effluent by 49.4%. *S. liquefaciens* has important potential for bioremediation of pulp and paper mill effluent [83].

Decolorization studies using industrial dye, Remazol Brilliant Blue R (CI 61200), on solid and liquid medium and *Trichoderma aureoviridae* show that the final visual decolorization was up to 76%. Bamboo degradation evaluations revealed a decrease in lignin content of about 50% [84].

17.8 KRAFT AND LIGNIN

Pulp and paper industrial effluent is rich in recalcitrant compounds and causes pollution. For the treatment of such compounds, an activated sludge process is frequently used. A bacterial mixture of *Klebsiella* sp., *Alcaligenes* sp., and *Cronobacter* sp. efficiently remove the pollutants from the waste-water of pulp and paper mills. The reduction of chemical and biochemical oxygen demand (BOD) is up to 72.3% and 91.1%, respectively. A significant reduction in color (55%), adsorbable organic halides (AOX; 45.4%), total dissolved solids (22%), and total suspended solids (SS; 86.7%) was also noticed, allowing discharge into the environment without any risks [85]. Pulp and paper mills are some of the main water- and energy-intensive industries responsible for large-scale water pollution. The problems associated with pulp and paper mill effluents are pH, color, high levels of BOD, COD, SS, AOX, etc. Paper manufacturing releases chlorinated lignosulfonic acids, chlorinated resin acids, chlorinated phenols (trichlorophenol, trichloroguicol, tetrachloroguicol, dichlorophenol, dichloroguicol, and pentachlorophenol), and chlorinated hydrocarbon into the effluent waste-water. These are the major contaminants formed in the effluent of pulp and paper mills. Some compounds in the effluents are resistant to biodegradation and can accumulate in the aquatic food chain.

The traditional water-treatment technologies that are used in paper mills (such as dissolved air flotation or biological treatment) are not able to remove recalcitrant contaminants. Therefore, advanced water treatment technologies, such as AOPs, are being implemented in industrial waste-water treatment chains, aiming to either improve water biodegradability or its final quality. In general, all AOPs

have been reported to achieve good organic removal efficiencies that are boosted if AOPs are combined with biological stages [86].

Pulp and paper mills are facing two major challenges: to increase energy efficiency and reduce the output of pollutants. Adopting integrated methods, together with a combination of biological (e.g., anaerobic digestion) and physicochemical (e.g., novel Fenton reactions) treatment methods, can be environmentally and economically preferable for minimizing environmental contaminants and increasing energy recycling [87].

Considerable interest has been focused on the use of biotechnology in pulp bleaching, as large numbers of microbes and the enzymes produced by them are known to be capable of preferential degradation of native lignin and complete degradation of wood. Certain white-rot fungi can delignify Kraft pulps increasing their brightness and their responsiveness to brightening with chemicals [88].

Waste-water containing kraft lignin (KL) discharged from pulp and paper industries cause serious environmental contamination. Some anaerobic bacteria capable of degrading KL are beneficial to both lignin removal and biofuel regeneration from the effluent. *Dysgonomonas* sp. WJDL-Y1 isolated from the sludge of a pulp and paper mill degrade KL and the optimal conditions are pH 6.8 and 33°C [89].

P. chrysosporium, *Corius versicolor*, and *T. versicolor*, among a few other white-rot fungi, are suitable for efficient degradation of recalcitrant chromophoric material in pulp and paper mill effluents.

Lignin from tobacco stalk and nicotine can be degraded effectively by *T. versicolor*, *Trametes hirsuta*, and *P. chrysosporium.* The main and highest ligninolytic enzymes produced by these strains are lignin peroxidase, MnP and Lac. The different oxidative enzymes and chemical products detected during the degradation indicated a possible pathway for the utilization of tobacco stalk [90].

17.9 ANIMAL WASTE MANAGEMENT

Waste from cattle, pigs, and poultry intensive husbandry is a valuable source of nutrients and renewable energy. However, most of the waste is still collected in lagoons or left to decompose in the open, which pose a significant environmental hazard. The air pollutants emitted from manure include methane, nitrous oxide, ammonia, hydrogen sulfide, volatile organic compounds, and particulate matter, which can cause serious environmental concerns and health problems [91].

In the past, livestock waste was recovered and sold as a fertilizer or simply spread onto agricultural land. The introduction of tighter environmental controls on odor and water pollution oblige some form of waste treatment. The biogas potential of animal manure can be harnessed both at small- and community-scale. There are thousands of digesters operating at commercial livestock facilities in Europe, United States, and Asia.

In the degradation and utilization of solid waste from anaerobic digestion of poultry litter by *Agrocybe aegerita*, lac, peroxidase, CMCase and xylanase activities were high [92].

The evaluation of enzyme activities and chemical changes in recalcitrant phenolic-rich waste (olive cake) showed that, after treatment with *P. ostreatus* or *Eisenia fetida*, 90% of phenols were removed. Lac and MnP activities appeared after treatment with *P. ostreatus*. The toxicity of the wet olive cake against the seeds of *Lepidium sativum* significantly decreased after degradation [93].

Panus tigrinus strains (M609RQY and M109RQY) with their lignolytic enzymes have degradation potential for abundant agro-industrial wastes under solid-state fermentation conditions [94].

Odor pollution is a big environmental problem caused by large-scale livestock production in several regions, and there is a pressing need to develop practical techniques to reduce these odors. A combination of lignin peroxidase and a peroxide strongly reduce the release of eight chemicals (propionic acid, isobutyric acid, isocaproic acid, isovaleric acid, phenol, *p*-cresol, indole, and skatole), NH_3 and H_2S as well as odor intensity from pig manure [95].

Lignin presents an abundant component of lignocellulosic biomass, which is currently largely unutilized. It has the potential to replace fossil-based non-sustainable resources as a renewable feedstock for production of valuable chemicals.

The ability of Lacs to attack and degrade lignin in conjunction with Lac mediators is currently viewed as one of the potential "breakthrough" applications for lignin valorization [96].

Compared to fungal Lacs, bacterial Lacs are generally more stable at different pH and temperatures. It should be underlined that, to date, fewer than 10 bacterial Lacs have been completely purified and characterized as opposed to more than 100 fungal Lacs [97,98].

The relatively recent demonstration of the huge capacities of Lacs to reduce several types of organic and nonorganic pollutants strongly suggests deep evaluation on the one hand of their activity toward such pollutants and on the other hand of the reduction of lignocellulosic content of farmed animals' manure.

17.10 CONCLUSIONS AND PERSPECTIVES

Classical waste disposal is based on incineration or chemical decomposition. These techniques cannot be applied to polluted soils and waters. Thus, there is a need for more easily applicable tools. Degradation by microorganisms, algae, and plants or enzymes derived from these organisms represents alternatives in the transformation of pollutants into harmless compounds.

Recently several Lacs and other oxidases have been isolated mainly from white-rot fungi showing activity at large pH values and a huge thermotolerance.

Obtaining such enzymes at an affordable cost is a major challenge, but given the promising results presented in the present review such efforts should be encouraged. Preservation of a sound global environment requires new approaches in order to prevent pollution.

Enzymatic treatment of animal waste with lignolytic enzymes appears to be a potential interesting option to be explored.

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CHAPTER

Chitinases

18

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18.1 INTRODUCTION

Chitin is a nitrogen-containing polysaccharide consisting of β-1,4-linked *N*-acetyl-D-glucosamine which is chemically analogous to cellulose, except that one of the hydroxyl groups of each glucoside residue is replaced by an acetylated or deacetylated amino group. As a lignocellulose, chitin is relatively resistant to degradation and it is not digested by animals, except certain fish. Chitin is the second most abundant natural polymer and is widely distributed as a structural component of crustaceans, insects, and other arthropods, as well as a component of the cell walls of most fungi and some algae. Approximately 75% of the total weight of shellfish, such as shrimp, crabs, and krill are considered as waste, and chitin comprises 20%−58% of the dry weight of the said waste. About 10^{11} tons of chitin is alone produced annually in the aquatic biosphere (Figs. 18.1−18.4).

Chitinase (EC 3.2.11.14) enzyme is capable of hydrolyzing insoluble chitin to its oligo- and monomeric components found in a variety of organisms including viruses, bacteria, fungi, insects, higher plants and animals, and it plays an important physiological role depending on its origin. Chitinases are constituents of several bacterial species such as some of the best-known *Aeromonas*, *Serratia*, *Vibrio*, *Streptomyces*, and *Bacillus*.

Chitinases belongs to glycosyl hydrolase family 18, an ancient gene family that is widely expressed from prokaryotes to eukaryotes. In humans, despite the absence of endogenous chitin, a number of chitinases and chitinase-like proteins (C/CLPs) have been identified. Chitinases with enzymatic activity have a chitin binding domain containing six cysteine residues responsible for their binding to chitin.

Chitinases are classified as endochitinases or exochitinases. Endochitinases cleave chitin at internal sites to generate multimers of GlcNAc. Exochitinases catalyze the hydrolysis of chitin progressively to produce GlcNAc, chitobiose, or chitotriose. Chitinase has a wide-range of applications, some of which have already been realized by industry, such as preparation of pharmaceutically important chitooligosaccharides and *N*-acetyl-D-glucosamine, preparation of single-cell protein (SCP), isolation of protoplasts from fungi and yeast, control of pathogenic

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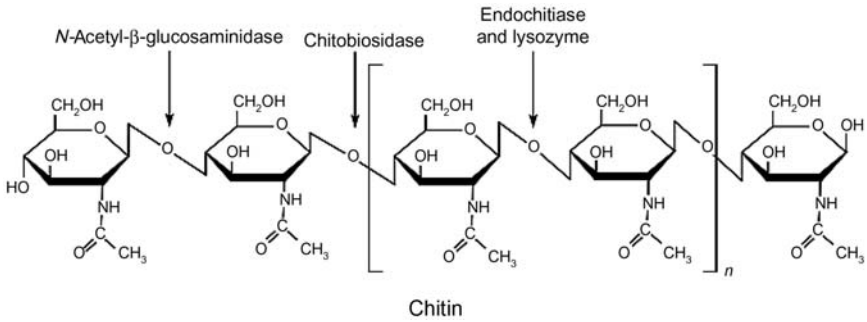

FIGURE 18.1

Schematic representation of chitin.

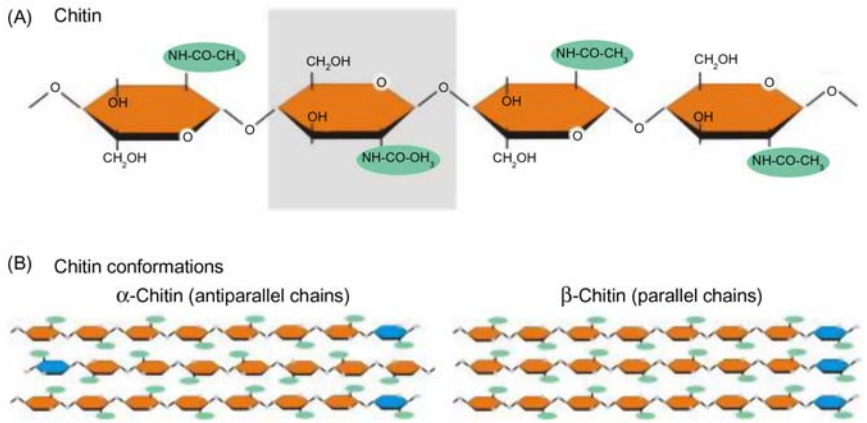

FIGURE 18.2

Schematic representation of chitin chains (A) Chitin, (B) Chitin conformations.

fungi, treatment of chitinous waste, and control of malaria transmission. Chito-oligomers produced by enzymatic hydrolysis of chitin are used in various fields such as in medical, agricultural, and industrial applications, such as antibacterial, antifungal, antihypertensive, and as a food quality enhancer. Chitinases can be exploited for their use in the control of fungal and insect pathogens of plants.

Possible future applications of chitinases are as food and feed additives, therapeutic agents for asthma and chronic rhinosinusitis fungal remedy, an antitumor drug, and as a general ingredient to be used in protein engineering.

In an effort to identify a microbial enzyme that can be useful as a fungicide and biodegradation agent of chitinous wastes, a chitinase (Chi242) was purified from the culture supernatant of *Streptomyces anulatus* CS242 utilizing powder of shrimp shell wastes as the sole carbon source. The chitinase was purified by

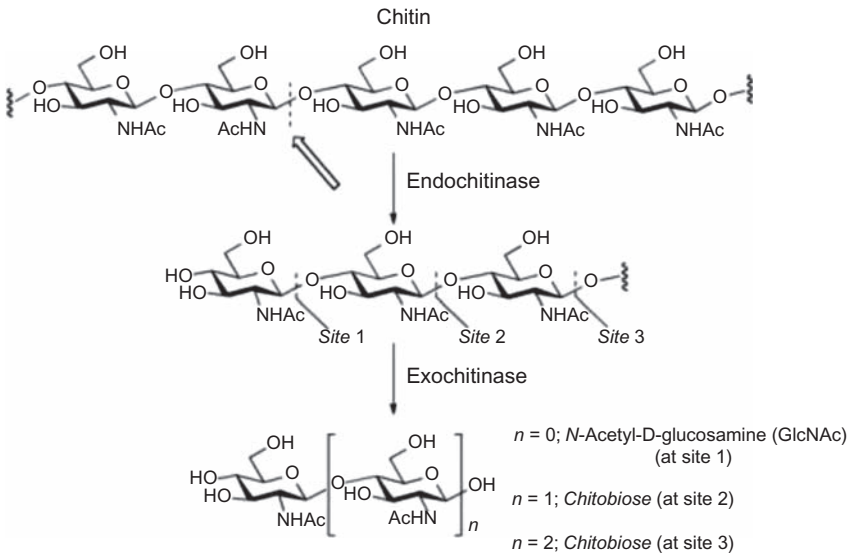

FIGURE 18.3

Schematic representation of endochitinase and exochitinase on chitin.

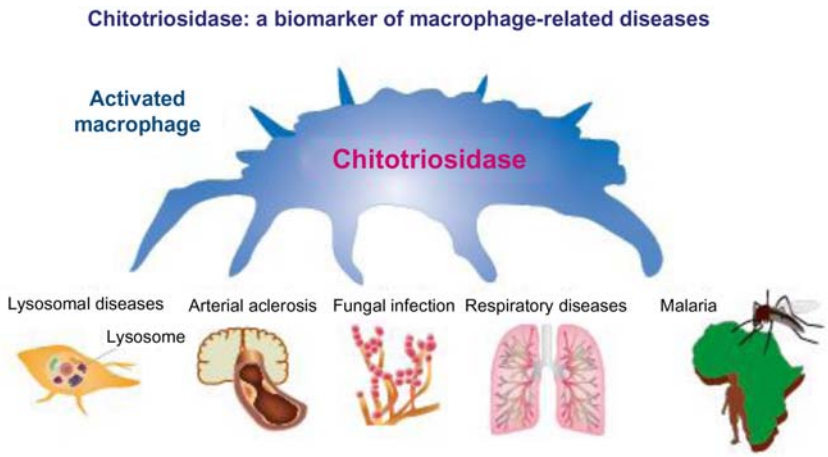

FIGURE 18.4

Chitotriosidase as a disease biomarker.

employing ammonium sulfate precipitation and gel permeation chromatography techniques. The molecular weight of the purified chitinase was ~38 kDa by SDS-PAGE. The N-terminal amino acid sequence (A-P-G-A-P-G-T-G-A-L) showed close similarity to those of other *Streptomyces chitinases*. The purified

enzyme displayed optimal activity at pH 6.0 and 50°C, respectively. It showed substantial thermal stability for 2 hours at 30−60°C, and exhibited broad pH stability in the range 5.0−13.0 for 48 hours at 4°C. Scanning electron microscopy confirmed the ability of this enzyme to adsorb on to solid shrimp biowaste and to degrade chitin microfibers. Chi242 could proficiently convert colloidal chitin to *N*-acetyl glucosamine (GlcNAc) and *N*-acetyl chitobiose (GlcNAc)2 signifying that this enzyme is suitable for bioconversion of chitin waste. In addition, it exerted an effective antifungal activity toward fungal pathogens, signifying its role as a biocontrol agent. Thus, a single microbial cell of *S. anulatus* CS242 justified its dual role.

Besides the huge potential of applications of chitinases, it should be underlined that such enzymes are involved in pathological reactions in the cases of allergy to the chitinase-protein know as latex−fruit syndrome. It appears that chitinase is the only enzyme harboring a dual character of positive and negative effects.

It is becoming increasingly clear that the function of chitinases is not exclusive to catalyzing the hydrolysis of chitin-producing pathogens, but also includes a crucial role in bacterial infections and inflammatory diseases [1].

18.2 APPLICATIONS OF CHITINASES

Chitinases have several field applications. Chitinases are attaining prominence in the field of biotechnology applied in waste management, pest control in agriculture, and human health care [2].

Heterologous expression and biochemical characterization of single-modular chitinase-D from *Serratia marcescens* (SmChiD) with unprecedented catalytic properties which include chitobiase and transglycosylation (TG) activities besides hydrolytic activity were performed by Vaikuntapu et al. [3]. Without accessory domains, SmChiD hydrolyzed insoluble polymeric chitin substrates like colloidal, α-, and β-chitin. The TG products were further hydrolyzed. SmChiD with its unique catalytic properties, could be a potential enzyme for the preparation of efficient enzyme cocktails for chitin degradation.

18.3 SELECTION AND PRODUCTION OF CHITINASES

Chitinases from *Paenibacillus laterosporus* (ChiL) and *Brevibacillus* (ChiB) are optimally active at pH 7.0 and 6.0, respectively, and show high-temperature stability up to 55°C. With their stability in a broad temperature range, ChiL and ChiB can be utilized for the industrial bioconversion of chitin wastes into biologically active products [4].

In *Thermococcus chitonophagus* a hyperthermophilic archaeon that can grow on chitin as the sole organic carbon source, a new chitinase, Tc-ChiD, has been identified. Tc-ChiD represents a new family of chitinases and the gene encoding Tc-ChiD contains regions corresponding to a signal sequence, two chitin-binding domains and a putative catalytic domain. The presence of the two chitin-binding domains significantly enhance the thermostability of the catalytic domain. The presence of multiple chitinases in *T. chitonophagus* with different modes of cleavage may contribute to its unique ability to efficiently degrade chitin [5]. da Silva et al. [6] overexpressed a *Lactococcus lactis* chitinase encoded by a new allele, ChiA1−2 in *Escherichia coli*.

Psychotolerant Antarctic fungus *Lecanicillium muscarium* CCFEE 5003 is a powerful producer of extracellular cold-tolerant enzymes, including chitin-hydrolyzing enzymes. The production of chitinolytic activities is induced by chitin and other polysaccharides and consists of a number of different cold-tolerance proteins. *L. muscarium* could have an important role for potential applications in the degradation of chitin-rich materials at low temperature and the biocontrol of pathogenic organisms in cold environments [7].

Aeromonas veronii TH0426 is a pathogen of the farmed yellow catfish *Pelteobagrus fulvidraco* but shows high-level expression of pullulanase and chitinase. Its genome sequence could help to reveal its pathogenic mechanism associated with yellow catfish, develop a vaccine to decrease economic losses for fish farming, and explore the potential application in producing pullulanase and chitinase [8].

Gangwar et al. [9] showed that *Streptomyces violascens* NRRL B2700 produce extracellular chitinase under submerged fermentation conditions. Maltose, xylose, fructose, lactose, soybean meal, and ammonium nitrate are good additional carbon and nitrogen sources to enhance chitinase yield.

Cohnella sp. A01 strain isolated from shrimp ponds has the highest chitinolytic activity among the other 12 strains of the same source. Their best incubation pH and temperature are 5.0 and 70°C, respectively. Growth of the bacterium is optimum on colloidal chitin, peptone, and K_2HPO_4 as best carbon, nitrogen, and phosphorus sources, respectively. Enzyme thermostability and morphological alteration of chitin treated by enzyme reveal cracks and pores on the chitin surface, indicating a potential application of this enzyme in several industries [10].

18.4 WASTE MANAGEMENT

Recombinant chitinases can be used to convert chitinous biomass into simpler useful depolymerized components, hence reducing water pollution. Chitooligomers obtained by the action of chitinases have a wide range of biotechnological applications in biochemical, food, and various chemical industries. Chitinase can also be used in the conversion of chitinous waste into biofertilizers.

Chitinous waste is effective in the production of SCP. In this approach chitinase degraded chitinous waste is used as the carbon or nutritional source for the production of biomass. Chitinase-producing bacteria and yeasts such as *S. !marcescens* and *Pichia kudriavzevii* can be used in aquacultures for SCP production.

A novel polysaccharide system in the soil Bacteroidete *Flavobacterium johnsoniae*, tailored for conversion of recalcitrant chitin, has been described [11]. The *F. johnsoniae* chitin utilization locus (ChiUL) consists of 11 contiguous genes encoding carbohydrate capture and transport proteins, enzymes, and a two-component sensor-regulator system. The key chitinase (ChiA) encoded has an extraordinary hydrolytic efficiency and derives from synergy between its multiple chitinolytic (endo- and exoacting) and previously unidentified chitin-binding domains. The analysis of the ChiUL will benefit future enzyme discovery and enzymatic intramolecular synergism comprehension.

The novel chitinase (PbChi67) from the marine bacterium *Paenicibacillus barengoltzii* CAU904 is most active at pH 3.5 and stable within pH 3.0−9.0. The optimal temperature of PbChi67 is 60°C and it is stable up to 55°C with a thermal denaturing half-life of 43 minutes at 65°C. The enzyme is an endo-type chitinase and has strict substrate specificity toward colloidal chitin and glycol chitin. Chitinase (PbChi67) properties make it a good candidate for recycling of chitin materials [12].

Cellvibrio japonicus is a Gram-negative soil bacterium known for its ability to degrade plant cell wall polysaccharides through carbohydrate-active enzymes (CAZymes). Several putative chitin-degrading enzymes are also found amongst these CAZymes, such as chitinases, chitobiases, and lytic polysaccharide monooxygenases (LPMOs). Characterization of the enzymes reveal tight and specific binding to chitin, thereby unraveling a new family of chitin-binding modules (CBM73). The full-length LPMOs shows no activity toward cellulose, but are able to bind and cleave both α- and β-chitin. Gene deletion show that LPMO10A is needed by *C. japonicus* to obtain efficient growth on purified chitin and crab shell [13].

Two recombinant chitinase, Tc-ChiD, proteins from the hyperthermophilic archaeon *T. chitonophagus* were produced in *E. coli*, one without the signal sequence, Tc-ChiD(ΔS), and the other corresponding only to the putative catalytic domain, Tc-ChiD(ΔBD). *T. chitonophagus* can grow on chitin as the sole organic carbon source. Tc-ChiD is an exo-type chitinase and the analysis with GlcNAc oligomers modified with *p*-nitrophenol suggest that Tc-ChiD recognizes the reducing end of chitin chains. As a thermostable chitinase Tc-ChiD may be useful to degrade and utilize chitin [14].

The algicidal mechanism of one chitinase-producing bacterium, *Thalassiosira pseudonana* (strain LY03), is based on chemotaxis to algal cells, fastening themselves on algal cells with their flagella, and then producing chitinase to degrade algal cell walls, and eventually cause algal lysis and death. The interaction between chitinase-producing bacteria and diatoms will be helpful in the protection and utilization of diatoms resources [15].

18.5 BIOCONTROL AGENTS

Chitinases are present in plants alongside various pathogenesis-related proteins as a part of plant defense mechanisms. Thus, overexpression of a combination of various chitinases in transgenic plants may aid against fungal pathogens. Chitinase can also be directly used as a biopesticide against various fungi and insects that can be an alternative to chemical pesticides. Other than being used directly as a biocontrol agent, chitinase can act as a target for biopesticide as chitin has a major role in insect metamorphosis as well as in gut of insects. A pseudo-trisaccharide allosamidin is an inhibitor of chitinase enzyme and it can be potentially used as biopesticide.

The resistance mechanism to *Verticillium dahliae*, a destructive and soil-borne fungal pathogen that causes massive losses in cotton yields is poorly understood. Evidence indicates that chitinases are crucial hydrolytic enzymes, which attack fungal pathogens by catalyzing the fungal cell wall degradation. Chitinase genes (Chis) from diploid *Gossypium raimondii*, *Gossypium arboreum*, and tetraploid *Gossypium hirsutum* and *Gossypium barbadense* can be phylogenetically classified into six groups and expression patterns of Chis indicate their various expressions in different organs and tissues, and in the *V. dahliae* response. Silencing some Chis genes in cotton significantly impairs the resistance to *V. dahliae*, suggesting these genes might act as positive regulators in disease resistance to *V. dahliae* [16].

After incubation in gelatin−chitin medium, *Paenibacillus ehimensis* produce several chitinase isozymes. The specific activity of the purified chitinase was 3.8 units/mg protein with a purification factor of 0.27. Inhibition rate of the conidial germination of *Colletotrichum gloeosporioides* was 87% in partial purified chitinase treatment compared with control [17].

Canola plants arboring co-expressed chit42 from *Trichoderma atroviride* with chitin-binding domain and polygalacturonase-inhibiting protein 2 (PG1P2) of *Phaseolus vulgaris* contaminated with *Slerotinia sclerotiorum* mycelia have disease resistance through delayed onset of the infection and restricted size and expansion of lesions as compared to wild type plants. They can be helpful in increasing the canola production [18].

Two chitinase genes from nonpathogenic bacterium *Listeria innocua* were expressed in *E. coli*. The genes were close homologs of chitinases from the pathogenic bacterium *Listeria monocytogenes*, in which chitinases and chitin-binding proteins play important roles in pathogenesis in mice-infection models. The purified recombinant enzymes exhibit optimum catalytic activity under neutral and acidic conditions at 50°C. Both enzymes produced primarily dimers from colloidal chitin as a substrate and they can hydrolyze oligomeric substrates in a processive exo- and nonprocessive endo-manner [19].

The *CHI2* gene from *Chitinolyticbacter meiyuanensis* SYBC-H1 encoding 383 amino acids has the same catalytic domain as the glycoside hydrolase family

19 chitinase. *CHI2* chitinase expressed in *E. coli* BL21 cells has optimal activity at 40°C and a pH of 6.5. Fe^{3+}, Fe^{2+} and Zn^{2+} inhibit CHI2 chitinase activity, while Na^{+} and K^{+} promote its activity. CHI2 chitinase is an *N*-acetyl glucosaminidase and its biochemical properties make it suitable for bioconversion of chitin waste [20].

Chitinases can be exploited for their use in control of fungal and insect pathogens of plants [21,22]. Chitinase activity also acts as an indicator showing the activity of fungi in soil [23].

A chitinase (*CHI42*) purified from the culture supernatant of *S. anulatus* displayed optimal activity at pH 6.0 and 50°C. Scanning electron microscopy confirmed the ability of this enzyme to adsorb onto solid shrimp biowaste and to degrade chitin microfibers, that it is suitable for bioconversion of chitin waste, and exerts an effective activity toward fungal pathogen signifying its role as a biocontrol agent [24].

The chitinase Chi9602ΔSP displayed on the cell surface of *Bacillus thuringiensis* BMB171 exhibits a high inhibitory effect toward the mycelial growth of two phytopathogenic fungi, *Fusarium oxysporum* FB012 and *Physalospora piricola* FB016. The cell-surface display system can enhance the stability of Chi9602ΔSP and the display system can be used to express biocontrol agents on the cell surface of *B. thuringiensis*, and provides an attractive method for inhibiting phytopathogenic fungi [25].

Entomopathogenic fungal chitinases and proteinases need to break down the insect's cuticle. Both enzyme classes support the infection process by weakening the chitin barrier followed by mechanically penetrating the weakened cuticle and reaching the insect's hemolymph, where it starts proliferating. Monitoring fungal enzyme production in dependence of several factors can facilitate in establishing optimal growth and harvesting conditions for a selection of isolates, with the aim of achieving maximum biocontrol activity [26].

Several *Pseudomonas* strains are interesting potential tools as new sources of producers of pathogen suppressors. *Pseudomonas fluorescens* group is particularly well-known for its plant-beneficial properties including pathogen suppression. Some strains of this group also cause lethal infections in insect larvae. Twenty-six *P. fluorescens* group strains, isolated from all around the world, exhibit lethal to avirulent activities toward lepidopteran larvae. Strains *Pseudomonas chlororaphis* and *Pseudomonas protegens* are highly insecticidal in animals and plants [27]. Analysis of selected genes reveal the importance of chitinase C and phospholipase C in insect pathogenicity. *Pseudomonas* strains with potent dual activity against plant pathogens and herbivorous insects have great potential for use in integrated pest management for crops. *P. fluorescens* (UMB20), *Pseudomonas aeruginosa* (KMB25), and *Pseudomonas asplenii* (BMB42) from rice plants have the ability to biocontrol fungi and promote plant growth. The fungal growth inhibition by the isolates ranged from 86.85% to 93.15% in volatile and 100% in diffusible metabolites tests [28].

P. protegens strain Pf-5 is a soil bacterium that has capacity to suppress plant diseases and has been shown to be lethal to certain insects. Rhizoxin, orfamide A, and chitinase production are required for full oral toxicity of Pf-5 against *Drosophila melanogaster*, with rhizoxin being the primary determinant [29].

Mycosphaerella fijiensis is the causal agent of the Black Sigatoka Disease (BSD) of banana. Chitinases from strain *Streptomyces galilaeus* CFFSUR-B12 have no inhibitory effects on *M. fijiensis* ascospore germination, but inhibit up to 35% and 62% of germ tube elongation and mycelial growth, respectively. Thus, *S. galilaeus* cultures are potential biocontrol agents of BSD [30].

A recombinant chitinase (RmChi44) from a gene of *Rhizomucor miehei* cloned and expressed in *E. coli* hydrolyzes colloidal chitin to yield mainly *N*-acetyl chitobiose. The activity of RmChi44 is of exo-type cleavage pattern and the enzyme displays β-*N*-acetylglucosaminidase activity. RmChi44 show antifungal activity against some phytopathogenic fungi [31].

A novel chitinase from the "Baozhu pear," a class III chitinase, hydrolyze chitin derivatives into di-*N*-acetylchitobiose (GlcNAc2) and GlcNAc2 into *N*-acetylglucosamine, which represents chitobiosidase and β-*N*-acetylglucosaminase activity. This chitinase exhibits strong antifungal activity toward agricultural pathogenic fungi and can be a potential fungicide in the biological control of plant diseases [32].

Chitinases find their use in other fields such as agriculture and mosquito control. Chitinases can also be exploited as additives in order to supplement frequently used insecticides and fungicides so that they can be more potent, and at the same time, the concentration of the chemically synthesized active agents in the ingredients can be minimized, which are otherwise harmful to health and environment [33]. Chitinases also have applications in the bioconversion of chitin waste to fertilizer [34].

18.6 MEDICAL APPLICATIONS AND BIOMARKERS

Chitinases have a significant function in human healthcare. An important medical use for chitinases has also been recommended in augmenting the activity of antifungal drugs in therapy for fungal diseases [35].

Consisting of the body's first line of defense against external agents, which also includes chitin-containing pathogens, various chitinase family proteins have constitutively shown their expression in macrophages, digestive tract, and in epithelial cells of lungs [36]. It has been reported that lungs show an increased expression of acidic mammalian chitinases (AMCases) in the development of Th2 inflammation in the human asthmatic airway and in allergic animal models as well. It has also been shown that AMCases have a very important role to play in the IL-13 effector pathway activation and in the pathogenesis of Th2 inflammation. It has been suggested from studies of cancer, arthritis, and liver fibrosis

that chitinase 3-like protein 1 (CHI3L1) also has an important role in tissue remodeling and inflammation [37].

AMCase is known to be induced by allergens and helminths, yet its role in immunity is unclear. AMCase-deficient mice have reduced numbers of group 2 innate lymphoid cells during allergen challenge, but this was not required for the establishment of type 2 inflammation in the lung in response to allergens or helminths. In contrast, AMCase-deficient mice show a profound defect in type 2 immunity following infection with the chitin-containing gastrointestinal nematodes, *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus bakeri*. Accordingly, AMCase functions as a critical initiator of protective type 2 responses to intestinal nematodes but is largely dispensable for allergic responses in the lung [38].

Recently, human proteins with chitinase activity and CLPs were identified and established as biomarkers for human diseases. Chitinases are becoming tools for diagnostics and prognostics for numerous diseases [39].

In humans, eight family 18 glycosyl hydrolases (GH18 chitinases) have been identified including active chitotriosidase and AMCase. Most of the human chitinases lack chitinolytic activity due to mutation of an essential glutamic acid residue at the catalytic domain, and they are termed "chitolectins." Human chitinases play a role in a variety of human diseases, such as asthma and Parkinson's disease [40].

Asthma comprises several related pathologies with similar clinical manifestations resulting from distinct underlying mechanisms. Novel biomarkers could lead to asthma stratification and thus improve upon the current stepwise approach.

Promising biomarkers are sputum eosinophils, serum periostin, and exhaled nitric oxide. Other biomarkers, mainly cytokines, leukotrienes, and chitinases, can be quantified in body fluids and could also be useful for asthma stratification [41].

Chitinase-1 over-producing cells, observed in Gaucher's disease, a genetic human lysosomal storage disorder in which glucocerebroside accumulates in certain organs, are not known to occur in other species. Chitinase 3-like-1, which is linked to human asthma, possesses several polymorphisms in humans [42].

Urinary chitinase 3-like protein 1 is a good biomarker for the prediction of acute kidney injury in medical intensive care units of adult patients [43].

CLPs retain structural similarity to chitinases but lack enzymatic activity. CLPs are upregulated in several human disorders that affect regenerative and inflammatory processes. The insect CLP (*Drosophila* imaginal disc growth factor 3, IDGF3) plays an immune-protective role during nematode infections. Nematodes force their entry into tissues creating wounds. IDGF3 has multiple roles in innate immunity serving as an essential component required for the clot of seal wounds, and Idgf3 mutants display an extended developmental delay during wound healing. Vertebrate and invertebrate CLP proteins function in analogous settings and have a broad impact on inflammatory reactions and infections [44]. The multifunctional role of CLPs in the regulation of inflammation and

intratumoral processes makes them attractive candidates for tumor therapy and immunomodulation [45].

A number of artificial medical articles such as contact lenses, artificial skin, and surgical stitches have been formed from chitin derivatives. These derivatives have an extensive medical use because quite a few of these chitin derivatives are known to be nontoxic, nonallergic, biocompatible, and biodegradable [46].

Chitinase is used as an antifungal agent in combination with antifungal drugs in therapy for various fungal infections. Human AMCase is found to increase in Th2 inflammation and is considered to play a role in asthma and allergic reactions. It is also involved in the effector pathway of IL-13. Chitinase is also being suggested to be used for the detection of invasive fungal infections in humans. Chitooligosaccharides also have an enormous pharmaceutical potential to be used in human medicines because of their antitumor activity (shown by chitohexaose and chitoheptaose), wound healing properties, and antihypertensive activity. *N*-Acetyl glucosamine, which is a monomeric unit of chitin polymer, is also reported to be an antiinflammatory agent.

Plasmodium falciparum is also reported to produce chitinase enzyme in the sporogonic cycle. Chitinase is produced by pathogen in the midgut of anopheline vector to disrupt the peritrophic membrane and let the parasite get to the salivary glands. Inhibition of chitinase can stop the sporogonic cycle, so it can be considered a good target. *P. falciparum* forms a membrane sac around the ingested blood meal, that is, chitin containing peritrophic matrix, and thus addition of exogenous chitinases to blood meal prevents the formation of peritrophic matrix.

Through deacetylation chitin can be converted to chitosan. Chitin and chitosan have the properties of biocompatibility, biodegradability, nontoxicity, ability to form films, etc. Chitin and chitosan have been largely used to produce polymer scaffolds. The use of chitosan to produce designed nanocarriers and to enable microencapsulation techniques is being thoroughly researched for the delivery of drugs, biologics, and vaccines [47].

Most probably, it will be possible to produce chitinases with novel health functions—novel therapeutic approaches for several diseases including asthma, and chronic rhinosinusitis, antitumor drugs since chitohexaose and chitoheptaose has shown an antitumor activity and enhancement of human the immune system.

This research can be directed toward the identification of the active sites of chitinases and the novel functions associated with them. We can exploit protein engineering for the production of chitinases with exclusive functions.

Chitin and chitosan have massive structural propensities to produce bioactive materials with innovative properties, functions, and diverse applications, particularly in the biomedical field. The specific physicochemical, mechanical, biological, and degradation properties offer an efficient way to blend these biopolymers with synthetic ones. The immense activities of chitin/chitosan are successfully utilized to enhance the bioactive properties of polyurethanes, particularly in the biomedical field [48].

18.7 OTHER APPLICATIONS

The exact function of human chitotriosidase (EC 3.2.1.14) or chitinase-1 (CHIT1) remains unclear, but its participation in immune defense is postulated. Activity of chitotriosidase may also be connected with inflammation. Diabetic angiopathies are the major cause of decreased quality of life and increased mortality of type 2 diabetes (T2D). Therefore a number of investigations on the role of CHITI in different pathological units, such as the causal factors that contribute to the development of vascular changes in diabetes are being carried out. It is not easy to evaluate whether increased CHIT1 activity is a cause or rather a consequence of T2D [49].

Chitinases have been exploited to isolate fungal protoplasts that are used as experimental means to study the synthesis of cell walls, synthesis and secretion of enzymes, and strain improvement for biotechnological applications.

The level of chitinases can also be used for the indirect determination of fungal biomass present in the soil. An enzyme of use in the food industry, tannase, is produced by *Aspergillus niger* but tannase binds to its cell wall reducing the yield. Chitinase is used for fungal cell wall degradation that releases tannase from cell wall and increases the yield.

Chitinase proteins are in the group of the pathogenesis-related proteins that are strongly induced when host plant cells are challenged by pathogen stress. Chitinases constitute an important arsenal of plants against fungal pathogens. Transgenic plants have been produced that overexpress chitinases alone or in conjunction with other defense-related proteins. The phenotypes of such plants show enhanced disease resistance in most of cases. Chitinases are also implicated in relationships between plant cells and fungi (e.g., mycorrhizae associations) and bacteria (e.g., legume/*Rhizobium* associations) as well as in plant abiotic stress responses as noted for osmotic, salt, cold, wounding, and heavy metal stresses. Chitinases play a role in developmental aspects of plants too (i.e., regulation of the plant embryogenesis process) [50].

18.8 ALLERGY TO CHITINASES AND LATEX—FRUIT SYNDROME

Most latex-allergic persons have cross-reactive antibodies to the antigens from some vegetable foods [1]. Increasing numbers of plant-derived foods are suspected to cross-react with latex antigens. Several fruits have cross-reactivity, known as "latex—fruit syndrome" [2,3]. The defense-related proteins of the rubber tree and of cross-reactive plants act as pan-allergens responsible for the large cross-reactivity.

There are three chitinase classes—I, IV, and V—with one or two chitin-binding domains more or less related to hevein and composing family 19 of

glycosyl hydrolases. A representative member of class V is the precursor of *Urtica dioica* agglutinin, with a C terminal domain similar to the catalytic domain of classes I and II chitinases and an N-terminal region with two chitin-binding domains. In natural rubber latex, besides hevein, there are two minor allergens with chitinase activities: hevamine, a bifunctional lysozyme/chitinase enzyme homologous to class III chitinases, and a class II enzyme [51,52].

The class I chitinase of numerous fruits (banana, avocado, potato, tomato, kiwi, chestnut, passion fruit, pear, grapefruit, mango, pineapple, papaya, peach, orange, strawberry, watermelon, coconut, apricot, cherry, nectarine, etc.) is a pathogenesis-related protein (PR-3), which is an important cross-reactive allergen for latex-sensitized people. Patatin has been identified as a major cross-reactive allergen in potato. Patatin is a defense-related protein with lipid acyl-hydrolase or esterase activity, and functions as an important storage protein in potato tubers. Latex allergens (Hev b 7) homologous to patatin have also been found in natural rubber. The establishment of latex–fruit syndrome is comparable with that of pollen–food allergy syndrome, where patients suffering from pollinosis also experience food allergies ranging from itching and pruritus around the oral cavity, known as oral allergy syndrome (OAS), to generalized urticaria and even anaphylaxis.

Only special food proteins stable to heat treatments and resistant to digestive enzymes can become food allergens. This is based on the fact that only such a special protein can reach the intestine without being fragmented and thus establish per-oral sensitization. This concept is called "class 1 food allergy" (or type I food allergy). In addition, food proteins responsible for such food allergies are now designed by "complete food allergens" or "class 1 food allergens."

Food allergy based on the cross-reactivity between the sensitizing antigen and the symptom-eliciting antigen is called "class 2 food allergy" or "type II food allergy."

Class 2 food allergy is usually confined to reactions around the oral cavity (namely OAS).

Although mammals lack endogenous chitin or chitin synthases, chitinases and CLPs are endogenously expressed in their lungs and other organs. In particular, CLPs have been described as dysregulated in a variety of diseases characterized by chronic inflammation and tissue remodeling, yet their potential role for humans has just recently begun to evolve. Chitin is a major component of a variety of allergy-triggering environmental components, including house-dust mites or fungal spores, and fungal asthma is increasingly appreciated as an underdiagnosed disease entity. Understanding of the complex immunological and pathophysiological implications of chitin–chitinase interactions in the human body is of high relevance for identifying new biomarkers and therapeutic targets for fungal diseases and other conditions, where chitin-coated microbial derivatives play a critical role.

IgE-mediated cannabis or marihuana (*Cannabis sativa*) allergy is on the rise. Exposure to cannabis allergens may trigger a *C. sativa* sensitization and/or

allergy. The entity, known as the "cannabis–fruit/vegetable syndrome" might also imply cross-reactivity with tobacco, natural latex, and plant-food-derived and alcoholic beverages. Therapy comprises absolute avoidance measures without knowing whether cross-allergies will be halted [51,52].

An elicited expression of AMCase may be involved in the pathogenesis of asthma. The specific suppression of elevated AMCase leads to reduced eosinophilia and Th2-mediated immune responses in a mouse model of allergic asthma. The exposure of chitin and the fundamental component of the major allergen mite and several of the inflammatory mediators, show significant enhancement on AMCase expression. These observations are the basis of developing a promising therapeutic strategy for asthma by silencing AMCase expression [53].

Chitotriosidase enzyme (EC 3.2.1.14) is the major active chitinase in the human body. It is produced mainly by activated macrophages, in which its expression is regulated by multiple intrinsic and extrinsic signals. Chitotriosidase is an essential element in the innate immunity against chitin-containing organisms such as fungi and protozoa. Its immunomodulatory effects extend far beyond innate immunity [54].

18.9 PERSPECTIVES FOR CHITINASES

To some extent, chitinases are surprising enzymes since they can show positive effects and deleterious ones as in the latex–fruit syndrome and related pathologies.

Nevertheless, they have the potential to become important tools in future "green" applications. New uses would include food preservation.

Chitinase, being the hydrolyzing enzyme for the second most abundant naturally occurring polymer, holds many applications in the field of environmental, therapeutic, and industrial biotechnology. Economical and practical utilization of chitinases can be achieved either by high yield or better catalytic activity. The main focus in chitinase research is on improving its catalytic activity. Protein engineering of the enzyme is providing a way to achieve this goal. Two major approaches for engineering protein are directed evolution and site-directed mutagenesis. In the directed evolution approach, chitinase can be randomly mutated by error-prone PCR and thereby generation of a mutant library. The chitinase gene library can then be screened by using fluorescent activated cell sorter and/or in 96-well plates. Mutants showing higher activity as compared to wild type can be selected and combination of these positive mutations can also be attempted to get the best catalytic activity.

So far evidence suggests that class I chitinases are the major panallergens associated with the latex–fruit syndrome. cDNA clones for some of these allergenic enzymes have been obtained and the corresponding recombinant proteins have been expressed in yeasts. These recombinant products may be useful for the diagnostic and potential immunotherapy of latex-related allergy.

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PART

VI

Enzymes and new or alternative food- and feedstuffs

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CHAPTER

Alternative and new sources of feedstuffs

19

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19.1 INTRODUCTION

In animal husbandry, feed is the greatest cost position [1], and in intensively farmed animals production cereals are the most widely fed energy sources in North America, Europe, and Australia.

Cereal grains such as triticale and sorghum, cereal grain coproducts, oilseeds, oilseed coproducts, and pulses are some of the present alternatives for corn, wheat, and soybean meal (SBM) although availability depends on local circumstances.

Plant-based alternative feedstuffs have a high proportion of antinutritional factors (ANFs) and fiber reducing the availability of several nutrients in the diets [2]. Therefore, adding supplemental to diets can increase the nutritive value of alternative feedstuffs [3].

19.2 FEATHER MEAL

The poultry industry has been steadily increasing each year and a byproduct of their harvests include a relatively high proportion of feathers that can be up to 5%–7% of their weight [4]. Disposing of these feathers could pose serious problems to environmental pollution, which has prompted investigations into their valorization, including as potential and inexpensive dietary protein sources in terrestrial and aquatic feeds [5,6]. However, since feathers are designed for insulation and locomotion, up to 90% of the total protein is in the form of keratin, known as scleroproteins. Keratin is insoluble and largely indigestible by most proteases (e.g., pepsin and trypsin) due to their cross-linking chains in their α-helix and β-sheets with disulfide bonds. Therefore, this necessitates the hydrolysis of feathers to improve their nutrient utilization and digestibility in terrestrial animals or fish [7]. This is often done with heat under pressure and/or with chemicals to produce hydrolyzed feather meal. This process substantially increases the amount of digestible protein by degrading keratin into smaller peptides and amino

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acids [4,8]. In addition, various minerals that are essential for animals or fish are also liberated [9]. Such a process, however, may destroy some heat-sensitive nutrients such as some essential amino acids (e.g., cysteine), while creating unnatural amino acids including lysinoalalanine and lanthionine [10]. Moreover, the use of high heat, pressure, and chemical treatments can be expensive and may not fully degrade keratin within feather meal [11]. For these reasons, the use of enzymes may potentially be a more effective process at not only improving the nutritive value of feather meal in terms of digestibility but also potentially the biochemical composition.

In an early study, Elmayergi and Smith [12] compared the use of dietary feather meal that was unfermented with feather meal fermented by *Streptomyces fradia* to chicks. It was found that although growth was unaffected, fermented feather meal had improved pepsin digestibility as well as higher amino acids that included methionine, tyrosine, lysine, and histidine, which are known to be limiting in feather meal [12]. This latter finding is in contrast with those of Papadopoulos et al. [10] since pretreating feather meal with "Maxatase" (a commercial enzyme produced from *Bacillus* sp.) at increasing levels (0.2%, 0.4%, and 0.6%) led to a decrease in many amino acids, while only tyrosine and phenylalanine significantly increased. Nevertheless, that pepsin digestibility and nitrogen solubility increased in a near linear manner with increasing enzymes and it was suggested this was likely due to breaking of the cystine-disulfide bonds from keratinase within the enzyme product. A potential contributor for this discrepancy in the amino acid findings could be the use of bacteria versus enzymes. For example, it has been shown that bacteria with keratinolytic activity can produce free amino acids (reviewed in Ref. [7]), and subsequently increased crude protein [13].

The amount and type of the released amino acids from feathers, however, appears to be dependent on the bacterial species. For example, amino acids were the main by-products produced by *Fervidobacterium islandicum* AW-1 and *Meiothermus ruber* H328, but proline, followed by valine was predominately produced by the former [14], while serine followed by valine were mainly produced by the latter bacterium [15]. On the other hand, when the bacteria *Kocuria rosea* or *Stenotrophomonas maltophilia* were incubated with feathers, the highest amount of amino acid production was of valine and lysine, respectively [16,17]. Moreover, one of the by-products from *K. rosea* incubation with feathers includes the production of carotenoid pigments [16], which would be appealing to both the salmon and crustacean aquaculture industry. Meanwhile, when Eaksuree et al. [18] compared the effects of incubating feathers with *Bacillus lichenformis* or *B. pumilus* (incubated at 250 rpm, 37°C for 24 hours), many of the proximate and amino acid contents were significantly improved compared to the untreated feather meal. The crude protein of feather meal, however, was significantly higher while crude fat, ash, and energy were lower when incubated with *B. pumilus*, compared with feathers incubated with *B. lichenformis*. Similarly, the concentration of methionine, lysine, and cystine generally increased significantly when incubated with either bacteria, with the highest levels occurring in the *B. pumilus*

treatment. Eaksuree et al. [18] confirmed keratinase activity during incubation, which was generally higher from *B. pumilus* despite similar colony-forming unit counts, and was suggested as a contributing factor. A complementary study by these authors further revealed that the inclusion of feather meal fermented with *B. pumilus* in the diets of chickens caused the highest nitrogen retention, apparent fat digestibility, and apparent metabolizable energy values, followed by feather meal treated with *B. lichenformis*, while the lowest values were from the control treatment (no bacterial fermentation) [18].

Another potential reason for differences in these bacterial treatments could potentially include the environmental conditions during incubation. For example, Williams et al. [19] found that the dietary inclusion of feather meal that was previously incubated with *B. lichenformis* anaerobically led to higher growth of broiler chicks, from day 6 to day 21 posthatch, compared to untreated feather meal or when aerobically incubated with *B. lichenformis*. Moreover, anaerobically treated feather meal also led to similar growth of the chicks compared to the SBM control diet, although it is important to note that lysine, methionine, and histidine were supplemented [19]. Meanwhile, it has been reported that *B. lichenformis* are thermophilic [20], which may allow for their incubation at high temperatures during conventional means of hydrolysis. Indeed, there is reportedly interest in thermophilic bacteria and strains capable of keratinolytic activity for this reason [21], and other examples include *B. pseudofirmus* FA30-01 [22] and *Brevibacillus thermoruber* T1E [23]. One of the reported benefits of including some species of *Bacillus* is the potential to act as probiotics to terrestrial animals and fish and their thermophilic capabilities may also improve their resistance to heat during pellet extrusion [24].

Preprocessing feathers with heat appears to be an important step to improve the efficacy of keratinolytic activity from some bacteria and/or enzyme treatments. For example, it was shown that *Bacillus megaterium* (F7-1) had no effect on degrading keratin from untreated feathers, but was only effective after feathers were hydrolyzed with heat (100°C for 1 hour) [25]. Meanwhile, enzymatically treating prepressed turkey feather meal (with Allzyme at 0.25%; proprietary mixture of protease, lipase, and amylase), followed by autoclaving, had no effect on the amino acid composition compared to only autoclaved feather meal. In addition, when these were included in the diets of poults, there was no significant difference in the growth, protein efficiency ratio, total metabolizable energy (TME), or true amino acid availability between feather meal with or without enzyme treatment [26]. It would seem that further investigations comparing the efficacy of including enzymes before or after feather meal hydrolysis could be worthwhile, particularly when enzyme cocktails are used.

Interestingly, the inclusion of a commercially available keratinase (Versazyme at 1 g/kg diet) produced from *B. lichenformis* in soybean-meal- or cottonseed-meal-based diets was shown to significantly improve the nitrogen retention, total tract apparent digestibility of starch, feed-conversion ratio, as well as growth of broiler chickens within the first 21 days [27]. Despite the absence of feather meal,

Wang et al. [27] suggested these positive effects were due to the keratinase degrading the plant protein sources into more available forms, as well as the enzyme significantly increasing the villus height to crypt depth ratio in the intestine, which may have facilitated nutrient absorption.

In addition to the ability of various bacteria to produce keratinase, this has also been demonstrated with fungi [28–30]. However, it was reported that *Chrysosporium* spp. did degrade keratin in feathers, but the majority of nitrogen was converted to ammonium [31]. Meanwhile, incubating feathers with *B. bacillus*, and to a greater extent various fungi *Aspergillus flavus*, *A. fumigatus*, and *Trichophyton* sp., gradually increased the protein concentration of feathers, but was then substantially reduced coinciding with a drastic increase in pH [13]. The increased pH was suggested to be from the production of ammonium resulting from amino acid catabolism [13] and may provide an indicator for the optimal fermentation duration.

Studies on the use of enzymes produced by fungi to dietary feather meal are limited and show mixed results [30]. It was shown that hydrolyzed feather meal incubated with Allzyme (enzyme produced by *Aspergillus niger*; 130 lipase units/g and 12,500 hemoglobin units tyrosine base/g) had no effect on dry matter, organic matter, and crude protein digestibility in Beagle dogs. This was despite confirming feather meal degradation based on electron microscopy. On the other hand, the digestibility of gross energy was significantly improved and the presence of lipase from the enzyme was suggested as the cause [30].

Despite the potential of keratinase to improve the nutritive value of feather meal, the majority of studies on enzymatic treatment of animals or fish have employed commercially available enzyme cocktails. In general, this has shown positive effects on protein digestibility. In one study, diets containing autoclaved feather meal, with or without pretreatment with a commercially available enzyme (Insta-Pro, enzyme extract from *Bacillus* sp. added at 2.5 g/kg to hydrolyzed feathers) were compared with meat bonemeal control diet to broiler chickens for 6 weeks [32]. It was shown that both feather-meal-based diets initially led to significantly lower growth after 35 days, but by the time the experiment ended at 42 days, there was no significant difference between the treatments [32]. Nevertheless, it was shown that the enzyme-treated feather meal did improve in vitro digestibility of crude protein compared to feather meal that was only autoclaved.

It was similarly shown with another commercial enzyme (Insta-Pro4) added at 25.6 mg/g of feathers for 12 hours at 21°C, improved the in vitro pepsin digestibility of hen feathers with or without being autoclaved compared to feathers that were untreated or treated with NaOH [9]. Moreover, compared to the control untreated feather meal or NaOH-treated feather meal, the enzyme-treated feather meal had significantly higher methionine, lysine, and histidine concentrations [9]. Despite this finding, when enzyme-treated feather meal (E-FM) was included as the dominant protein source in the diets of White Leghorn cockerels, there was no difference in the protein efficiency ratio or net protein ratio compared to those

fed diets with untreated feathers (C-FM) or treated with NaOH (N-FM) [9]. On the other hand, Kim and Patterson [9] found that the apparent metabolizeable energy of E-FM to the cockerels was significantly higher than the C-FM or N-FM diets and it was suggested that this was likely due to the higher lipid content (over 10-fold higher) in the E-FM diet. As pointed out by the authors, however, this was likely due to contamination from the hen carcasses when both the feathers and dead hens were being pretreated. Indeed, other studies found that enzyme-treated feather meal had little to no effect on the lipid content [26,32,33]. It seems possible that the increase in amino acids was also due the potential digestion of the dead hens. On the other hand, in a later study by Kim and Patterson [9], both enzyme- (Insta-Pro) treated hen meal and Na-OH-treated hen meal, at similar conditions as in Ref. [9] before being fermented and autoclaved reduced the crude protein and some essential amino acids including methionine and cystine. Although this did improve digestibility for dry matter and crude protein compared to hen meal that was only autoclaved [9] the discrepant results in amino acids is unclear.

There is some evidence indicating that the health of animals can be improved by enzymatically treating feather meal. In two separate trials, the dietary supplementation of enzyme-treated feather meal (added at 1.5%) to the growth, digestibility, and intestinal health of nursery cultured pigs was compared with spray-dried porcine plasma (1.5%) or dried porcine soluble (2.0%), while the control diet did not contain any of these ingredients [34]. The enzyme type was not specified, and amino acids were supplemented in the diets to satisfy the requirements of the pigs. It was found that, with the exception of the spray-dried porcine plasma diet, pigs fed the diet with the enzyme-treated feather meal had significantly higher growth after 28 days compared to the other diets as well as a higher villus height/crypt depth and better fecal consistency (lower fecal score). Pan et al. [34] suggested that this might be due, in part, to increases in the volatile fatty acids within certain sections of the intestine that may have facilitated water and sodium absorption. It is known that volatile fatty acids are by-products of bacterial fermentation of certain indigestible carbohydrates, and may improve the intestinal health of animals [35] and indeed was suggested as possibly increasing the immunoglobin G levels in the pigs fed the enzyme-treated feather meal. While the authors emphasized the benefits of including this ingredient in the diets of pigs, further research into comparing treated and untreated feather meal with different enzyme types may further elucidate these findings.

Investigations into the potential use of enzymatically treated dietary feather meal have also extended to various fish species [33,36–38], and perhaps the earliest study was on white-leg shrimp *Litopenaeus vannamei* [36]. In this study, feather meal was incubated with Insta-Pro (a proteolyic-enzyme premix number 1955), without water, at 5.13% (wt/wt basis with feather meal) at 30, 60 or 120 minutes. The soluble protein content, which was used as an indicator of hydrolysis, was shown to be significantly higher than ground feather meal (untreated with enzyme or heat) in all the enzyme treatment durations. Moreover,

when the enzyme treatment duration was 60 or 120 minutes, the soluble protein content was significantly higher than SBM. In two feeding trials with *L. vannamei*, the two main findings were that the inclusion of the enzyme-treated feather meal slightly, but not significantly, improved *L. vannamei* growth compared to steam-treated feather meal and that 20% of enzyme-treated feather meal could replace a soybean and fishmeal mixture without compromising growth [36]. These authors therefore recommended the use of enzymatically treated feather meal in the diets of *L. vannamei*. Further studies on other fish species also showed some benefit when feather meal was enzymatically treated, but the conditions of incubation as well as enzyme type were not specified since these were often obtained from a commercial feed company. In one such experiment, enzyme-treated feather meal obtained from a feed company did lead to slightly improved growth in tilapia compared to heat hydrolyzed feather meal [33]. A similar finding was also found in a later study on tilapia [37]. Meanwhile, Davies et al. [38] compared the use of enzyme or heat hydrolyzed feather meal on the digestibility in three different fish species that included European sea bass, *Dicentrarchus labrax*, gilthead sea bream, *Sparus aurata*, and turbot, *Psetta maxima*. It was shown that there was no improvement to the crude protein, lipid, or energy digestibility, but methionine digestibility tended to be better when feather meal was enzymatically treated [38]. Considering the positive findings with incubating feather meal with bacteria, this would appear to be a worthwhile direction.

19.3 INSECT MEALS

The use of insect meals is believed to be an attractive option in the diets of terrestrial animals and fish based on the ability of various insect species to efficiently and cheaply convert low-value biological waste into high-quality protein under various environmental conditions. Examples of these include black solder fly larvae (*Hermetia illucens*), housefly maggot and pupae meal, mealworms (*Tenebrio molitor*), silkworm pupae meal, as well as locusts, crickets, and grasshoppers [39,40]. The success of their inclusion in the diets of terrestrial animals and fish appear to be dependent on the host and insect species, which has been covered in two recent comprehensive review papers [39,40]. One of the suggested limitations to the utilization of insect meal includes a relatively high amount of chitin $(C8H13O5N)_n$, at between 11.6 and 137.2 mg/kg dry weight [41]. Chitin is a polymer of unbranched poly-β-(1→4)-*N*-acetyl-glucosamine, and is similar to keratin since they both provide structural support, are insoluble in water and can be resistant to digestion. However, specific enzymes in the class of chitinases, can cleave their β-1,4 glycosidic bonds resulting in chitin-oligosaccharides and their derivatives that may be more nutritionally accessible. Not all animals or fish, however, appear to harbor this enzyme or intestinal bacteria capable of chintinolytic activity, which might explain species-specific differences to dietary

chitin [39,40]. There is evidence, however, indicating some adaptation, since bacteria with chitinolytic activity can increase in lambs and cattle when consuming diets with chitin, but the chitin was from crustacean shells [42,43].

Interestingly, among three different shrimp species, including *Penaeus vannamei*, *P. setiferus*, and *P. duorarum*, chitin digestibility ranged between 33% and 52% [44], but was much higher at 90% for the carnivorous Atlantic cod (*Gadus morhua*) and the relatively high levels of chintinase in the stomach were believed to be the cause [45]. Although Atlantic cod are known to consume a variety of crustaceans in the wild [46], chitin may not provide nutritional value to the fish. This is because it was recently shown that dietary chitin of up to 5% had no effect on the growth of Atlantic cod or another carnivorous fish, Atlantic halibut [47]. In a separate study, the highest tested dietary chitin of 1% also had no effect on the growth of common carp [48]. In contrast, the presence of dietary chitin of up to 10% increased the growth of Japanese eel (*Anguilla japonica*), yellowtail kingfish (*Seriola quinqueradiata*), and sea bream (*Pagrus major*) [49]. Meanwhile, dietary chitin has been shown to act as an immunostimulant to some fish species [48,50,51].

In addition to species-specific differences, there have also been somewhat contrasting findings between studies examining only chitin or dietary insect meal in the same species. For example, Kempaka et al. [52] found that for broilers, the inclusion of dietary chitin at 2.6%−3.9% significantly reduced dry matter and ash digestibility as well as nitrogen retention. More recently, however, De Marco et al. [53] found that the inclusion of two different insect meals (*H. illucens* and *T. molitor*) at 25%, had no effect on broilers since growth or digestibility was unaffected compared to the control diet (no insect meal). Meanwhile, Shiau and Yu [54] found that all dietary supplementation of chitin at 2%, 5%, or 10%, negatively influenced the growth and nutrient digestibility in tilapia. Interestingly, however, it was later demonstrated that tilapia possess chitinase within their intestine and stomach, but it is particularly high in their serum [55], and studies on various insect meals to this fish species have generally been positive as a partial replacement to fishmeal (reviewed in Ref. [39]).

A lack of intestinal chintinase activity or bacteria capable of chitinolytic activity in juvenile turbot (*P. maxima*) was suggested for decreasing their growth, digestibility, feed intake as well as whole-body crude lipid and energy content of fed diets with increasing amounts of black soldier fly (*H. illucens*) (from 0% to 76%) and since various crustaceans constitute the natural diets of turbot, such activities could have been lost over time when they were kept in captive conditions. It is perhaps worthwhile to note, however, that chitinase tends to be located in the stomach and serum (and not the intestine) while the composition of chitin in crustaceans is different from that of insects [39]. It was nevertheless recommended that supplementations of chitinase or bacteria with chitinolytic activity might lead to better results. It was shown that that the dietary inclusion of *Streptoccoccus milleri* (at 0.12 g/animal per day) that have chitinolytic activity in diets with 25% shrimp shell waste, significantly improved the growth of broilers compared to those not receiving these bacteria [43].

Indeed, when chitinase-supplemented diets (at 18.64 IU) were fed to 14-week-old cockerels, this was reported to significantly improve the TME compared to unsupplemented diets [56]. A similar finding was reportedly obtained with broilers [57]. On the other hand, the use of two different enzyme cocktails, including various proteases (Ronozyme ProAct) at 0.2% or xylanase and β-glucanases (Ronozyme Multigrain) at 0.1% in the diets of European sea bass (*D. labrax*) containing 25% of yellow mealworm (*T. molitor*) actually decreased the apparent digestibility of acid detergent fiber [58]. These authors suggested that a cause for this finding could include the enzymes decreasing beneficial bacteria in the intestinal tract of the fish that assist with fermenting certain indigestible carbohydrates. A reportedly effective chitinase enzyme, that produced both endo- and exochitinase activity was obtained from *Aeromonas veronii* B565 that was capable of degrading shrimp-shell chitin, colloidal chitin, powdered chitin, and β-1,3-1,4-glucan [59]. Moreover, there was a synergistic effect of protein digestion in the presence of trypsin, which was suggested to be an effective supplement in fish or animal diets [59]. When shrimp bran was included in the diets of tilapia at 5%, and supplemented with 0, 16.2, or 162 U/kg of the enzyme, the optimal growth, feed-conversion ratio, and nitrogen digestibility in tilapia was found to be when 16.2 U/kg was used. Moreover, this diet also significantly reduced mortalities when subjected to the bacterial pathogen *A. hydrophila* and was suggested to be due to improved immunological responses, based on a decrease in gut IL-1 β expression and heat shock protein (Hsp) expression, which is a mediator of inflammatory responses [59]. The dietary inclusion of 162 U/kg was shown to upregulate Hsp expression, and this finding, along with a reduction in protein digestibility and resistance to *A. hydrophila* challenge, suggested that products from excessive chitin digestion was harmful and required more investigations [59]. The authors emphasized the importance of these findings considering dietary chitin negatively affected the growth of tilapia in a previous study [54].

Based on these results, supplementations of chitinase and/or bacteria with chitinolytic activity in the diets of terrestrial animals and fish could be beneficial. This may not be restricted to only trying to minimize chitin acting as an ANF since there is evidence indicating that some by-products of enzymatically degraded chitin, including chito-oligosaccharides (COS), acetylglucosamine (GlcNAc), or chitosan, act as prebiotics in both terrestrial animals and fish (reviewed in Ref. [59]). In addition, antioxidant, antimicrobial (inhibitory action to some viruses, fungi, and bacteria) and hypocholesteromic properties of these by-products have also been documented [59].

Chitinases have several field applications and are attaining prominence in the field of biotechnology waste management, pest control in agriculture, and human healthcare [60]. Details on chitinases can be found in Chapter 18, Chitinases. Clearly, more research is needed in this area to not only improve the utilization of dietary insect meal as a more sustainable resource but may also potentially provide superior productivity.

19.4 ALGAE AND SEAWEEDS

19.4.1 FEED APPLICATIONS

Algae are a diverse group of photosynthetic organisms that can be broadly categorized as microalgae and macroalgae. Microalgae are planktonic unicellular organisms while macroalgae, also known as seaweeds, are macroscopic multicellular organisms that include brown algae (*Phaeophyceae*), red algae (*Rhodophyceae*), and green algae (*Chlorophyceae*) as the most commercially important in the feed industry [61,62]. Macroalgae are by far the most aquacultured group in the world, largely due to their relative ease of culture with minimal feed input and technology as well as their high demand from various industries around the world [63]. Their applications include their use in medicine, feeds as binders, fertilizers, as well as supplements in livestock feeds. In terms of nutrition, they often have a high organic mineral content that are in an available form to animals and fish, but the protein content can be low, reportedly at 5%−12% for brown and green macroalgae but higher at 18%−50% for red macroalgae. Nevertheless, the amino acid composition is relatively well balanced, and for some species such as *Macrocystis pyrifera* and *Ulva* spp., has a higher methionine plus cystine content than some plant protein sources such as SBM [61]. Similarly their lipid content can be low, but is often comprised of long-chain polyunsaturated fatty acids that are deficient in various plant oil sources. A recent literature review indicates that their inclusion in the diets of livestock is relatively minor (1%−10%), which is due in part to their lower protein content that other protein sources [61]. Other factors include an excessive mineral content, including toxic metals such as arsenic, and potentially less-well-digested carbohydrates including carrageenan, agar, fucoidands, laminarins (mostly in green macroalgae), and floridean starches (red macroalgae).

Nevertheless, a high mineral content may be beneficial to certain fish, particularly freshwater fish, since this may decrease energetic cost for osmoregulation, but this area remains relatively unexplored. Meanwhile, carrageenan and agar are sometimes purposefully added to fish diets to act as binders but may also enhance growth and/or innate immunity as shown in basa fish *Pangasius bocourti* [64] and more recently in red drum *Sciaenops ocellatus* [65]. It has also been shown that focoidan, but particularly laminarin, enhanced piglet growth and gut health [66,67]. Other bioactive compounds within seaweeds may also potentially improve immunological function and disease resistance [63]. Therefore, understanding these relationships may be important towards not only using the most appropriate macroalgae type but also the potential application of exogenous enzymes. Research in this area, however, is still scarce but the results so far have been promising [68,69].

In a study on egg-laying hens, different processing methods of brown marine seaweed were compared which included sundried seaweed, boiled seaweed, or autoclaved seaweed at two different levels (3% or 6%) with or without enzyme

supplementation [67]. The enzymes consisted of Xylem 500 (extracted from *Bacillus subtilis* consisting of X-amylase 8000 U/g and 1,4 β-xylanase 1260 U/g) and Amcozyme 2× enzyme (Amylase 2,500,00 U/g, protease 2,000,000 U/g, lipase 150,000 U/g, β-glucanase 30,000 U/g, xylanaze 500,000 U/g, cellulose 15,000 U/g) at a 1:1 ratio. This was then supplemented in the diets at 1 g/kg and fed to 23-week-old hens for 19 days. It was shown that treatments receiving the enzyme supplementations had significantly higher egg-laying rates, a greater egg mass, but lower body weight gain and ovarian weight as compared to hens fed the unsupplemented diet. It was further shown that while the nutritional quality of the eggs was unaffected by the enzyme, however, the amount of calcium and phosphorus were significantly higher in the egg shells of hens fed the enzyme-supplemented diet, while sodium and potassium were significantly lower in the unsupplemented diet. It was suggested that the enzyme improved mineral availability, while the effect of significantly increasing their intestinal length could have also been a contributor that may have explained the lower pancreatic weight [67]. It was concluded by the authors that a combination of autoclaving brown algae in the presence of the enzyme at 6% could improve the production of eggs but also eggshell quality.

Recently, Zhu et al. [69] compared the use of a nonstarch polysaccharide (NSP) degrading enzyme at 0%, 0.05%, and 0.10% in the diets of white-spotted snapper *Lutjanus stellatus* containing *Gracilaria lemaneiformis* (GL) that was sundried, baked (60°C for 5 hours), and ground into powder at 15%. The enzyme including xylanase (5000 IU/g, glucanase 500 IU/g, and pentosanase and cellulase both at 50 IU/g) was added to distilled water and incubated with SBM and GL for 1 hour at 40−45°C. The experimental duration was 60 days and the fish were kept within floating cages. It was shown that fish receiving diets with 0.1% enzyme supplementation had significantly higher growth, feed efficiency ratio, and muscle ash content compared to diets with 0% or 0.05% enzyme supplementation. Meanwhile, both the muscle and whole body protein content was significantly higher for fish in the 0.10% enzyme diet compared to the control treatment. Al-Harthi and El-Deek [68] found no effect on pepsin or lipase in the stomach or trypsin and lipase in the intestine, although there was a significant increase to amylase activity within the stomach and intestine, which was suggested to be a contributor to the positive effects of the enzyme. Red algae are known to contain high amounts of floridean starches, and although they reportedly have no amylose [67], it is a polymer of glucose consisting of α(1,4)-links that can be broken with α-amylase [70]. Moreover, Zhu et al. [69] pointed out that a similar enzyme in another study also hydrolyzed NSP in the brown macroalgae, *Sargassum thunbergii* that are known to contain a high amount of fucoidands [67]. However, since Zhu et al. [69] incubated both SBM and GL with the enzyme at the same time, it is possible a contributor to this response was also NSP degradation in SBM. Further research into incubating only macroalgae as well as with other enzymes may be worthwhile.

19.4.2 OTHER APPLICATIONS OF ALGAE AND DERIVATIVES

In order to survive in a highly competitive environment, freshwater or marine algae had to develop defense strategies that result in a tremendous diversity of compounds from different metabolic pathways. Recent trends in drug research from natural sources have shown that algae are promising organisms to furnish novel biochemically active compounds such as fatty acids, steroids, carotenoids, polysaccharides, lectins, mycosporine-like amino acids, halogenated compounds, polyketides, and toxins [71].

Macroalgae constitute a renewable resource used by the food industry, as feed for animals, to produce phycocolloids, as cosmetic ingredients, and for pharmaceutical applications. The moss, *Physcomitrella* patens, which is a bryophyte, occupies a key evolutionary position bridging the gap between green algae and higher plants [72,73]. Because the protonema stage of the species grows quickly and simultaneously, it can be cultivated in a bioreactor as a genetically stable cell suspension. *Physcomitrella* can be easily manipulated using standard molecular biology methods and is haploid in a vegetative state, making it a highly suitable system for engineering strains towards industrial applications [74,75].

A promising group of phototrophic organisms for biotechnology applications are the macroalgae (brown, *Phaeophyta*; red, *Rhodophyta*; and green, *Chlorophyta*) as they constitute diverse sources of natural products. Trosset and Carbonell [76] suggest and discuss the application of systems biology to red macroalgal species that have been identified as rich sources of diverse and novel bioactive compounds for drug development. Many of these molecules have high commercial value for nutritional supplements, specialty pigments, industrial polysaccharides, and aquaculture [77]. Others produce bioactive compounds including ones with antibacterial, antitumor, antiviral, and antifungal activities [78]. For example, many brown (e.g., *Laminaria saccharina*) and red (e.g., *Agardhiella subulala*) marine macroalgae produce bioactive compounds such as eicosanoids, which have substantial bioactivity against inflammation, asthma, heart diseases, and cancer. A few other macroalgal species, such as members of *Ochtodes*, *Plocamium*, and *Portieria*, produce halogenated monoterpenes that may provide antitumor activities [79]. These macroalgal species are potential candidates for cell factory development. Thus far, stable and transient genetic transformations have been developed for seven macroalgal species to change or enhance their bioactivity. These species are: *Pyropia yezoensis*, *Porphyra miniata*, *Kappaphycus alvarezii*, and *Gracilaria changii* from the red algal phylum; *Saccharina japonica* and *Undaria pinnatifida* from the brown phylum; and the green alga *Ulvalactuca* [80]. These species are prime candidates for future cell factory developments due to the availability of their genetic information; this is an advantage that can offer a better understanding for genetically engineering them. While the genomic knowledge about macroalgae is limited, the complete genomes of *Ectocarpus siliculosus*, *P. yezoensis*, *Gracilariopsis lemaneiformis*, *Chondrus crispus*, and *S. japonica* have been sequenced; these can serve as model species to established

genetic engineering in macroalgal species [81]. According to the Food and Agriculture Organization of the United Nations [82], algae constitute a large fraction of the global source for food production, pharmaceuticals, cosmetics, and fertilizers, and are processed to extract thickening agents or used as additives to animal feed with a high commercial value. Algal farming is practised for 37 different species in about 50 countries with a total estimated annual value of US$6.4 billion. Macroalgae such as *Pyropia* spp. have high nutritional value and are being used extensively for, consumption especially in Asian countries. Farming is producing up to 27 million tons of macroalgae for commercial use, and it has been expanded by 8% per year over the past decade.

Alginate is a biologically synthesized polymer that is commonly used as a food additive, for biomedical applications, including tissue constructs [83], microfluidic device manufacture [84], and in some bandages to promote wound healing. Alginates are mixed polysaccharides composed of α-L-guluronate residues and β-D-mannuronate residues linked by β-1−4 glycosidic bonds, produced by brown algae and by bacteria of the genera, *Pseudomonas* and *Azotobacter*. The properties of alginate, which are important for its biotechnological applications, are dependent on the alginate structure and on its molecular weight [85,86].

Alginate is a naturally occurring anionic polymer typically obtained from brown seaweed, and has been extensively investigated and used for many nutritional and biomedical applications. Its biomedical applications are based on its biocompatibility, low toxicity, relatively low cost, and mild gelation by addition of divalent cations such as Ca^{2+} [87]. Alginate hydrogels can be prepared by various cross-linking methods, and their structural similarity to extracellular matrices of living tissues allows wide applications in wound healing, delivery of bioactive agents such as small chemical drugs and proteins, and cell transplantation. Alginate wound dressings maintain a physiologically moist microenvironment, minimize bacterial infection at the wound site, and facilitate wound healing. Drug molecules, from small chemical drugs to macromolecular proteins, can be released from alginate gels in a controlled manner, depending on the cross-linker types and cross-linking methods. In addition, alginate gels can be orally administered or injected into the body in a minimally invasive manner, which allows extensive applications in the pharmaceutical arena. Alginate gels are also promising for cell transplantation in tissue engineering. Tissue engineering aims to provide man-made tissue and organ replacements to patients who suffer the loss or failure of an organ or tissue [88]. In this approach, hydrogels are used to deliver cells to the desired site, provide a space for new tissue formation, and control the structure and function of the engineered tissue [89].

Diatoms are becoming more and more prominent microalgae. They have enhanced importance and usefulness in commercial and industrial applications such as agrofuels, pharmaceuticals, health foods, biomolecules, material relevant to nanotechnology, and as bioremediators of contaminated water [90]. An interesting process is the ornithine-urea cycle which is absent in green algae and plants and is essential for diatom growth and their contribution to marine productivity [91].

Although not all the compounds in diatoms are known, due to the importance of these organisms, there is continual research to find, identify, and examine their properties. In the marine diatom *Haslea ostrearia*, a water-soluble blue pigment named marennine was identified and further experiments have shown its allelopathic, antioxidant, antibacterial, antiviral, and growth-inhibiting properties [92]. While this unusual pigment occurs only in selected species, there are some which are included in the more general group of photosynthetic pigments and are common among diatoms. These also have great benefits in medicine, pharmacy, cosmetics, food, and supplements. However, the pigment profile of diatoms is quite different than that found in plants' light-harvesting function [93].

More than 700 types of carotenoids have been identified in nature [94]. They are commonly synthesized by plants, algae, and some microorganisms. Seven kinds of carotenoids were found in diatoms with β-carotene as an example of carotenes.

Phytoremediation has emerged as the most desirable technology which uses plants for removal of environmental pollutants or detoxification. Many living organisms can accumulate certain toxicants to body concentrations much higher than present in their environments. The use of plants for the decontamination of several pollutants has attracted growing attention because of several problems associated with pollutant removal using conventional methods. Bioremediation strategies have been proposed as an attractive alternative owing to their low costs and high efficiencies. Recently, there has been a growing interest in using algae for biomonitoring eutrophication, organic, and inorganic pollutants [95].

19.4.3 ALGAE AS INVASIVE SPECIES

Algal blooms can be dramatic and are a result of excess nutrients from fertilizer, waste-water, and stormwater runoff, coinciding with lots of sunlight, warm temperatures, and shallow, slow-flowing water. Algal blooms are sometimes natural phenomena, but their frequency, duration, and intensity are increased by nutrient pollution, resulting from an overabundance of nitrogen (N) and phosphorus (P), particularly when the water is warm and the weather is calm. Some species of algae grow in clumps covered in a gelatinous coating and have the capability to float, allowing cells to stick together into large surface scums in calm weather. Other algae form thick mats that float on or just below the surface along the shoreline. In several places, blooms are most often composed of cyanobacteria, also known as blue–green algae [96].

The N and P pollution elements enter waterways from point sources (such as industrial and waste-water treatment plant discharges), nonpoint sources (such as septic tanks and stormwater runoff from farms, urban areas, and residential areas), and from nutrient-enriched rainfall. Masses of algae are often observed in stormwater ponds during warmer seasons.

Projects for nutrient reduction have included redirecting waste-water discharges from water bodies, stormwater collection and retention improvements,

sanitary sewer collection improvements, and repairing or removing malfunctioning septic tanks. At the same time, the district encourages best management practices and has constructed regional stormwater treatment facilities to reduce nutrient runoff from agricultural areas, particularly from husbandry facilities, and spread of fertilizers.

Algae are a natural component of the aquatic food chain and are typically not harmful to people. However, the overabundance of algae in a bloom can be aesthetically unappealing and harmful to the environment. If the types of algae that produce toxins reach high concentrations, then native aquatic organisms, livestock, pets, and even people who come in contact with the toxins, can be affected. When algal blooms block vital sunlight from reaching beneficial underwater plants that provide food and a place to live and grow for fish and other animals, the ecosystem can be negatively impacted. Algae become stressed and die when they deplete the nutrient supply or move from freshwater into saltier waters. Decomposition of dying algae can reduce levels of dissolved oxygen in the water, which fish and other aquatic animals breathe. Some fish species with little tolerance for low dissolved oxygen levels may die. In addition, some algal species can cause fish kills directly either by production of algal toxins or by clogging the gills.

While the possibility of algal toxins in the environment is a serious concern, the more common problems associated with harmful algal blooms are environmental damage and the impact on recreational activities and commerce due to the unsightly green scum and the accompanying unpleasant odor.

19.5 NONEDIBLE PLANT BIOMASS (LIGNOCELLULOSE)

Lignocellulose is the most abundant complex of polymers on Earth and it is continuously produced. Enormous quantities of organic wastes from agricultural and food-processing industries which are lignocellulosic in nature are generated annually. It is estimated that a ton of cereals harvested is accompanied by the production of two to three tons of lignocellulose-rich residues. Cellulose is part of the lignocellulose together with hemicellulose and lignin. Cellulose is composed of glucose monomers linked in chain by β-1−4 bonds. β-links are not hydrolyzed by humans and animals, with the exception of some species of termites. Cellulose is nontoxic and is resistant to degradation. Lignin is the most abundant renewable aromatic polymer on Earth and is composed of nonphenolic (80%−90%) and phenolic structures. It is essentially a polymerized aromatic substance, very resistant to hydrolysis. In wood it represents 20%−30% of the plant.

The cost-effective transformation of nonfood lignocellulose could revolutionize agriculture and reshape the bioeconomy, while maintaining biodiversity, minimizing agriculture's environmental footprint, and conserving freshwater.

A large proportion of these wastes are dumped or left to rot near the factories, burnt, or shredded and composted and used for landfill or improving soil quality.

However, with the application of appropriate bioconversion technology, these wastes represent a valuable resource, especially for areas with agricultural-based economies.

In recent years more than 150 laccases have been isolated from fungi, bacteria, and archea, including from extremophiles. Most of them have been well characterized and expressed. It is expected that improved enzymes will soon be available. Selective lignin degradation has potential applications in biotechnology when lignin elimination is fundamental for obtaining cellulose. It is not possible to define the precise structure of lignin since all show a certain variation in their chemical composition. However, the definition common to all is a dendritic network polymer of phenyl propene basic units. There are two principal categories of lignin: those which are sulfur bearing and those which are sulfur-free. The sulfur-bearing lignins are those which have to date been commercialized. These include lignosulfonates (world annual production of 500,000 tons) and Kraft lignins (under 100,000 tons p.a.).

Cellulases are responsible for cellulose depolymerization allowing the liberation of cellobiose and glucose. Cellulose hydrolysis already plays an important role in the conversion of lignocellulolytic biomass into agroethanol. It is foreseen that in the future it will be one of the most interesting natural and sustainable resource materials. It can be assumed that there is a clear need for valorization, not only for feed but also for food, particularly in the regions where chronic misery and undernutrition conditions prevail.

Cellulases play a fundamental role in food biotechnology. Complexes of macerating enzymes (cellulases, xylanases, and pectinases) are currently utilized in several agroindustries, including that of olive oil extraction. Cellulases are also used as human therapeutic factors in the treatment of phytobezoars. These enzymes are used in detergents, textiles, paper, waste treatment, and agroindustries, and in a small range as nutritional additives. The combination of cellulolytic, hemicellulolitic and pectinolytic activities works synergistically on the recalcitrant lignocellulose complex which is difficult to degrade.

In the food industry, laccase is already used for the elimination of undesirable phenolic compounds in baking, juice processing, wine stabilization, and bioremediation of waste-water. In the beer industry, laccase not only provides stability, but also increases the shelf life of beer

So far in the food and feed industries, laccases are almost not used in practice. Thus, the final conclusion should be to strongly suggest the intensification of studies in this field.

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CHAPTER

Tyrosinases—physiology, pathophysiology, and applications

20

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20.1 INTRODUCTION

Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase catalyzing the o-hydroxylation of monophenols to the corresponding catechols (monophenolase or cresolase activity) and the oxidation of monophenols to the corresponding o-quinones (diphenolase or catecholase activity). It is involved in the biosynthesis of melanin and catalyses the ortho-hydroxylation of tyrosine (monophenol) to 3,4-dihydroxy-L-phenylalanine or L-DOPA (o-diphenol) and the oxidation of L-DOPA to dopaquinone (o-quinone). The o-quinone can then be transformed into melanin pigments through a series of enzymatic and nonenzymatic reactions. Melanogenesis fulfills a number of physiological roles in different organisms. Melanins are responsible for browning occurring during postharvest storage or wounded tissue when exposed to air. In agriculture this browning poses a significant problem with huge economic impact, making the identification of compounds that inhibit melanin formation extremely important. In other cases (such as raisins, tea, and cocoa), the tyrosinase activity is needed for the production of distinct organoleptic properties. For fungi, it has been established that melanin is connected with the formation of the reproductive organs and spore formation, the virulence of pathogenic fungi, and tissue protection after damage. Mushrooms are considered a cheap source of tyrosinase.

The best-characterized tyrosinases are derived from *Streptomyces glaucescens* and the fungi *Neurospora crassa* and *Agaricus bisporus*. Molloy et al. [1] recently demonstrated that bacterial tyrosinase from *Ralstonia solanacearum* can be modified through engineering which in turn is used for the improved catalytic efficiency towards D-tyrosine using random and site-directed mutagenesis. In invertebrates, apart from providing pigmentation, melanin is also involved in three physiologically important processes: defense reactions (immunity), wound healing and cuticular hardening (sclerotization), and structural proteins resulting in the formation of the hard cuticle. In mammals, the melanin is produced in specialized pigment-producing cells known as melanocytes, which originate in the neural crest during embryogenesis and are spatially distributed throughout the organism during

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development. This distribution is under strict genetic control and may lead to interesting skin patterns, as in the case of zebras and leopards. The pigments are synthesized in membranous organelles called melanosomes and play several diverse and important roles, including thermoregulation, camouflage, and sexual attraction. In humans, the main role of the melanins is photoprotection of the skin by absorbing UV radiation that causes DNA damage and the formation of reactive oxygen species (ROS). The plant *Portulaca grandiflora* (*Portulacaceae*) is a potent source of tyrosinase [2]. Whereas tyrosinases are widely distributed in mushrooms, plants, invertebrates, and mammals, much of the modern significance in the development of pharmaceutical, food bioprocessing, and environmental applications has focused on the use of microbial tyrosinases.

Tyrosinases have a strong preference for phenolic and diphenolic substrates and are somewhat limited in their reaction scope, always producing an activated quinone as product. Despite this fact, they have potential in several biotechnological applications, including the production of novel mixed melanins, protein cross-linking, phenolic biosensors, production of L-DOPA, phenol and dye removal, and biocatalysis. Although most studies have used *Streptomyces* sp. enzymes, there are several others of these proteins that are also of potential interest. A solvent-tolerant enzyme has been described, as well as an enzyme with both tyrosinase and laccase activities [3].

20.2 PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL ROLES OF TYROSINASE

The role of tyrosinase may vary in different kingdoms. In invertebrates they play a fundamental role in defense and sclerotization mechanisms [4].

Tyrosinase is a key enzyme in melanin biosynthesis. Melanin is the most important pigment synthesized through melanogenesis in the melanocytes and their activity is the major determinant of the color of the hair and skin. Melanocytes are produced in the neural crest and travel to the basal layer of the epidermis and the hair matrices during embryogenesis. Their role is pivotal in protecting the skin from UV light by absorbing UV rays of sunlight and removing ROS [5].

Due to exposure to sunlight, the number of melanosomes increases, thus increasing their melanin content and their transfer to keratinocytes. Melanin is a class of compounds, which is also found in the plant, animal, and Protista kingdoms, where it serves predominantly as a pigment and is synthesized within melanosomes, a membrane-bound granule [5].

There are two types of melanin pigments that can be produced by the melanocytes, namely, eumelanin (black or brown) and pheomelanin (red or yellow). Both amount and type of genes operate under incomplete dominance [6].

Overactivity of tyrosinase leads to overproduction of melanin leading to hyperpigmentation of the skin. Underactivity leads to disorders such as vitiligo (depigmentation spots that occur of the skin) and whitening of hair. In skin aging, the wrinkling of skin occurs along with certain pigment abnormalities such as hypo- or hyperpigmentation [7]. For the treatment of hyperpigmentation, tyrosinase inhibitors have been investigated. Tyrosinase is a rate-limiting enzyme in the conversion of tyrosine to melanin [8]. Tyrosine inhibitors thus play an important role as skin lightening agents [9]. Plant sources such as *Glycyrrhiza glabra* (golden shower) [10] or green tea [11] are used in cosmetic applications as nutrition and whitening agents. *Cassia fistula* is recognized for its antidiabetic properties [12].

C. fistula flower extract displays a high potential for antiaging in normal skin fibroblast cells and can prevent skin aging via an increase in collagen and hyaluronic acid production as well as by inhibiting collagenase, matrixmelloproteinase-2 (MMP-2), and tyrosinase activity, all of which are involved in skin aging [13]. Wu [14] recently described 236 compounds obtained from terrestrial and marine plants, animals, microorganisms, and fungi, which have been shown to inhibit tyrosinase activity.

It should be underlined that some chemical and fungal derived skin lightening agents have been proven to have chronic, cytotoxic, and mutagenic effects in humans [15].

Betalains are vacuolar pigments composed of a nitrogenous core structure, betalamic acid [4-(2-oxoethylidene)-1,2,3,4-tetrahydropyridine-2,6-dicarboxylic acid]. Betalamic acid condenses with imino compounds (cyclo L-3,4-dihydroxy phenylalanine/its glucosyl derivatives), or amino acids/derivatives to form variety of betacyanins (violet) and betaxanthins (yellow), respectively. About 75 betalains have been structurally unambiguously identified from plants of about 17 already known families. Betalain synthesis starts with hydroxylation of tyrosine to L DOPA and subsequent cleavage of the aromatic ring of L DOPA resulting in betalamic acid formation. This pathway consists of two key enzymes, namely bifunctional tyrosinase (hydroxylation and oxidation) and L DOPA dioxygenase (O_2-dependent aromatic ring cleavage). Various spontaneous cyclization, condensation, and glucosylation steps complement the extended pathway, which has been presented here comprehensively. The biosynthesis is affected by various ecophysiological factors including biotic and abiotic elicitors that can be manipulated to increase pigment production for commercial scale extraction. Betalains are completely safe to consume, and contribute to health [16].

20.3 APPLICATIONS OF TYROSINASES

Tyrosinases are type-3 copper proteins involved in the initial step of melanin synthesis. The tyrosinase from the mushroom *A. bisporus* is best characterized

because of enzymatic browning problems during postharvest storage. Fungal tyrosinases are generally associated with the formation and stability of spores. They also play an important role in defense, virulence mechanisms, and in browning and pigmentation. More recently, tyrosinases were found in several other fungi with relevant and very promising properties for biotechnological applications. The limit of these applications remains due to the fact that native fungal tyrosinases are generally intracellular and produced in low quantity [17].

20.3.1 TYROSINASES—FOOD AND FEED APPLICATIONS

Tyrosinases are proposed for application in food processing, not only for the melanogenic reaction it catalyzes, e.g., in tea production [18], but also for its cross-linking activity that can modify the structure of food. Tyrosinases of different origins have been tested as cross linking agents on a wide variety of proteins from milk, meat, and cereals. In contrast to traditional cross linking agents, tyrosinases are characterized by high specificity in the reaction they catalyze and, furthermore, they utilize food matrix components such as proteins in their reaction and do not require the addition of any chemical or food additive. The improvement of the textural properties of food can be achieved by using carbohydrate based gelling agents. Recently, a comparative study assessed the ability of different tyrosinases from fungi and bacteria, e.g., common mushroom *A. bisporus*, the plant pathogen *Botryosphaeria obtusa*, and the Gram negative *Verrucomicrobium spinosum*, to cross link the commonly used gelling agent gelatine and revealed that the addition of phenolic compounds to the reaction mixture significantly accelerated the reaction [19]. Tyrosinase is useful in cross linking the proteins of food matrices. In dairy and meat applications it can be applied in the production of low calorie and low fat food [20]. Caseins are generally good substrates for tyrosinase because their structure is flexible [21] and more accessible for the action of the enzyme. The addition of tyrosinase from *Trichoderma* has been reported to improve the firmness of gels from raw milk and sodium caseinate [22]. This tyrosinase is also able to form protein−oligosaccharide conjugates, where the protein is alpha casein [23], but the reaction is not very efficient. This tyrosinase is also effective in improving the firmness of gels contained with low meat chicken breast and creates a network between the collagen molecules [24]. In baking, *Trichoderma* tyrosinase has also been able to enhance the hardness and reduce the extensibility of dough and to modify the structure of baked bread by cross linking the cereal proteins [25,26].

The applications of tyrosinase to animal feed are recent. Aiming at the improvement of the nutritional value of animal feed, mushroom tyrosinase has recently been able to increase the bioavailability of iron from phytase treated fava bean-based preparations [27]. Acetone extract of *Ascophyllum nodosum* has antioxidant, inhibition of tyrosinase, and strong antimicrobial activities, and is proposed as a natural antioxidant and antimicrobial for nutraceuticals and feed [28].

20.3.2 TYROSYNASES, REMOVAL OF PHENOLIC COMPOUNDS, AND BIOREMEDIATION

Tyrosinases have several industrial applications and potential for new ones. A novel use of tyrosinase for the in vitro conjugation of the protein gelatin to the polysaccharide chitosan has been described [29]. Recently, methods for the recombinant production of bacterial tyrosinase became available. These are utilized for the formation of biomaterials such as cross linked proteins and melanin. Tyrosinase is used in the production of tailor made melanin and other polyphenolics from various phenols and catechols materials, and they have a wide application, e.g., in the development of organic semiconductors or photovoltaics. Tyrosinase can also be used to generate cross linked proteins, allowing enzyme biocatalysts such as lipase to be easily recycled.

Phenolic compounds are present in the wastewater of a number of industries such as coal conversion, resin and plastics, petroleum refineries, textiles, dyes, iron and steel, and pulp and paper [30].

Phenols are toxic and coloration pollutants in industrial wastes imposing decontamination of such compounds and tyrosinase application has become very effective in the removal of phenol and its derivatives from wastes [31,32]. The application of bacterial tyrosinase to the treatment of contaminated waste waters can be performed either with tyrosinase producing microbial strains or with the enzyme in an immobilized form as protagonist [33,34]. Tyrosinase has the advantage over other enzyme systems for phenol removal since molecular oxygen rather than hydrogen peroxide is the oxidant, theoretically reducing the potential cost of applying the technology [35].

Polyphenol oxidase (PPO; e.g., tyrosinase) from *A. bisporus* and *Terfezia leonis* are relatively stable [36]. They are able to oxidize mono-, di-, and triphenols. L tyrosine and pyrocatechol are their best phenolic substrates. Preferable pH for both is close to neutrality and optimal temperature is 30–40°C. Short treatment at 70°C totally inactivates the enzymes. *A. bisporus* PPO immobilized by encapsulation in calcium alginate beads catalyze efficaciously phenol oxidation and can be reutilized [37].

20.3.3 INDUSTRIAL APPLICATIONS

Different methods are utilized for the immobilization of tyrosinase such as electrochemical transducer, cross linking, on the surface of electrodes, entrapment in polymer films and hydrogels, carbon paste matrix, graphite epoxy composite electrodes, electropolymerization, self assembled monolayers, silica sol gel, alumina sol gel, and nanoparticles [2]. The use of the tyrosinase polysaccharide bioelectrode for monitoring environmental pollution can be initiated as composite material (agarose and guar gum) has a good film forming and adhesion ability, as well as being nontoxic and biocompatible, thus increasing its use for tyrosinase

entrapment and subsequent session fabrication. Biosensors based on tyrosinases were designed for measuring phenols, polyphenols, and pesticides [38], as well as toxic metals [39].

20.3.3.1 Tyrosinases and dye production

Taking into account the role of tyrosinase in the synthesis of melanin from tyrosine, the enzyme has been applied to the synthesis of dyeing and coloring solutions. For example, tyrosinase from *Rhizobium etli* has also been used for melanin production in *Escherichia coli* [40]. Tyrosinase was proposed in 1947 for the dyeing of animal fibers [41]. More recently, a coloring composition containing the enzyme from *A. bisporus* and L DOPA has been proposed for coloring hair [42]. Nevertheless, tyrosinase from other mushrooms has also been tested for coloring goat hair, but the effect was not promising [43].

20.3.3.2 Tyrosinases for medical applications

Tyrosinases are omnipresent in nature and are considered one of the fundamental enzymes involved in several biological functions and defense mechanism (especially in melanogenesis). The substrate specificity of most tyrosinases is generally wide and substrates include various mono-, di-, and poly phenolic compounds. This qualified tyrosinase for the production of *ortho* diphenols, also called substituted catechols, essential intermediates in the synthesis of pharmaceuticals, plastics, antioxidants, and agrochemicals. L DOPA, the product of the tyrosinase reaction with tyrosine, has a high value since it is the main drug for the treatment of Parkinson's disease (PD) [44]. Pathophysiology of PD involves loss of nigral dopaminergic neurons. Levodopa, dopamine precursor, remains the gold standard for the relief of the symptoms. Continuous dopaminergic stimulation has been shown to dramatically improve motor fluctuations and dyskinesia [45]. Continuous dopaminergic stimulation is a therapeutic for the management of PD in which dopaminergic agents provide continuous stimulation of striatal dopamine receptors and will ameliorate, delay, or prevent the onset of levodopa related motor complications [46]. Several studies have been targeted at providing an alternative enzyme based process using free or immobilized tyrosinase [47]. Nevertheless, the technique using the enzyme tyrosine phenol lyase that does not catalyze the further oxidation of L DOPA might be more promising [48]. Tyrosinase from mushrooms is applied to the synthesis of natural compounds with estrogenic activity such as coumestan and derivatives that are generally isolated from plant material [49]. The same enzyme has also been used for the production of the antioxidant hydroxytyrosol in the presence of ascorbic acid (vitamin C), and the final reaction mixture could be used directly as a food additive [50,51]. Other medical applications of tyrosinase include the production of melanins as natural antibacterial compounds for the treatment of wounds, i.e., the local application of melanin precursor and tyrosinase in the form of a cream or ointment [52].

20.3.3.3 Other applications of tyrosinases

In recent years, many amperometric biosensors based on the inhibition of the activity of tyrosinase enzymes have been used for the determination of triazine and phenylurea herbicides in the environment. The use of these herbicides is persistent in spite of the danger they cause to the environment, as they are widely used due to their low environmental persistence but they contribute to the high acute toxicity. They cause a potent hazard to human health as their presence can be detected in surface and ground water. Different techniques are used extensively for the detection and quantitative determination of the toxic levels. Typically, liquid chromatography or gas chromatography is used, but nowadays electrochemical enzyme sensors are also considered an alternative method to the conventional spectrometric techniques for pollutant determination due to their simplified sample. To enhance the biosensor response and sensitivity, nanoparticles together with immobilized alkaline phosphates have been incorporated into a sol gel/chitosan biosensor membrane. These nanoparticles used in electrochemical biosensors have the ability to provide a favorable microenvironment for biomolecules such as proteins to exchange electrons directly with an electrode. Also, different enzyme based biosensors involving laccase, tyrosinase, glucose oxidase, horseradish peroxidase, and nanoparticles have been used for the construction of electrochemical biosensors. A tyrosinase electrode is also employed to monitor phenolic and catecholic compounds. It is one of the effective transducers for the detection of phenols or catechols [53].

20.4 CONCLUSIONS AND PERSPECTIVES

Tyrosinases have a large set of applications, based essentially on their oxidizing capacity. They have a strong preference for phenolic and diphenolic substrates and display potential in several domains of applications. There are several of these proteins with a strong potential interest. Also, two families of mono oxidases, tyrosinses and laccases, display similar activities in different fields [54].

Environmental, demographic, and socioeconomic reasons demand alternative, more environment friendly, food and especially feedstuff productions, which take care of our soil resources. Therefore, the evaluation of combinations of tyrosinases and laccases, which may fulfill that aim, is suggested.

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CHAPTER

Probiotics and enzymes in the gastrointestinal tract

21

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21.1 INTRODUCTION

Microorganisms are normally present in the gastrointestinal (GI) tract of humans and animals, including insects. The number of species found there is of more than 400 [1,2], and half of the weight of the colon digesta is represented by the bacterial cells with a concentration of 10^{12} colony-forming units per gram of content.

The GI tract is sterile until birth when the microbial colonization starts. The mammalian gut is colonized by a highly complex, diverse, and dynamic microbiota. Although considered sterile during gestation, at delivery the gut is exposed to microbes during passage through the birth canal. "Environmental" bacteria are then ingested from the vagina, feces, skin, and the early-life environment [3]. These bacteria undergo important changes in function due to several factors such as age, environment, diet, health status, etc. [4,5].

The establishment and development of the normal gut microbiota requires continuous microbial exposure during the early stages of life, and this process is compromised under conditions of excessive hygiene [6]. Following birth, bacterial transfer to the neonatal intestine is continuous throughout the suckling and nursing periods [7]. The resulting microbiota is very diverse and reflects the microbial communities associated with the birth and rearing environments, as well as maternal contact [7,8]. The mucosal immune system of the pig undergoes rapid changes during the neonatal period, similar to humans [9,10].

As a general concept, high microbial diversity is thought to be beneficial to the ecosystem by reducing the opportunity for colonization by infectious agents [10].

The microflora of the digestive tract is a complex microbial ecosystem, well balanced, which in an aboral direction undergoes specific changes as to the ratio of aerobic and anaerobic microorganisms, the functions of which supplement each other. The aerobes ensure for the whole ecosystem the scavenger effect. The microbial profile of the digestive tract is typified by the absence of anaerobic microorganisms in the stomach and conversely their absolute predominance in the distal colon [11].

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The basic physiological functions of the microflora of the digestive tract can be characterized as follows: (1) microbial barrier against pathogens and potential pathogens; (2) formation of products of the microflora and their influence on the blood supply of the intestinal mucosa and peristalsis; (3) stimulation of the immune system in the gut; (4) reduction of bacterial translocation; (5) production of vitamins. Deserved attention has been paid to this problem for many years in the world literature. Awareness of the problem has opened the door to manipulation of the microbial flora of the digestive tract by the use of diet or prebiotics, probiotics, as well as antibiotic treatment.

"Probiotic" literally means "prolife." The term was introduced in 1965 by Lilly and Stillwell [12] in opposition to the term "antibiotic." In 1989, Fuller [13] underlined the concept of probiotic viability and the idea of beneficial effects to the host. The history of probiotics started centuries ago with the production of fermented foods, particularly milk. Such "technologies" were well known to the Greeks and Romans, who recommended their consumption [14]. The fermentation of dairy foods represents one of the oldest techniques for food preservation [15].

A consensus definition of the term "probiotics," based on the available information and scientific evidence, was adopted after a joint expert consultation in 2001 by the Food and Agricultural Organization (FAO) of the United Nations and the World Health Organization (WHO). The delivered definition of "probiotics" was—"live microorganisms which, when administered in adequate amounts, confer a health benefit on the host."

The modern hypothesis of the positive role played by certain bacteria was introduced in 1907 by Metchnikoff [16], who suggested that it would be possible to modulate the GI microflora and to replace harmful microbes with useful microbes [17], and underlined that proteolytic bacteria such as *Clostridia*, which are part of the normal gut flora, produce toxic substances including phenols, indols, as well as ammonia from proteolysis. These compounds were considered to be responsible for the so-called "intestinal autointoxication," which would cause the physical changes associated with old age [18].

During the beginning of the last century it was observed that milk fermented with lactic-acid bacteria inhibits the growth of proteolytic bacteria by the low pH produced by the hydrolysis of lactose. The pioneer researchers also noted that some rural populations in Bulgaria and the Russian steppes, who had milk fermented by lactica-cid bacteria as their main food, were exceptionally long lived. In Paris, e.g., physicians began prescribing the sour-milk diet for their patients [19]. *Bifidobacteria* was first isolated, in 1900, from a breast-fed infant and was found to be dominant in the GI microflora of breast-fed babies. It appeared that diarrhea incidence was much lower in such infants and that bifidobacteria ingestion had therapeutic positive effects against childhood diarrhea [8]. Later on, intestinal lactic acid bacterial species with alleged health-beneficial properties were introduced as probiotics, including *Lactobacillus rhamnosus*, *Lactobacillus casei*, and *Lactobacillus johnsonii* [20]. The most commonly utilized probiotics are strains of lactic acid bacteria such as *Lactobacillus*, *Bifid bacterium*, and *Streptococcus*. The first two resist gastric acid,

bile salts, and pancreatic enzymes, adhere to colonic mucosa, and readily colonize the intestinal tract. Moreover, lactic acid bacteria have been demonstrated to inhibit the in vitro growth of many enteric pathogens, including *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli*, *Clostridium perfringens*, and *Clostridium difficile*. The yeast *Saccharomyces boulardii*, a patented yeast preparation, also possesses properties that make it a probiotic agent. It inhibits the growth of several microbial pathogens, its temperature optimum is 37.8°C, it survives transit through the GI tract, and it is unaffected by antibiotic therapy.

Basically, an equilibrated GI microbiota play a fundamental role in nutrition physiology by the digestion of compounds not hydrolyzed by the endogenous enzymes and by the synthesis of hydrosoluble vitamins, as well as in health protection by the barrier effect against potentially allochthonous pathogens [21].

"Digestive health" is considered to be a central part of physical and even emotional well-being. However, the GI microbiota can be fragile and, for instance, a treatment with antibiotics or stress due to a bad diet can impact the thousands of different species that live in the GI tract. This is the main basis for the concept of those advising the use of probiotics. Nevertheless, the expectations on probiotics are very often placed too highly. They are not, on the one hand, a fantastic universal therapeutic and, on the other hand, make up for poor lifestyle in humans and weak zoo technical practices.

The interest of researchers and of food and feed additives producers in probiotics has strongly increased in the last 20 years. Accordingly, there are today hundreds of published papers [22–27] of diverse content quality as well as several books available on the theme, e.g., Refs. [28–32].

The fundamental aspects of probiotics will be summarized, with emphasis on those related to the interactions with the enzymology of the GI tract.

21.2 BENEFICIAL EFFECTS OF PROBIOTICS IN HUMANS AND ANIMALS AND VARIABILITY OF RESULTS

Probiotics are said to improve human health and well-being as well as health and zoo technical performance of farmed animals, including fish. Pets' health is also mentioned as a target for the utilization of probiotics. In humans, it is claimed that they can contribute to the treatment of diverse pathologies from eczema to hypercholesterolemia and Crohn's disease.

Probiotics are used to contribute to the treatment of several GI and non-GI disturbances. It should be underlined that the results supporting their utilization are often conflicting, especially for non-GI-associated diseases. The general demonstration supporting the use of probiotics is related to the treatment of diarrhea and intestinal diverticulosis. The use of probiotics in non-GI illnesses is very weakly supported by available data. Probiotics may have some beneficial effects outside the GI in atopic eczema in children and genitourinary infections.

The knowledge of the mechanism(s) of action of probiotics is still very embryonic. It is proposed that when ingested they attach to the intestinal mucosa preventing epithelial attachment of pathogenic bacteria. Some bacteria, especially *Lactobacillus* and *Bifidobacterium*, produce lactic acid, acetic acid, and propionic acid, which lower the pH and inhibit the growth of pathogenic bacteria. Another proposed mechanism of action is their immunomodulatory effects. Many of the claims made by probiotic manufacturers are not supported by clinical data and thus require deep further evaluations. In the United States, probiotics are considered a health food and are therefore not regulated by the Food and Drug Administration (FDA) as a drug.

- Antibiotic-associated diarrhea (AAD): There is some reasonable evidence that taking high doses of some probiotics (*L. rhamnosus* or *S. boulardii*) while taking antibiotics can prevent children getting AAD. Without any help, antibiotics can often reduce the protective gut bacteria, resulting in diarrhea. Probiotics given with antibiotics may also reduce the risk of developing a *C. difficile* infection. *C. difficile* is potentially dangerous bacterium that can cause diarrhea and life-threatening complications. It can infect the gut if the balance of gut bacteria is disturbed by antibiotics. Probiotics are thought to directly kill or more probably inhibit the growth of harmful bacteria, stopping the production of toxic substances.
- Probiotics may treat infectious diarrhea: There is some evidence that probiotics can shorten an episode of diarrhea caused by a GI disorder. Nevertheless, the results are not clear enough to make any treatment recommendations.
- Probiotics may help to protect premature babies: Some babies born prematurely are at risk of a serious condition, necrotizing enterocolitis (NEC). This is when tissues in the baby's gut become inflamed and start to die. There is some a little evidence that probiotics may reduce the likelihood of premature babies developing NEC, although there are still some uncertainties, and routine use of probiotics in premature babies is not currently recommended.
- Probiotics may help with irritable bowel syndrome (IBS): Probiotics may help to reduce bloating and flatulence in some patients suffering from IBS. However, the extent of the benefits, nor the most effective type of probiotic are not known.
- Probiotics may help with lactose intolerance (LI): LI is a common digestive problem in which the ingested lactose is not or very poorly digested. Some studies have found that certain probiotics, such as *Lactobacillus acidophilus*, may help to reduce the symptoms of LI, which include stomach cramps, flatulence, and diarrhea.
- Probiotics may help pouchitis: Some people with ulcerative colitis need to have resection of part of their bowel and a loop of hingut constructed in its place. This loop, or pouch, can sometimes become inflamed, leading to

diarrhea and other problems. A few small studies have shown that probiotics can help treat pouchitis. Basically, much more research is needed before it can be recommended as an effective treatment.

- Probiotics do not seem to help babies with colic: It has been suggested that probiotics may be a useful treatment for babies with colic, but there is no clear evidence.
- Do probiotics boost the immune system? Adverts for Danone probiotic yogurts used to claim they could "boost the immune system," but such claims were ruled unproven by the European Food Safety Authority (EFSA) and can no longer be made. There is a lack of evidence for probiotics benefiting the immune system, and research found no effects in healthy children.
- Probiotics cannot be recommended for vaginal conditions: There have been suggestions that probiotics can help in the treatment of bacterial vaginosis, without any experimental evidence.
- Probiotics cannot be used for inflammatory bowel disease: There is a lack of evidence to make any conclusions about the effectiveness of probiotics for relieving symptoms of Crohn's disease or ulcerative colitis.
- Probiotics are not useful in the treatment of eczema. Some authors have suggested that giving probiotics to young children may reduce their risk of developing eczema, but the evidence is not very strong.
- Other uses: Many other claims are made for probiotics, e.g., that they lower blood pressure and cholesterol, alleviate skin conditions, treat ulcers and urinary tract infections, prevent colon cancer, ease anxiety and depression, and ward off traveler's diarrhea. It should be underlined that clear evidence to support these claims is lacking.

The high variability and general inconsistence of the results on the utilization of probiotics in human applications is also present in that performed in farmed animals, including fish. Very often, in feeding, probiotics are presented as an alternative to antibiotics growth promoters. In my opinion, this is a nonrealistic and too optimistic claim, because up until now it has never been demonstrated that the probiotics effects support the comparison regarding the health and performance improvements.

In a deep evaluation of the published results obtained in 120 experiments with bacterial probiotics in piglets, it was concluded that in 80% of the trials there were no measurable positive effects, and in some of them negative ones were observed [33]. Very often the sporulation capacity of the bacterium is proposed as an argument pro, since the microorganism will not suffer, without any formulation, with the technological treatment of the feed and to the lytic action in the GI tract. It is obvious that a reasonable germination capacity should take place, to obtain a result. *Bacillus* are under this criterion the first choice. However, not all the *Bacillus* strains share such qualities. One of the arguments allowing a physiological explanation of the beneficial effects of probiotics are their metabolic effects. However, it should be underlined that it not always possible to do so. As

an example, the probiotic based on the strain *Bacillus cereus* CIP 5832 has a negligible germination in vivo and accordingly has no effects on the absorption of nutrients, observed with the strain *Sporolactobacillus* P44 [24,25]. The strain CIP5832 has no effects on the zoo technical performances (daily weight gain and feed-conversion ratio) in piglets [33]. However, there is some published information on the beneficial effects of the strain *B. cereus* var. *toyoi* on the zoo technical performances of piglets as well as a significant reduction of the incidence of liquid feces and post weaning diarrhea [34].

The numerous and diverse biological activities of yeasts make them promising candidates for a wide range of applications not limited to the food sector. They have a long tradition as major contributors to the production of fermented foods and beverages. Their antagonistic activities toward pathogenic bacteria, yeasts, and fungi are now widely recognized. In addition to their role in food technology, the yeasts are used in feed and veterinary practices, as well as in human medicine and biomedical and pharmaceutical industries. One of these roles consists primarily of antagonizing other microorganisms such as undesirable yeasts, molds, and bacteria. However, there have been relatively few studies that try to identify the mechanism(s) of inhibition by yeasts. Also, only *Saccharomyces cerevisiae* var. *boulardii* has been studied in detail and its inhibitory mechanisms relatively well defined, antagonistic activities are associated with other genera or species, and exploration of their potential industrial and biotechnological applications is expected [35].

21.3 ISSUES ON THE UTILIZATION OF PROBIOTICS

Probiotics are thought to help restore the natural balance of bacteria in the GI tract when it has been disrupted by stress, illness, or treatment. Probiotics may be helpful in some cases, but there is little evidence to support many of the health claims made for them. For example, there is no evidence to suggest that probiotics can help treat eczema in humans. There are several issues that the potential user of probiotics needs to be aware of. Firstly, probiotics are generally classed as food rather than medicine, which means they do not undergo the rigorous testing that medicines do.

Taking into account the way probiotics are regulated, one cannot always to be sure that:

- the product actually contains the bacteria stated on the food label;
- the product contains enough bacteria to have an effect;
- the bacteria are able to survive long enough to “work” in the gut.

It is also worth noting that there are many different types of probiotics that may have different effects on the body, and little is known about which types are best. Also, there is likely to be a huge difference between the pharmaceutical-grade probiotics that show promise in clinical trials and the yogurts and supplements sold in shops.

Today, probiotics are found in more places than in yogurt and in the supplements aisle. "Good bacteria" are turning up in everything from toothpaste and chocolate, to juices and cereals, and most of them have high prices.

To separate the reality from the hype, the following should be kept in mind about probiotics in humans consumed in foods or ingested as supplements:

1. Probiotics are not regulated like drugs, although supplement makers cannot make disease-specific health claims without the FDA's or the EFSA's consent. Also, there are no standardized amounts of microbes or minimum levels required in foods or supplements.
2. Mild side effects are possible. Taking probiotic supplements induces a tendency to develop gas and bloating in the first few days.
3. All foods with probiotics are not created equal. Beyond the dairy products, probiotics are also found in pickles packed in brine, sauerkraut, kimchi, tempeh, and miso.
4. Although the majority of probiotics found in foods are safe for most people, the bigger concern is whether the organism is actually present when the person consumes the food.
5. Probiotics might not be safe for everyone. There are definitely some people who should avoid probiotics in foods or supplements, including individuals with weakened immune systems, cancer under chemotherapy, organ transplants, people with short GI tracts, infected by HIV, and with abnormal heart valves.
6. Probiotics should be utilized before their expiration dates to maximize the potential benefits. Some supplements needs refrigeration.
7. The amount of probiotics in a food is often unclear. Labels on supplements should specify the genus, species, and strain, in that order. Microbe counts are listed as colony-forming units, which are the number of live organisms in a single dose, typically in the billions.
8. Probiotics are some of the most expensive dietary supplements, with one dose often costing more than US$1 a day, and a higher price might not necessarily reflect a higher-quality supplement, or a reputable manufacturer.
9. It is recommended to check high-quality studies published in reputable medical journals that show positive results and follow the uptake advised.

21.4 MARKET SIZE FOR PROBIOTICS AND MAIN PRODUCERS

Proponents claim that probiotics (meaning "for life," as opposed to antibiotics) confer health benefits primarily by rebalancing the normal microflora in the large intestine (colon).

It has been mentioned that the results supporting the utilization of probiotics are often conflicting, not only in humans but also in animals. However, the

additives based on microorganisms have a very big market success history. The sales of probiotics in 2014 were worth US $34.33 billion, they rose to US $42 billion by 2016 and it is foreseen that the market will grow by US $13.58 billion to reach US $47.91 billion in 2019, driven by the sector of functional foods and beverages, the expansion into new sectors, intensive R&D, and product innovations conducted by the top vendors.

There are many general types of bacteria used as probiotics (two common ones are *Lactobacillus* and *Bifidobacterium*), and many different species as well as strains within species. They have different physiological effects—and thus possibly different health benefits (as well as possible risks). Some yeasts, such as Saccharomyces are also used as probiotics.

In the whole volume of sales in 2014, functional foods represented 82.9%, dietary supplements represented 8.53%, animal feed 8.16%, and others 1.02%.

The main suppliers of probiotics are:

- Attune Foods—established in 1908, entered the probiotic bars segment in 2009 by acquiring Erewhon, Uncle Sam, New Morning, and Skinner's.
- Bifodan—founded in 1992 in Denmark, provides probiotic solutions to the dietary supplements industry.
- BioCare Copenhagen—Danish bioventure company specializes in microbial active ingredients. The company offers products related to women's healthcare, digestive health, immunity, and weight management. BioCare Copenhagen primarily works with yeast cells and lactic acid bacteria.
- BioGaia—established in the 1980s, BioGaia is a leading probiotic biotechnology company headquartered in Sweden.
- Ch. Hansen—offers probiotics in dairy products, infant formula, and dietary supplements. All their products are clinically tested, and a key focus area for the company is on R&D.
- Daflorn—Founded in 2001 in Bulgaria. It creates *Lactobacillus bulgaricus* and other fermented milk probiotic microorganisms.
- Danisco—a Denmark-based subsidiary of DuPont. Was awarded the 2014 North American Award for New Product Innovation for introducing a new probiotic for animal feed. The probiotic ingredient can be used as an additive to antibiotics to improve gut health in poultry.
- Danone—in 2012, the company formed an association called the Global Alliance for Probiotics along with other key players like Yakult, Danisco, and BioGaia. The major aim of this association was to seek a 3-year moratorium from the European Commission and EFSA. In 2014, the company filed for a patent for one of its strains (*Bifidobacterium animalis* ssp.) after studies revealed that this strain can help treat IBS and inflammatory bowel disease.
- Deerland Enzymes—established in 1990 as an enzyme consulting company in the United States. It is regarded as one of the most popular manufacturers of enzymes and probiotics for dietary supplements.

- Lallemand—Canadian company founded in 1934, specializes in probiotic ingredients for gut health, oral health, better immunity, and women's health.
- Nestlé—despite being a conglomerate, the company has a wide presence in the field of probiotics. The company is considered to be the pioneer in developing infant formula with probiotics, including *Bifidobacterium lactis* in combination with docosahexaenoic acid and arachidonic acid (Omega 3 and 6). This product is widely available in the United States and Canada. The company also launched a probiotic substitute for curd under the brand name Nestlé Actiplus Dahi in the Indian market in 2011.
- Probi—probiotic producer established in 1991. The company focuses on R&D in the following segments: GI health, immunity, metabolic health (including cardiovascular disease, type 2 diabetes, and obesity) and nutrition uptake.
- Winclove Probiotics—founded in 1991 in Amsterdam. Offers probiotics solutions, including its flagship brand Ecologic.
- Yakult—a Japanese company considered to be one of the pioneers of probiotic products. It specializes in probiotic beverages, and the organization has published several research papers on the health benefits of its products.

21.5 SAFETY ISSUES OF PROBIOTICS

The metabolic activity that is the enzymatic activity associated with the eventual production of toxic substances by probiotics should be underlined. Probiotic bacteria should not produce harmful substances by metabolic activities. One test is to determine whether the bacteria convert food components or biological secretions into secondary substances harmful to the host. For example, some intestinal bacteria act on proteins and their digested products to produce ammonia, indol, phenols, and amines [36].

Although *Lactobacillus* and *Bifidobacterium* species have not been reported to produce very harmful compounds, the data on the production and consumption of ammonia are interesting. Araya-Kojima et al. [37] measured the enzyme activities related to the consumption and generation of ammonia in *Bifidobacterium* sp. of human origin. Compared with other bacteria of the intestinal flora, *Bifidobacterium* sp. have a lower deaminase activity involved in the production of ammonium from amino acids but a higher ammonia assimilation activity. Secondary bile acids are important harmful substances that are produced by intestinal bacterial actions on liver secretions. They may exhibit carcinogenicity by acting on the mucous-secreting cells and promoting their proliferation, or they may act as promoters of carcinogenesis [38]. Many intestinal bacteria, including *Bifidobacterium* and *Lactobacillus* species, can deconjugate conjugated bile acids. However, *Bifidobacterium* species [39], *Lactobacillus* (five species), *Leuconostoc lactis* subsp. *lactis*, and *Streptococcus thermophilus*, have been reported to lack

the 7α-dehydroxylase activity that is related to the production of secondary bile acids [39,40]. *Enterococci* possess properties that can be ascribed roles in pathogenesis and have increasing importance as nosocomial pathogens. They produce aggregation substances, surface carbohydrates, or fibronectin-binding moieties that may facilitate adherence to host tissues. *Enterococcus faecalis* appears to have the capacity to translocate across intact intestinal mucosa in models of antibiotic-induced super-infection. Extracellular toxins such as cytolysin can induce tissue damage as shown in an endophthalmitis model, increase mortality in combination with aggregation substance in an endocarditis model, and cause systemic toxicity in a murine peritonitis model [41].

Very often *Lactobacilli* infections appear in patients with a deficient immune system [42]. It has been demonstrated that clinic samples of *L. rhamnosus* have a binding capacity to fibroctin and that the thrombocytes aggregation is higher than that of the other *Lactobacillus* strains [42]. Several studies have highlighted the risk of translocation with subsequent sepsis and bacteremia following probiotic administration but there is still a lack of investigations on the dose effect of these compounds, as well as on the potential pathologic issues [43]. *Lactobacillus jensenii, casei, paracasei, rhamnosus, acidophilus, bulgaricus, lactis, curvatus, delbruecki, plantarum* and *gasseri*, have been responsible for several hundreds of diseases, sometimes dramatic, such as endocarditis, bacteremia, bloodstream infectious, hepatic abscess, catheter related bacteremia, purpura fulminans, sepsis, pneumonia, septicemia, septic urinary infection, meningitis, pyelonephritis, endocarditis, etc. [43–45].

Great care is thus required in the choice of the proper *Lactobacillus* species, their genetic stability and the translocation risk, mainly related to inflammatory disease-induced gut mucosa enhanced permeability. It should be remind that the use of *Lactobacillus* or other probiotic agents can cause invasive disease in certain populations.

In animal utilization of probiotics, issues also appeared. A clear representative example is that of the probiotic Paciflor based on a strain of *B. cereus* (Collection de l'Institut Pasteur—CIP 5832) developed in 1989 by Nguyen et al. [23] and recommended for piglets, hens, ducks, and rabbits. This probiotic has already be mentioned for its weakness of physiological and zoo technical effects [24,25,33]. The German company BASF acquired it later on, and the product had a certain market success. In the meantime, several alerts on the safety, i.e., toxin production, of the strain appeared and the European authorities (Scientific Committee on Animal Nutrition—SCAN) reexamined the Paciflor strain for toxin production. In the SCAN official opinion delivered in May 2001 it was mentioned that the Paciflor strain belongs to a group of bacteria commonly implicated as a cause of food poisoning in humans and known to produce enterotoxins and/or an emetic toxin. In the slaughtering process, there is a risk that meat products may be contaminated with Paciflor spores and spread to humans via the food chain. The demonstration of the toxinogenic activity of *B. cereus* led to the withdrawal of Paciflor from the market and the recommendation that *Bacillus* strains be fed to food-producing animals as an additive must,

therefore, be shown not to be able to produce toxins damaging to human health [46]. The concerns regarding the safety of some *Bacillus* strains on their human applications have been recently underlined [47].

21.6 PROBIOTICS AND ENZYME ACTIVITIES IN THE GI TRACT

Bacteria are known to be producers of extracellular enzymes but the information on this is still lacking. Nevertheless, we know that the microorganisms of the GI produce enzymes having a fundamental role in the whole digestive and metabolic process and in the pathogenesis of infectious diseases. For example, it has been demonstrated that *Bacillus* JAQ04 and *Micrococcus* JAQ07 can produce extracellular protease and lipase. However, the *Micrococcus* strain was unable to secrete α-amylase exogenously.

LI, also known as lactose malabsorption, is the most common type of carbohydrate malabsorption. It is associated with the inability to digest lactose into its constituents, glucose and galactose, due to lack or low levels of endogenous lactase. At birth, lactase activity is at its highest and it declines after weaning. The undigested lactose is hydrolyzed by the hingut bacteria and produces gas, hydrogen, and methane, as well as short-chain fatty acids. The consequent symptoms include: bloating, cramping, flatulence, and liquid faeces. LI is very frequent in Asian and Africans populations, whilst it is rare in Scandinavian populations [48].

Two possible interventions in the case of LI are the supplementation of commercially available lactase (tablets) or the addition of probiotics.

The consumption of lactase as a food supplement may assist in restoring adequate levels of the enzyme needed for hydrolysis of lactose, especially for patients with low, or nonexistent levels of lactase. On the other hand, lactase products are problematic since not all lactase preparations are of the same concentration. Moreover, it is difficult to assess the amount of lactase tablets needed in order to fully hydrolyze lactose in each dairy mill.

It has been clearly demonstrated that people with LI tolerate the lactose in yogurt better than the same amount of lactose in milk. The assumption is that the presence of lactase-producing bacteria in the yogurt, especially *L. acidophilus*, contributed to the digestion and absorption of lactose. Also, it has been shown that *L. bulgaricus* and *S. thermophilus* ameliorate LI by their ability to produce lactase. The consumption of milk containing *Bifidobacterium longum* results in significantly less hydrogen production and flatulence as compared to the consumption of control pasteurized milk. Thus, the administration of probiotics may assist with the consumption of dairy products containing lactose.

Azo dyes are organic colorants characterized by the presence of one or more azo groups. They are used widely in several industries since they are easy to synthesize and have chemical stability. However, the release of these compounds into the environment is undesirable, not only because of their color, but also

because many azo compounds and their breakdown products are toxic and/or mutagenic. The use of biological treatment of azo dyes by the action of bacteria is increasing [49,50].

Protein-enzymes with azoreductase activity have been identified and characterized from a wide variety of bacteria, such as *C. perfringens, Pigmentiphaga kullae, Xenophilus azovorans, E. faecalis, S. aureus, E. coli, Bacillus* sp., *Pseudomonas aeruginosa*, and *Rhodobacter sphaeroides*.

Thus, azoreductases are well represented in the GI tract and their metabolic products can be an important source of toxic or mutagenic compounds. Accordingly, probiotics reducing the azoreductase production strains appear as important targets [51,52].

21.7 CONCLUSIONS

The sales of probiotics are growing. Probiotics are said to improve human health and well-being, as well as the health and zoo technical performances of farmed animals including fish. Pets' health is also a target for probiotics. In humans, the general demonstration of the use of probiotics is related to the treatment of diarrhea and pouchitis. The evidence for the use of probiotics in non-GI illnesses is very weak. The high variability and general inconsistency of the results on the utilization of probiotics in human applications is also evident in animals. In this field, yeast-based probiotics seem to be more efficacious than those based on bacteria. Besides these concerns, safety issues related to the production of harmful substances and direct pathologies induced by some probiotic strains are present in the literature. Some probiotics can be interesting due to their role in the production of enzymes helpful for digestion and protection of the GI. For feed applications, it is suggested on the one hand to evaluate the probiotic potential of sporulating microorganisms from soil origin, and on the other hand, combinations of probiotics and feed enzymes.

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CHAPTER

Formulation of enzymes

22

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22.1 INTRODUCTION

Ideally enzymes and even any other food or feed additive should not need formulation which is a supplementary step in the production and has a relatively high cost. However, near all enzymatic products are handicapped by weakness in one or more of the following ways: dust production, low mixability capacity, weak resistance to the harsh conditions of composed feed preparation (pressure, moisture, and temperature), poor resistance to acidic denaturation and proteolytic hydrolysis in the stomach.

Although enzymes have been in use for a long time in several applications such as detergents, textiles, baking, brewing, etc., their use in the animal feed has been a more recent phenomenon. Feed enzymes need to be robust to withstand variations in pH and temperature. High-temperature stability allows the enzyme to withstand pelletization and also to have a long shelf life. Over the years, forms of feed enzymes have been going through an evolution from liquid to powder to granules in order to make them more and more heat stable.

Formulation is often considered as an art, and generally the precise details of the methods used to stabilize enzyme preparations are kept secret. Sometimes it is only the formulation of an enzyme that gives a manufacturer the competitive edge over rival companies. It should be remembered that most industrial enzymes contain relatively little active enzyme (<10% w/w, including isoenzymes and associated enzyme activities), the rest being composed of inactive protein, stabilizers, preservatives, salts, and the diluent, which allows standardization between production batches of different specific activities. The key to keeping enzyme activity is maintenance of conformation, thus preventing unfolding, aggregation, and changes in the covalent structure.

22.2 BASIS OF ENZYMES FORMULATION

Several types of enzymes' formulation have been developed. T-granulates (tough—"dusty frees") were initially developed for the detergent industry allowing physical

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strength and minimization of enzyme dust levels. T-granulates are manufactured with a combination of high-shear granulation and several coating technologies.

Bright-field/stabilizer-field (BF/SF)-granulates (small granulates) deliver mainly for bakery enzymes that have a much smaller particle size distribution than the products for the detergent industry. The small particle size distribution allows the products to be mixed homogeneously into flour. Big granulates/small granulates (BG/SG)-granulates are produced by spray-drying and fluid-bed agglomeration.

Spraying and drying the enzyme on a core particle in a fluid-bed led to a final product, micogranulte, free of fine dusty particles and accordingly much more safe for use in food industries. The fluid-bed technology is very flexible in handling different core materials for different industries.

Formulation by resistant coating (CT—coated and tough) make the enzymes able to withstand the rather high temperatures, pressure, and moisture experienced when the enzyme/feed mixture is processed into feed pellets.

Immobilized enzymes are mainly for the starch and oils/fats industries, but are also used within biocatalysis. The enzyme is immobilized on a carrier or in a matrix, which increases the enzyme's stability and prevents it from leaking into the substrate/product during application. The immobilized enzyme product is designed to perform the hydrolysis over several months in either batch or continuous process, allowing high productivity and lower costs.

Liquid formulations are largely used and are obtained and stabilized with polyols such as glycerol, sorbitol, or monopropylene glycol, sugars, and/or salts. The trick is to lower the water activity sufficiently to ensure that the solution is not only enzymatically but also microbiologically and physically stable. The key quality parameters for solid formulations are essentially low dust, particle size, and stability of the granulate in the final product formulation, e.g., enzyme feed additive. The main points in the liquid products formulations are enzymatic, physical, and microbial stabilities.

It is difficult to give an overview of the current food and particularly feed enzymes taking into account the very large number of such additives available in the market place. They are extremely diverse, regarding source, enzyme activities, stability, formulation, efficacy, and other characteristics [1]. To ensure that a candidate enzyme will be stable in the entire distribution and feed production chain, namely storage or processing of the feed, and in premixes and finished feeds. The ability to resist the often harsh conditions of the feed process is particularly important. Appropriate formulation technology such as granulation has proven to be the way to obtain the most stable pelleting products. In addition, granulation technologies can ensure that the products are safe and easy to handle (e.g., low dust and high flowability).

22.3 STABILIZATION AND IMPROVED RESISTANCE OF PROTEIN-ENZYMES

There are three possible approaches to improve the stability of nutritional enzymes: the use of additives, controlled use of covalent modification, and enzyme immobilization.

In general, proteins are stabilized by increasing their concentration and the ionic strength of their environment. Neutral salts compete with proteins for water and bind to charged groups or dipoles. This may result in the interactions between an enzyme's hydrophobic areas being strengthened causing the enzyme molecules to compress and making them more resistant to thermal unfolding reactions. Not all salts are equally effective in stabilizing hydrophobic interactions, some are much more effective at their destabilization by binding to them and disrupting the localized structure of water (the chaotropic effect). This is illustrated by the fact that ammonium sulfate and potassium hydrogen phosphate are powerful enzyme stabilizers, whereas sodium thiosulfate and calcium chloride destabilize enzymes. Many enzymes are specifically stabilized by low concentrations of cations which may or may not form part of the active site, e.g., Ca^{2+} stabilizes α-amylases and Co^{2+} stabilizes glucose isomerases. At high concentrations, salt discourages microbial growth due to its osmotic effect. In addition, ions can offer some protection against oxidation to groups such as thiols by salting-out the dissolved oxygen from solution.

Low molecular-weight polyols (e.g., glycerol, sorbitol, and mannitol) are also useful for stabilizing enzymes, by repressing microbial growth, due to the reduction in the water activity, and by the formation of protective shells which prevent unfolding processes. Glycerol may be used to protect enzymes against denaturation due to ice-crystal formation at subzero temperatures. Some hydrophilic polymers (e.g., polyvinyl alcohol, polyvinylpyrrolidone, and hydroxypropylcelluloses) stabilize enzymes by a process of compartmentalization whereby the enzyme–enzyme and enzyme–water interactions are somewhat replaced by less potentially denaturing enzyme–polymer interactions. They may also act by stabilizing the hydrophobic effect within the enzymes. Many specific chemical modifications of amino acid side chains are possible which may (or, more commonly, may not) result in stabilization. A useful example of this is the derivatization of lysine side chains in proteases with *N*-carboxyamino acid anhydrides. These form polyaminoacylated enzymes with various degrees of substitution and length of amide-linked side chains. This derivatization is sufficient to disguise the proteinaceous nature of the protease and prevent autolysis.

Important aspects regarding the molecular basis of thermostability have been evidenced by comparison of enzymes from mesophilic and thermophilic organisms. A frequently found difference is the increase in the proportion of arginine residues at the expense of lysine and histidine residues. This may be possibly explained by noting that arginine is bidentate and has a higher pK_a than lysine or histidine. Consequently, it forms stronger salt links with bidentate aspartate and glutamate side chains, resulting in more rigid structures. This observation, among others, has given hope that site-specific mutagenesis may lead to enzymes with significantly improved innate stability. In the meantime, it remains possible to convert lysine residues to arginine-like groups by reaction with activated urea. It should be noted that enzymes stabilized by making them more rigid usually show lower activity (i.e., V_{max}) than the "natural" enzyme.

Enzymes are very much more stable in the dry state than in solution. Solid enzyme preparations sometimes consist of freeze-dried protein. More usually they are bulked out with inert materials such as starch, lactose, carboxymethylcellulose, and other poly-electrolytes which protect the enzyme during a cheaper spray-drying stage. Other materials that are added to enzymes before sale may consist of substrates, thiols to create a reducing environment, antibiotics, benzoic acid esters as preservatives for liquid enzyme preparations, inhibitors of contaminating enzyme activities and chelating agents. Additives of these types must, of course, be compatible with the final use of the enzyme's product.

Enzymes released onto the market should conform to a number of quality procedures including regulatory requirements, which are legal and mandatory. This is provided by the quality assurance within the company. Enzyme products must be consistent as appropriate to their intended use. This may be ensured by good manufacturing practice and further checked by quality control.

Whole pancreas extracts are administered to patients suffering from mucovicidose as a complementary therapy for their exocrine pancreatic insufficiency. The administrated dosages are very high to overcome the destruction in the stomach of the ingested one. Without protective formulation near 50% of the amylase, trypsin and chymotripsin activities and around 90% of that of lipase disappear at the pylorus level [2]. In patients suffering from cystic fibrosis there is limited evidence of the benefit from enteric-coated microspheres when compared to nonenteric-coated pancreatic enzyme preparations. There is no evidence on the long-term effectiveness and risks associated with pancreatic enzyme replacement therapy [3].

Developing enzyme products that are efficacious, stable, and safe has always been the main aim of enzyme suppliers. Formulation of hydrolases is the fourth phase of the production process after selection, fermentation, and recovery. Although much effort focuses on finding the right enzyme molecules for a given application, formulation plays a big role in the final use and usefulness of the product. The nature of the enzyme protein is the starting point of all formulation work, and knowledge about parameters such as solubility and compatibility is indispensable. A new enzyme molecule with excellent performance can fail in the application if the enzyme is not stable during transportation and storage as well as efficacious in the application level. The right formulation can bypass these inconveniences. Except for enzymes that are stable and efficient as they are, the importance of performing the right formulation is mandatory. Different needs of formulated enzymes are covered by liquid and several solid forms, including immobilized ones.

The retention of the activity is the main objective of the producers of enzymes after desired purification degree and concentration. Often a freshly supplied enzyme sample will have a higher activity than that stated by the manufacturer. This is done to ensure that the enzyme preparation has the guaranteed storage life.

The manufacturer will usually recommend storage conditions and quote the expected rate of loss of activity under those conditions. It is of primary

importance to the enzyme producer and customer that the enzymes retain their activity during storage and use. Some enzymes retain their activity under operational conditions for weeks or even months. However, most enzymes do not. Most industrial enzyme preparations contain a relatively small amount of active enzyme, the rest being due to inactive protein, stabilizers, preservatives, salts, and the diluent which allows standardization between production batches of different specific activities. The key to maintaining enzyme activity is maintenance of conformation, so preventing unfolding aggregation of the enzyme molecules and changes in the covalent structure. Three approaches are possible for that objective: use of additives, controlled use of covalent modification, and enzyme immobilization.

Formulation cost is a fundamental determinant factor in the use of enzymes in a technological process. Hundreds of enzymes are available at very different prices, some are much cheaper and many are much more expensive. As enzymes are catalytic molecules, they are not directly used up by the processes in which they are used. Their high initial cost, therefore, should only be incidental to their use. However, due to denaturation, they do lose activity with storage time. They should be stabilized against denaturation to be utilized in an efficient manner. When they are used in a soluble form, they retain some activity after the reaction which cannot be economically recovered for reuse and is generally wasted. This activity residue remains to contaminate the product and its removal may involve extra purification costs. In order to eliminate this wastage, and give an improved productivity, simple and economic methods must be used which enable the separation of the enzyme from the reaction product. The easiest way of achieving this is by separating the enzyme and product during the reaction using a two-phase system; one phase containing the enzyme and the other phase containing the product. The enzyme is imprisoned within its phase allowing its reuse or continuous use but preventing it from contaminating the product; other molecules, including the reactants, are able to move freely between the two phases. This is known as immobilization technology and may be achieved by fixing the enzyme to, or within, some other material. "Immobilization" does not necessarily mean that the enzyme cannot move freely within its particular phase, although this is often the case. A wide variety of insoluble materials, also known as substrates (not to be confused with the enzymes' reactants), may be used to immobilize the enzymes by making them insoluble. These are usually inert polymeric or inorganic matrices.

Immobilization of enzymes often incurs an additional expense and is only undertaken if there is a sound economic or process advantage to the use of the immobilized, rather than free (soluble), enzymes. The most important benefit derived from immobilization is the easy separation of the enzyme from the products of the catalyzed reaction. This prevents the enzyme from contaminating the product, minimizing downstream processing costs and possible effluent handling problems, particularly if the enzyme is noticeably toxic or antigenic. It also allows continuous processes to be practicable, with a considerable saving in

enzyme, labor, and overhead costs. Immobilization often affects the stability and activity of the enzyme, but conditions are usually available where these properties are little changed or even enhanced. The productivity of an enzyme, so immobilized, is greatly increased as it may be more fully used at higher substrate concentrations for longer periods than the free enzyme. Insoluble immobilized enzymes are of little use, however, where any of the reactants are also insoluble, due to steric difficulties.

Emulsification of oleoresin of *Nigella sativa* L. (Black cumin) in an aqueous solution containing gum Arabic/maltodextrin (1:1 w/w) followed by encapsulation in powder form by spray-drying show that the encapsulation efficiency of the whole oleoresin in the powder range from 84.2% to 96.2% and that of the volatile oil fraction was 86.2%. This formulation of *N. sativa* oleoresin powder can be used in the fortification of processed food and nutraceuticals [4].

Lipases are known for their versatility in addition to their ability to digest fat. They can be used for the formulation of detergents, as food ingredients, and as biocatalysts in many industrial processes. Because conventional enzymes are frangible at high temperatures, the replacement of conventional chemical routes with biochemical processes that utilize thermostable lipases is vital in the industrial setting. Theoretical studies on enzymes have provided numerous fundamental insights into the structures, folding mechanisms, and stabilities of these proteins. The studies corroborate the experimental results and provide additional information regarding the experimental structures [5].

22.4 NUTRITIONAL ENZYMES AND SPECIFIC REQUESTS OF FORMULATION

Use of exogenous enzymes in animal diets has attracted considerable interest over last 20 years. Microbial phytase is one of the most commonly used enzymes in monogastric animal diets [6]. Feedstuffs of plant origin form the major constituents of monogastric diets. About two-thirds of P of plants is present as phytate. Phytate-P is unavailable to monogastrics [7]. In addition, phytate-P chelates several important minerals as well to form complexes with proteins, thereby reducing their bioavailability. The addition of microbial phytase to the diet has been reported to improve the utilization of phytate phosphorus in rainbow trout *Oncorhynchus mykiss* [8] and common carp *Cyprinus carpio* [9]. Availability of protein, P, and other elements from fishmeal, soy protein concentrate and phytase-treated soy protein concentrate-based diets to Atlantic salmon, *Salmo salar* was found to be different [10]. In *Pangasius pangasius* phytase supplementation significantly improved weight gain, protein efficiency, apparent net protein utilization, and feed-conversion efficiency in fingerlings [11].

NSP enzymes should act in the intestine and thus should keep the activity after crossing the stomach.

Plant protein sources are more in use as alternatives to costly and scarce fishmeal in the aquafeed. A major concern with the plant ingredients is the presence of a wide variety of antinutritional substances. Phytic acid is one such antinutritional factor found in most of the feedstuffs such as barley, rice, sorghum, wheat, maize, gram, groundnut, rapeseed, soybean, cottonseed, and sesame. High protein content make soybean meal a potential promising alternative protein source in aquafeed. However, about two-thirds of the P in soybean meal is present as phytate, which is not efficiently utilized by monogastric animals, including fish. Because of its high density of negatively charged phosphate groups, phytate forms mixed salts with mineral cations called phytin. During germination, phytin is degraded by the action of phytases, which provides the growing seedlings with phosphate, mineral cations, and myo-inositol. Apart from its storage function, phytate has also been assumed to play an important role in P homeostasis, buffering cellular P levels. Phytate-P makes complexes with other nutrients such as minerals, protein, carbohydrate, and lipids, thereby rendering them unavailable. Hence, phytate-rich ingredients have become major concern among researchers and nutritionists while formulating aquafeed.

Aquaculture is one of the fastest developing sectors in the world, now accounting for nearly 50% of the world's food fish and aquafeed, making is an important industry in the world. In feed pellet production, two types of aquafeed processing technology are widely employed: pelleting (mainly produce sinking aquafeeds) and extrusion (can produce sinking or floating extruded aquafeeds). According to their buoyancy, fish feed can also be divided into sinking, slow sinking, and floating aquafeed. Floating aquatic feed is suitable for tilapia, crucian, ranidae, weever feeds, etc., and sinking aquatic feed for *Siganus guttatus* feed, etc. Pelleting is the process to compress small particles into larger solids with a given shape and texture, which involves the combination of moisture, heat, and pressure. The quality of pelleted feed is influenced by the quantity of fat, fiber, and starch in the formula. In the pelleting process, steam can be added into the ingredients for starch gelatinization, which is conducive to ingredients binding. Generally, an amount of steam is added to the mash to increase its moisture content to approximately 16% and temperature to about 85°C before passing through the pellet die. Extrusion requires higher levels of moisture, heat, and pressure than pelleting. Temperature higher than 100°C is needed in order to achieve expansion of the feed as it leaves the die. Extrusion consists of wet extrusion and dry extrusion. Pelleted aquafeed has lower stability in water, lower digestibility, and lower processing cost than extruded aquafeed. The manufacturing process in slower for extruded aquafeed. Extrusion can be either dry or wet. Preconditioning is a very important part of the wet extrusion process for steam or water injection into either the conditioner and or the extruder barrel. The heart of the extruder machine working is: preconditioning, cooking, and die shaping. A high-temperature and high-pressure process can sanitize the final product. Wet extruder is usually employed for high quality pellet production on a large scale.

Without utilizing preconditioning, dry extrusion employs lower moisture levels. Therefore it is suitable for processing low moisture and high fat feed material in small and medium sized production.

Diets for farmed fish require diets containing 30%−55% of crude protein and an amino acid supply precisely adapted to meeting the needs for optimal growth. Fishmeal is an ideal protein source for aquafeed because it has a high protein content (65%−72%) and an ideal profile of indispensable amino acids. Finding alternatives to fishmeal has become an absolute and urgent need in order to drastically reduce the pressure on natural marine fish stocks. Animal by-product meals are used as alternative protein sources in aquafeed in non-European countries. Particularly in Europe, more attention has been given to the use of plant protein sources that are available in large amounts. The limitations with regard to their incorporation in aquafeed are related to the levels of protein, the amino acid profiles, the presence of antinutritional factors and the lower digestibility coefficients. Numerous studies were and are being performed with different fish species leading to improved knowledge on the potential of various plant resources for fish nutrition [12,13].

Until now, fish diets with a high level of soybean meal supplemented with different enzyme cocktails resulted in lower performance of rainbow trout, compared to animals fed fishmeal [14]. In shrimp and carnivorous fish species, the replacement of fishmeal by plant protein sources has only achieved limited success. Carnivorous fish species require good quality and highly digestible protein source in their feed. Among plant protein ingredients, soybean meal is considered as the most promising plant protein source. However, the high concentration of antinutritional factors limited the inclusion levels of soybean meal in aquafeed. In soy-protein concentrate, the antinutritional components are eliminated. Soybean meal, soy-protein concentrates, soybean oil, and other vegetable proteins and oils, can replace from one-third to one-half of the fishmeal in feeds for many farmed species, reducing the need for wild-caught fish for fishmeal. Soybean meal costs significantly less than most animal meals, including fishmeal. Reducing feed costs is critical to improving efficiency and maintaining sustainability in aquaculture operations. Because the nutrient requirements of farmed fish and shellfish are so complex, each feed ratio is formulated based on the individual species' needs. Most farm raised fish and shellfish can easily digest soybean meal, which helps the fish to transform ingested protein into body muscle more efficiently.

Compared to fishmeal, soy-protein concentrate has the advantage of high protein and amino acids digestibility coefficiency, it is readily available, and is of consistent quality. Literature studies demonstrate that soy protein concentrate is a good alternative to fishmeal in shrimp and fish diets. In shrimp diet, 40% of fishmeal can be replaced by soy protein concentrate without negative influence on growth performance. In fish, 40%−100% of fishmeal can be replaced by soy protein concentrate. In conclusion, soy protein concentrate is a good protein source for aquafeeds. However, it appears that research efforts should be made with the aim of stabilizing the right mixture of enzymes.

Lipase is particularly sensitive to gastric denaturation and proteolysis [2]. Accordingly, since it should act in the first segment of the small bowel, its formulation should be particularly protective of the enzyme activity. For extruded feeds, the best technique for incorporation of enzyme supplements is pulverization post-extrusion, preferably in vacuum mixers, allowing deep penetration of the enzyme in the extrudates. Also, with pulverization, negative effects of denaturation and of Maillard reactions are avoided [15].

22.5 BUFFERS

Buffers comprise an integral component of enzymes formulations. Not only do they function to regulate shifts in pH, they also can stabilize enzymes by a variety of mechanisms. Addition of buffers can result in increased conformational stability of enzymes, whether by ligand binding or by an excluded solute mechanism. In addition they can alter the colloidal stability of proteins and modulate interfacial damage [16].

Polymers are often used in enzymes formulation as well as in that of several oral drugs [17]. One of the characteristics of some multifunctional polymers (i.e., chitosan, polyacrylic acid) is their increased buffer capacity. Anionic and cationic polymers act like ion-exchange resins, maintaining the constant pH value inside the polymer network for a certain period of time. This may contribute to the increase in stability of the incorporated enzyme and/or drug, limiting its pH-dependent denaturation or enzymatic degradation. Similarly, in order to prevent degradation of the enzyme in the intestine, polyacrylates in their acidic form may be used [18] that inhibit the activity of proteases, such as trypsin and chymotrypsin, which are active only at pH values greater than 4. Polymers in enzyme and pharmaceutical technology constitute an important class of excipients of constantly increasing significance. Multifunctional polymers constitute a group of compounds which, apart from their basic functions, may additionally play an important role in formulating intelligent enzymes and drugs delivery systems, thus contributing to the improvement of achieved effects.

22.6 NANOFIBERS

Nanofibers have wide applications in medicinal pharmacy, textiles, tissue engineering, and in various drug delivery systems (DDSs). In oral DDSs, nanofibers can be delivered as nanofiber scaffolds, electrosponge nanofibers as oral fast delivery systems, multilayered nanofiber loaded mashes, surface-modified cross-linked electrospun nanofibers. Nanofibers are of 50–1000 nm in size, having large surface area, high porosity, small pore size, and low density. Various approaches for formulation of nanofibers are molecular assembly, thermally

induced phase separation, and electrospining. Most commonly utilized electrospining polymer nanofibres with different range can be produced collective usage of electro spinning with pharmaceutical polymers offers novel tactics for developing DDSs. Different polymers used in the preparation of nanofibers include biodegradable hydrophilic polymers, hydrophobic polymers, and amphiphilic polymers. Electrospun nanofibers are often used to load insoluble drugs for enhancing their dissolution properties due to their high surface area per unit mass. Besides the water-insoluble drugs, freely water-soluble sodium can also be spun into the fibers. The most commonly used polymers for nanofibers are gelatin, dextran, nylon, polystyrene, polyacrylonitrile, polycarbonate, polyimides, polyvinyl alchol, and polybenzimidazole. Delivery systems reviewed rely on temporal control, changes in pH along the gastrointestinal tract (GIT), the action of local enzymes to trigger drug release, and changes in intraluminal pressure. Dissolution of enteric polymer coatings due to a change in local pH and reduction of azo-bonds to release an active agent are both used in commercially marketed products. In vitro and in vivo studies have demonstrated that the release rates of drugs from these nanofiber formulations are enhanced compared to those from original drug substances. This review [19] is focused on the different types of polymers used, different used in the preparation of nanofibers, cytotoxicity studies, and application of nanofibers by using oral drug delivery.

22.7 GELATIN, MICROENCAPSULATION, AND REPLACEMENT

Gelatin is a protein of animal origin consisting of 84%–90% protein. That of pig is banished by two groups of humans because it is considered as not being Kosher or Halal. There are different alternatives to animal gelatin. The more important are: agar-agar, a binding and gelling vegetable perfect for replacing the animal gelatin; carrageenan, also known as Irish moss, polysaccharide (galactan) extract of red algae; pectin or more broadly the pectin substances are polyosides, attached to the carbohydrate from gooseberries, apples, quinces, and citrus seeds and zest; xanthan gum, polysaccharide (consisting of a combination of four compounds: glucose, mannose, glucuronic acid, and pyruvic acid) obtained from the action of *Xanthomonas campestris*; cellulose gum occurs as granular or fibrous powder.

Gelatin is generally regarded as safe. Although, at the beginning of the bovine spongiform encephalopathy scare in Europe, the popular media brought suspicion on all products of bovine origin. Besides being precipitated by polymers competing for water, gelatin is amphoteric. Fish skin gelatin is available commercially and can be produced for kosher use. The low gel-strength gelatin has been used to emulsify vitamin A before spray-drying to give another type of microencapsulated product using gelatin.

Gelatin is recognized in many industries as a stabilizer, a texturizing agent, a film former, and as a colloidal support medium. Depending on the concentration

and temperature, the emulsion can be free flowing, semisolid, or solid. Gelatin is used to produce microencapsulated oils for various uses, both in nutritional and pharmaceutical applications [20]. The traditional method of encapsulation is known as coacervation in which the dispersed oil is encapsulated by gelatin at the interface between the aqueous phase and the nonaqueous phases. Typical microcapsules range in size from 5 to 500 μm.

22.8 PERSPECTIVES AND CONCLUSION

The positive effects of feed enzymes cannot be questioned. It is expected that use of enzymes will increase because alternative feedstuffs, often with poor digestibility by the endogenous enzymes of monogastric animals, will be increasingly included in feed, and one can even suggest that sooner or later also in food. For the moment, crops used in swine and poultry feeding are in direct competition with humans. Regarding aquafeed production, the limit for fish capture needed for fishmeal production is recognized to be excessive and dramatic for the environmental equilibrium of the oceans. Also, 70% of the total extracted quantities by the industrial fisheries is utilized for meal production and reasonable are edible by Humans. By the alternative feedstuffs will require exogenous efficacious enzymes. The usefulness of such enzymes will increase and facilitate by appropriate enzymes or enzymes cocktails with some reinforced properties: strong natural activity, improved heat resistance, low production cost, long storage life, resistance to denaturation and proteolysis, broad pH range activity. Expanded aquaculture production will require more fish feed, which will in turn require higher quantities of alternate protein sources to substitute fishmeal. These proteins will be supplied from a variety of sources, most of which will require special processing or enzyme supplementation to explore their full potential. Increasing the use of phytase in aquaculture offers a tremendous opportunity to allow the use of low-cost plant meals.

Enzymes aimed at the hydrolysis of the very resistant fractions of the biomass, particularly laccases, will have a hugely important role in the near future. Several white-rot fungi thermoresistant laccases have been isolated and expressed recently. The potential for producing biochemicals, biofuel, and nutritional compounds will require strong research activity into their formulation.

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CHAPTER

Analytics of enzymes

23

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23.1 INTRODUCTION

Enzymes as feed additives play an important role in modern animal husbandry today. They are used to improve the health status of the animals and to optimize the feed conversion. The most prominent enzymes, presently used as feed additives, are phytases, xylanases, beta-glucanases, proteases, and amylases.

In human nutrition, enzymes are used as dietary supplements to improve human health or to overcome intolerance discomfort, which some people have to particular foodstuffs. Some examples are lactase as a digestive aid to eliminate lactose intolerance discomfort, or protease that can help people who are sensitive to gluten. Phytase hydrolyzes the antinutrient phytate, releasing the bound minerals iron, zinc, calcium, and phosphorus, which are important micronutrients for the human body.

All these enzymes require analytical tools in order to quantify the enzyme concentrations in straight products and in more complex matrices such as feed, food, or premixes. In order to follow enzyme activity during processing of the feed or food the analytics has to be accurate, robust, and as specific as possible. Sheehan [1] discussed the methods found in the literature and addressed some analytical problems which occur with the feed matrix.

In this chapter, the challenges in method development for enzymes and possible approaches are discussed for those not familiar with enzyme analytics because most of the enzyme analytical methods used by the industries are not in the public domain. In addition, most enzyme analytical methods were developed for a particular enzyme and cannot be used as they are for other enzymes belonging to the same class.

23.2 ENZYME ACTIVITY AND DEFINITION OF ENZYME ACTIVITY

Enzymes are highly specific molecules which act as catalysts for biochemical reactions. They show an inherited particular profile such as substrate specificity or pH and temperature optimum. The profile for an enzyme activity varies

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depending on its origin. EC 3.2.1.8, e.g., is the official nomenclature for endo-1,4-beta-xylanses. Under this EC number, numerous different microorganisms are listed (see, e.g., BRENDA database) which have an endo-1,4-beta-xylanse. However, all these endo-1,4-beta-xylanses may have different biochemical characteristics, which can have implications on the analytics. An analytical method developed for a particular enzyme may not necessarily be applicable for other enzymes belonging to the same EC number.

The reaction rate of the enzyme is used for the quantification of enzymes. The Nomenclature Committee of the International Union of Biochemistry [2] recommended that the reaction rate should be expressed in moles per second in order to adhere to the Système International (SI) units. In addition, they proposed katal as a new unit of enzyme activity, which is in line with the SI. In most cases, the catalytic activities are expressed in microkatals (μkat), nanokatals (nkat), or picokatals (pkat). However, this recommendation has met with little response within the food and feed industries. The most frequently used declaration of enzyme activity in the food and feed industries is still given in units according to the International Union of Biochemistry [3]. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the transformation of 1 μmol of the substrate per minute under standard conditions. The relation between enzyme units (U) and katals is given by 1 μmol per minute (U) corresponds to 0.01667 μkat (μmol/second = 1/60 U), respectively, 16.67 nkat. In addition, there are many other unit definitions and unit abbreviations in use by the feed and food enzyme industries, which are not related to the definitions mentioned above. The expression of the active enzyme content in units is therefore only useful for analytical purposes of a particular product. Comparing declared units of different products with the same enzyme activity cannot draw any conclusions of, e.g., the potency of a product, especially since the reaction conditions may be different from one enzyme to another.

23.3 ENZYME SUBSTRATE AND ITS INFLUENCE ON ANALYTICS

Enzymes are typically highly specific, catalyzing chemical reactions on a specific substrate. The substrate binds to the active site of the enzymes leading to an enzyme–substrate complex. After releasing the product, the enzyme is again ready to bind the next substrate molecule.

The substrates used for enzyme analytics are either purified from natural sources or synthesized. Synthesized substrates for feed or food enzymes are usually small molecules having very high purity when compared to natural substrates. The advantage of synthesized substrates is the constant high quality of the substrates, which show no batch-to-batch variation. However, some of them are poorly soluble in buffer solution. Natural substrates can have very complex structures like nonstarch polysaccharides (NSP). Choct [4] described the definition and

classification of NSP and gave some information about the chemical structure and the levels found in different cereals. The quality of natural substrates is a critical issue for the analytics. The substrates often show high batch-to-batch variations, which influence the quality of the analytical data. Changing the substrate batch can be destructive for established analytical methods. For routine enzyme analytics in the feed and food industries it is recommended to have the appropriate substrates in adequate supply and quality available. In addition, there are only a few suppliers of such substrates. The limited availability of different batches of a substrate makes the screening for the best substrate batch difficult.

Not all substrates available can be used for the determination of enzyme activity in complex matrices like feed, food, or premixes. Xylanases or beta-glucanases, e.g., cleave arabinoxylan and beta-glucan, respectively, and release reducing sugar equivalents. The increase of reducing sugar equivalents during the enzyme reaction is often measured as described by Miller [5]. However, these analytics can only be used for products but not for the analytics of NSP enzymes in complex matrices such as feed, which contains high levels of reducing moieties. In contrast, the phytase substrate, phytic acid, where the release of phosphate is monitored, can be used for the analyses in all matrices. McCleary [6] and Biely et al. [7] described the preparation of dye-labeled polysaccharides for the assay of endohydrolases and discussed the advantages and disadvantages of such substrates. Also, these substrates show high batch-to-batch variations as discussed by McCleary [6]. Dye-labeled substrates or synthesized substrates with a chromophore can be used for all matrices. There are soluble and insoluble dye-labeled substrates commercially available. The soluble substrates have some advantage over the insoluble substrates. In particular, well-defined substrate concentrations can be chosen with soluble substrates, which ensure a linear reaction during the defined enzyme reaction time. In addition, the analytical methods can be optimized economically and for high sample throughput.

23.4 DIRECT AND INDIRECT METHODS

In this chapter, “direct” methods refer to methods that use standards, which correspond to the product of the enzymatic reaction, such as phosphate for the quantification of phytase activity. Other examples are glucose and fructose standards, respectively, for the quantification of reducing equivalents, released from beta-glucan or arabinoxylan by glucanases and xylanases, respectively.

“Direct” methods have the advantage that the standards are commercially available. This allows everyone skilled in enzyme analytics to establish such methods in their laboratory. However, these methods are highly dependent on the substrate batch used in the original method. The use of other substrate batches can lead to inconsistent data. König et al. [8] described a method for the determination of xylanase, beta-glucanase and cellulase activity, and compared the data

obtained with different wheat arabinoxylan and beta-glucan substrate batches. They showed that similar batches could be identified. The batch-to-batch variation was usually in the range of $\leq 15\%$.

The "direct" methods are sensitive to changes in the procedure. Deviations of incubation temperature, incubation time or pH are reflected in the results, since the standards cannot compensate for these changes. Therefore, skilled people and accurate equipment is required for these methods.

"Indirect" methods refer to methods that employ a validated enzyme standard of the enzyme under investigation for quantification. "Indirect" methods are less sensitive to changes in the procedure, since the enzyme standards undergo the same reaction conditions as the samples. Different substrate batches may have less impact on the data compared to the "direct" methods. However, the enzyme standards are commercially not available. The dependency on the validated enzyme standards could cause some difficulties to establish such methods in an external laboratory.

23.5 DETERMINATION OF ENZYME ACTIVITY IN FORMULATED PRODUCTS

For the determination of enzyme activities in products "direct" as well as "indirect" methods are used. The ingredients used for the formulation of the enzymes usually do not interfere with the enzyme assay. For the quantification of the enzyme activity in products, synthetic, natural or dye-labeled substrates are used. For phytase products, phytic acid is used as substrate and the release of phosphate is quantified with a phosphate standard curve. For the quantification of polysaccharide endo-hydrolase activities, natural substrates are often used based on the methodology described by Miller [5]. The polysaccharide endohydrolases cleave the substrate releasing moieties with reducing ends. The reducing moieties are oxidized in an alkaline milieu by forming orange–yellow compounds with the 2-hydroxy-3,5-dinitrobenzoic acid (dinitro salicylic acid). These orange–yellow compounds are measured at a wavelength of 530 nm. The released reducing ends are quantified with glucose and xylose standard curves, respectively. Other enzyme producers use dye-labeled substrates, where the released soluble dye-labeled fragments are separated by centrifugation, when nonsoluble substrates are used. The dye-labeled fragments released from soluble chromogenic polysaccharide substrates require a precipitation step with organic solvent after the enzyme reaction, as described by McCleary [6]. The dye-labeled fragments in the supernatant are measured at 590 nm and quantified with the corresponding enzyme standard curve. The use of "indirect" methods for quality control has the advantage that small changes of the defined reaction conditions do not significantly change the result. For accurate measurements of enzyme activity in products, the original method set up by the producer of the enzyme should be used.

23.6 SAMPLE PREPARATION

Extraction of samples is a crucial step in enzyme analytics. For a good extraction yield of the enzymes from complex matrices like feed or food, the samples have to be strongly stirred during extraction. Adequate equipment, as described, e.g., in method ISO 30024 [9], should be used. For each enzyme the best extraction procedure has to be investigated. This includes the buffer system, concentration and pH of the buffer, extraction temperature, and extraction time. An optimized extraction procedure ensures reproducible data with regard to the extraction, but does not ensure 100% recovery of the enzyme activity from the feed or food. Lower recoveries are mostly due to inhibition of the enzymes by certain proteins found in cereals. Some examples for inhibition are proteins from wheat that inhibit xylanases [10,11], or protease inhibitors [12] or alpha-amylase inhibitors from wheat [13]. There are many more inhibitors in cereals. The degree of inhibition depends on the origin of the enzymes. Nonoptimized extraction procedures can also result in lower recoveries.

Engelen et al. [14] proposed grinding 100−150 g of feed sample and 5 g from the ground feed were taken for the extraction. In our experiments, grinding of the feed samples before analysis did not necessarily result in an homogeneous distribution of the product in the feed sample leading to data with low standard deviation. It even turned out that grinding of feed samples could result in losses of enzyme activity. Therefore, grinding of feed samples is not only labor-intensive, but it also can have a negative impact on the data. It is recommended to omit grinding for the determination of enzyme concentrations in feed samples. However, the sample size used for extraction has to be increased. As a compromise, we propose the use of 50 g of nongound feed for the extraction in 500 mL extraction buffer. This sample size is manageable and high analysis throughput is ensured.

The inhomogeneity observed in feed samples can have several causes. Either the enzyme product itself was not homogeneous, or only small amounts of enzyme product were added to the feed. Low particle number per gram enzyme product or the feed mixing, when not optimally set up, could contribute to inhomogeneity. As a comparison, feed supplemented with liquid enzyme products using well-adjusted spraying equipment led to highly homogeneous feed. This was reflected in the analytical data obtained with such feed samples, which showed very low variation.

Besides the feed preparation, the variation in the data can also be influenced by the analytical procedure used. It is important to use optimized extraction procedures and enzyme reaction conditions to obtain accurate and reproducible data.

23.7 ENZYME ASSAY AND SPECIFICITY OF ENZYME ASSAYS

The determination of enzyme activity in sample extracts (e.g., feed extracts) requires an enzyme assay, which is as sensitive as possible, especially when the enzyme activity is inhibited by components in the extract. The enzyme substrate

plays an important role in the sensitivity of an assay. If several substrates come into consideration, all substrates should be tested for the selection of the most adequate one. Natural substrates, dye-labeled or not, show high batch-to-batch variation. Therefore, it is recommended to test different batches, if available. The availability of a substrate should also be considered for the final selection of a substrate. Once the substrate is selected, it is advantageous to have adequate quantities of the substrate batch available. The substrate batch used in an established enzyme assay cannot be easily replaced by a new batch of the substrate. If the substrate batch has to be replaced by a new batch, the analytical method has to be revised in such a way that the data obtained with the new substrate batch are in agreement with the former batch. The batch-to-batch variation may be less pronounced in the data, when a validated enzyme standard is used for the quantification (indirect method).

For some enzymes (e.g., lactases, proteases) synthetic substrates are commercially available. Examples of soluble synthetic substrates are *N*-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide for serine proteases, *N*-carbobenzoxy-glycyl-L-proline-4-nitroanilide for prolyl endopeptidases or o-Nitrophenyl-β-D-galactopyranosid for β-galactosidases. Synthetic substrates are highly purified small molecules that show no batch-to-batch variations. There are also synthetic substrates of low solubility in water or buffer available. These substrates are not suitable for routine enzyme analytics.

After selection of the appropriate substrate, optimal reaction conditions have to be set up for accurate and reproducible data. The aim is to find reaction conditions where the assay shows the highest sensitivity. Yoon and Robyt [15] described the influence of Triton X-100, polyethylene glycols, and polyvinyl alcohols on activation and stability of 10 starch-degrading enzymes. All of the additives gave activation and stabilization of the 10 enzymes, but there was one specific additive and concentration for each enzyme that gave a maximum degree of activation. Zheng et al. [16] described Tween 20 (polysorbate 20) followed by Tween 80 (polysorbate 80) as the most effective nonionic detergent activator for the cellulose under investigation. Tween 20 was most effective for the activation and stabilization of our enzymes. However, for each enzyme the appropriate Tween 20 concentration had to be investigated.

McComb and Bowers [17] showed that optimum buffer conditions could drastically increase the sensitivity of the enzyme assay. They tested 23 compounds as buffer systems for human serum alkaline phosphatase. The best buffer at optimal buffer concentration and pH could increase the sensitivity of the assay by a factor of at least 100 compared to the buffer system with the lowest activity. Thompson et al. [18] tested the bovine alkaline phosphatase in six aminoethanol buffers. They showed that progress curves in ethanolamine and 2-(methylamino)-ethanol buffers were linear from zero time to at least 60 minutes, whereas progress curves in the other buffers became nonlinear in a short time due to product decomposition. They also showed that the optimal buffer concentration and the addition of magnesium ions enhanced enzyme activity.

The optimal reaction pH and temperature depend on the enzyme under investigation. Each enzyme has its characteristic pH and temperature optimum where the enzyme reaches the highest reaction rate. Temperature above the optimum will lead to denaturation of the enzyme. Therefore, the enzymatic reaction temperature should not exceed the optimum temperature. A reaction temperature slightly below the optimum should be chosen, in order to ensure the stability of the enzyme during the reaction time. Raising the reaction temperature by 10°C will increase the reaction rate of most enzymes by 50%−100%. This increase in activity improves the sensitivity of the assay, but it is not an essential increase, except for assays, which show a very low sensitivity. When the enzyme is inhibited, an increase in the incubation temperature may help to reduce or even eliminate the inhibition. The sensitivity of an enzymatic assay can be drastically improved by the reduction or elimination of inhibition.

Each enzyme assay requires an appropriate standard for the quantification of the enzyme. For enzymes, which do not interfere with components in the sample extracts, an external standard curve in the assay buffer can be set up. Depending on the substrate used for the enzymatic reaction, a direct or an indirect method can be used. Phytases presently on the market are not inhibited by components in the feed extract. For the quantification of the phytases, a direct method was chosen as described in method ISO 30024 [9]. The phytases are quantified with a phosphate standard curve. Also, for lactases a direct method was chosen as described in the Food Chemicals Codex [19]. The lactases are quantified with an *ortho*-nitrophenol standard curve. For enzymes reacting on complex substrates such as NSP, indirect methods were set up with validated enzyme standards for the quantification of the enzymes.

Validated enzyme standards are also required for the quantification of enzymes, which are inhibited by components in the sample extracts, or enzymes not following a first-order reaction.

An enzyme assay developed for a particular enzyme is not specific for this enzyme. Endogenous enzymes, acting on the same substrate, will also contribute to the final data. Therefore, the data obtained with such feed are the sum of added enzyme and endogenous enzyme activity. In addition, the assay cannot distinguish between enzymes of the same type that come from different origins.

23.8 HOW TO OVERCOME INTERFERENCES

Modification of enzyme activity by matrix components is often seen with xylanases, amylases, and proteases. However, listing may not be complete with these three types of enzymes. Inhibition can be, depending on the degree of inhibition, a throwback for the analytics. The sensitivity of many assays is not very high and inhibition reduces the sensitivity of the assay additionally. The methods have to be optimized in such a way that the inhibition is reduced to a minimum. The tools

for improvement are limited to temperature, pH, buffer system, and dilution of extracts. In some cases, increasing the temperature during the extraction of the sample can inactivate the inhibitor, if the inhibitor is a protein that does not survive the extraction temperature in contrast to the enzyme. Some inhibitors are sensitive to low or high pH. This can be used for the extraction procedure as well as for the enzymatic reaction, depending on the enzyme.

The following example with an endoprotease demonstrates the possibilities available for method development. Charney and Tomarelli [20] described a colorimetric method for proteolytic activity using azocasein as substrate and DelMar et al. [21] described *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitrianilide (Suc-AAPF-pNA) as a sensitive substrate for chymotrypsin. Comparison of the reaction rates with our endoprotease revealed that the substrate Suc-AAPF-pNA was much more sensitive compared to the azocasein substrate. Therefore, the Suc-AAPF-pNA substrate was chosen for the further method development. The optimal extraction pH for endoprotease-supplemented feed samples was determined. Feed was spiked with 5 mg endoprotease protein per kg and 25 g feed were extracted at room temperature for 60 minutes with 250 mL 0.1 M citrate buffer, containing 0.01% Tween 20, with the appropriate pH ranging from pH 1.2 to 5.0. The final pH of the feed extract was measured. Feed extracts were 50-fold diluted with 0.1 M citrate buffer pH 8, containing 0.01% Tween 20. The diluted samples had a pH between 6.8 and 7.2. The reaction conditions used for the determination of the activity were 1 mM Suc-AAPF-pNA pH 7.0 at 37°C for 30 minutes. The data are summarized in Fig. 23.1. The optimal extraction pH for feed samples containing the endoprotease was found to be between 2.3 and 3.3. Within this pH range 40%−43% of the endprotease activity was found in the feed extract.

With the aim of improving the recovery of the enzyme, the optimal extraction temperature was investigated. Twenty-five grams of feed, supplemented with endoprotease, was extracted for 60 minutes with 250 mL 0.1 M citrate buffer pH 1.6 containing 0.01% Tween 20 at different temperatures. The extraction temperatures were between room temperature (22°C) and 50°C. The final extraction pH was between pH 2.7 and 2.9. For the enzymatic reaction, the samples were 50-fold diluted with 0.1 M citrate buffer pH 8, containing 0.01% Tween 20. The diluted samples were incubated at pH 7.0 with 1 mM Suc-AAPF-pNA for 30 minutes at 37°C. The data are shown in Fig. 23.2. Increasing the extraction temperature also increased the recovery of the protease in the extract. The highest recovery of about 70% was reached between 40°C and 45°C. Therefore, the extraction temperature was set at 40°C.

The endoprotease under investigation had its pH optimum between 9.5 and 10.5. In order to improve the sensitivity of the method further, pH 10.0 was chosen for the enzyme reaction. The higher reaction pH may reduce interference, like inhibition, with the endoprotease. In addition, the higher pH may also eliminate endogenous protease activity, if relevant, since the method cannot distinguish between endogenous protease and added protease. Endogenous enzymes hydrolyzing the Suc-AAPF-pNA substrate would contribute to the final data. Citrate

5 mg Enzyme protein/kg feed extracted with 0.1 M citrate buffer + 0.01% Tween 20	pH extract	pH of extract 1:50 diluted with 0,1 M citrate buffer + 0,01 Tween 20, pH 8,0	ΔOD_{405} mean	% Recovery
Standard		7.1	1.491	**100**
Extraction temperature: RT (22°C)				
buffer pH 1,2	**2.3**	**6.8**	**0.638**	**43**
buffer pH 2,0	**3.1**	**6.9**	**0.614**	**41**
buffer pH 2,5	**3.3**	**6.9**	**0.589**	**40**
buffer pH 3,0	**3.7**	**7.0**	**0.456**	**31**
buffer pH 3,5	**4.0**	**7.0**	**0.226**	**15**
buffer pH 5,0	**5.2**	**7.2**	**0.080**	**5**

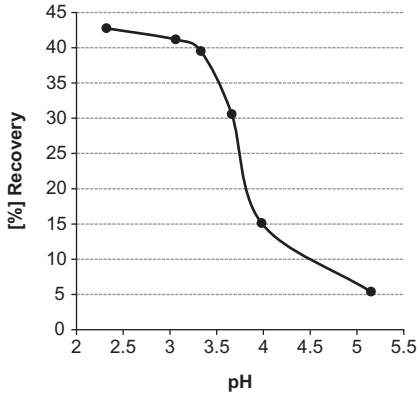

FIGURE 23.1

Optimal feed extraction pH for the endoprotease with 0.1 M citrate buffer containing 0.01% Tween 20.

5 mg Enzyme protein/kg feed extracted with 0.1 M citrate buffer + 0.01% Tween 20 pH 1.5	pH extract	pH of extract 1:50 diluted with 0,1 M citrate buffer + 0,01 Tween 20, pH 8,0	ΔOD_{405} mean	% Recovery
Standard		6.9	1.425	**100**
Extraction temperature °C				
22	2.8	6.9	0.619	**44**
35	2.7	6.8	0.949	**67**
40	2.7	6.9	1.031	**72**
45	2.8	6.9	1.018	**71**
50	2.9	6.9	0.867	**62**

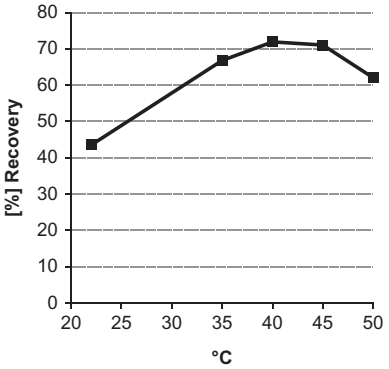

FIGURE 23.2

Optimal feed extraction temperature for the endoprotease with 0.1 M citrate buffer pH 1.6.

5 mg Enzyme protein/kg feed extracted with 0.1 M glycine buffer + 0.01 % Tween	pH extract	ΔOD_{405} mean	% Recovery
Standard		0.226	**100**
Extraction temperature: 40 °C			
buffer pH 1,8	**1.8**	0.178	**79**
buffer pH 2,0	**2.0**	0.215	**95**
buffer pH 2,2	**2.2**	0.221	**98**
buffer pH 2.4	**2.4**	0.223	**99**
buffer pH 2.6	**2.7**	0.229	**101**
buffer pH 2.8	**2.9**	0.231	**102**
buffer pH 3.2	**3.2**	0.221	**98**
buffer pH 3.4	**3.6**	0.216	**96**

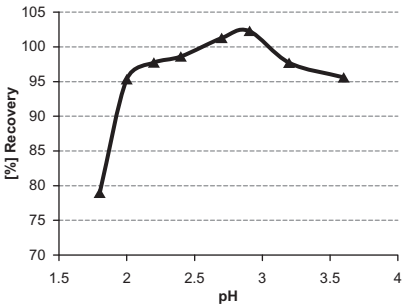

FIGURE 23.3

Optimal feed extraction pH for the endoprotease with 0.1 M glycine buffer containing 0.01% Tween 20.

buffer is not suitable for such high reaction pH. Therefore, the citrate buffer was replaced by 0.1 M glycine buffer, containing 0.01% Tween 20. Glycine with pKa values of 2.4 and 9.6 gave a good buffering at low and at high pH. Therefore, the glycine buffer could be used for the extraction of the feed at low pH and the enzymatic reaction at high pH. The optimal extraction pH for protease-supplemented feed samples was verified with the glycine buffer. Twenty-five grams of feed, containing 5 mg endoprotease per kg, were extracted at 40°C for 60 minutes with 250 mL 0.1 M glycine buffer containing 0.01% Tween 20, with the appropriate pH ranging from 1.8 and 3.6. The sample extracts were 400-fold diluted with 0.1 M glycine buffer pH 10, containing 0.01% Tween 20. The diluted samples were incubated at pH 10 with 1 mM Suc-AAPF-pNA for 30 minutes at 37°C. The data are shown in Fig. 23.3. The optimal extraction pH with the glycine buffer was between 2.4 and 3.2. In this pH range 100% of endoprotease activity was found in the feed extract. The change in the incubation pH from 7 to 10 and the eightfold higher sample dilution compared to the citrate buffer showed a positive effect on protease recovery. Most likely, the inhibitor did not survive these changes.

Glycine buffer pH 1.6 with 0.01% Tween 20 was chosen as final feed extraction buffer, which gave a final pH of the feed extract of between 2.2 and 3.2. Since 100% of the endoprotease activity was found in the extract, an external standard could be used for the quantification of the endoprotease in feed samples.

For an optimal method it is worthwhile to test all possible parameters. Unfortunately, the optimized method is only valid for the endoproteas tested. For other proteases, new methods have to be developed or the method has to be at least adapted to the new enzyme. This is true for all enzyme activities except for phytases, where method ISO 30024 [9] was established. This method works for all phytases presently on the market.

In most cases, the interference of the feed matrix with the enzyme persists, also under optimal extraction and assay conditions. A reason may be the low sensitivity of assays obtained with labeled complex substrates. Also restrictions in

Standard	Calculated Activity [FXU / ml]	ΔOD_{590}
A	**0.0010**	0.542
B	**0.0008**	0.453
C	**0.0005**	0.371
D	**0.0004**	0.335
E	**0.0003**	0.294

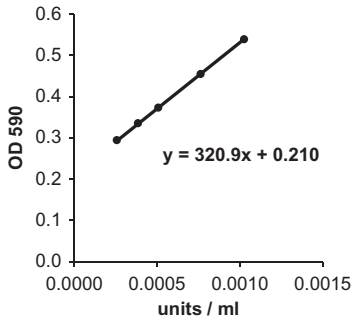

FIGURE 23.4

Xylanase standard in xylanase-supplemented feed extract.

the assay development, like limitations in pH or temperature selection, which are specific for each individual enzyme, can limit the sensitivity of an assay. Sometimes the inhibition can be reduced by dilution of the feed extract. However, low-sensitivity assays cannot be diluted to such a degree that the inhibition is significantly reduced. If the interference with the feed/food matrix cannot be eliminated, an internal standard has to be set up for quantification of the enzymes. This is so far no hurdle to the development of a method, as long as blank feed (mash and pellets), from the same feed batch as that used for the enzyme supplementation, is available. In reality, the appropriate blank feed, especially feed pellets, are not available. In these cases, feed supplemented with the enzyme has to be used for the standard curve, in order to quantify the enzyme in the feed.

Our experiments showed, that all xylanases tested interfered with the feed matrix. The xylanases in the feed extracts were inhibited. The optimization of the method did not eliminate the inhibition completely. Therefore, feed supplemented with the xylanase was used for the preparation of the standard curve for the quantification. Feed with the lowest xylanase concentration was chosen for the standard curve. A 50-g feed sample was extracted for 1 hour with 500 mL 0.1 M acetate buffer pH 4.5 containing 0.02% Tween 20. The feed extract was spiked with different concentrations of the xylanase standard. The standards were at least 20-fold diluted and incubated with 0.8% soluble azo-xylane from birchwood for 3 hours. The highest possible incubation temperature (65°C) was used to reduce interference of the xylanase with components in the feed extract. Fig. 23.4 shows an example of a standard curve, obtained with spiking of supplemented feed extract. The trendline was a linear regression with the formula $y = mx + b$. The intercept b of 0.21 was due to the xylanase present in the spiked feed extract. For the determination of the activity in feed samples the following formula was used: activity = ΔOD590*D/(m*W) where ΔOD590 was (OD590 sample—OD590 blank), D was the dilution of the sample, m was the slope of the standard curve and W was the weight from the sample. The intercept b was ignored since the value refers to the content of xylanase present before spiking.

The standard curve can be used for all feed samples, which were prepared with the same feed batch, the same xylanase, and which had the same treatment after xylanase supplementation.

Determination of the xylanase content in mash or pelleted feed requires an independent standard curve made in the corresponding feed form extract. Processing of the feed can modify the matrix and therefore the interaction with the enzyme. This is valid for all enzymes, which require such standard curves for the quantification. The standard curves and the feed extracts have to be prepared freshly each day.

23.9 DETERMINATION OF ENZYME ACTIVITY IN PREMIXES

There are many different premix formulations on the market. The customers usually define the composition of the premix and the exact composition is, in most cases, not known. The premixes can contain ingredients that heavily interfere with the analytical procedure. In particular, soluble minerals, which are in high amounts in the premix, can interfere with the analytics. Also, carrier material used for premixes can interfere with enzymes.

For the determination of enzyme activity in premixes, special attention has to be given in the extraction of the premixes. High concentrations of soluble minerals may inactivate enzymes irreversibly during extraction. Some procedures use ethylenediaminetetraacetic acid (EDTA) in the extraction buffer. EDTA binds metal ions and forms a metal complex, which remains in solution, but does not interfere with the enzymes. One example is an extraction buffer which contained 1.5% EDTA. Extraction of 5 g premix with 100 mL of this extraction buffer gave good recoveries, if the premix contained no minerals or when nonsoluble minerals were used. Premixes with soluble minerals showed recoveries below 80%. Most likely, the chelating capacity of the EDTA in the buffer was not sufficient for such high concentrations of metal ions. An alternative procedure was when the premix was mixed with a carrier such as corn meal before extraction. Corn meal is a preferred carrier for the enzyme tested, since it showed no endogenous enzyme activities. One gram premix was mixed with 50 g corn meal (ground to 2 mm) and extracted with 500 mL feed extraction buffer. Since the premix was highly diluted with corn meal, no EDTA was required in the extraction buffer. A standard curve made in corn meal extract was used for the quantification of the enzyme activity in the premix. With this procedure 100% of the activity was found in the extract. For the blending of the premixes carriers other than corn meal can be used. The carrier used for blending should be tested for endogenous enzyme activity. Endogenous enzyme activity should be considered in the calculation of the enzyme activity in the premix. Some premixes showed low recoveries with both methods independent of the enzyme tested. These premixes contained sepiolite as carrier. Sepiolite is a nonswelling, porous clay that has a

high sorption capacity. The low recoveries of the enzymes in these premixes might be partially due to absorption or adsorption of the enzymes by the sepiolite, which were not released any more during extraction.

23.10 OUTLOOK

The enzyme analytics frequently used by the feed and food industries have their weaknesses, not only in the nonspecific assays, but often also in the low sensitivity of the assays. The requirement of specific substrate batches necessary for many enzyme assays, specific analytical equipment, and skilled people for the execution, makes the transfer of such methods to other labs not simple. Feed manufacturers ask for enzyme assays, which are simple, fast, and accurate. More and more enzyme manufacturers go for enzyme-linked immune sorbent assays (ELISA) for the quantification of enzymes. The ELISA uses a primary antibody, which binds specifically to the active enzyme and a conjugated secondary antibody that binds to the primary antibody−enzyme complex. For the quantification of the enzyme, the appropriate substrate is added and incubated for the color reaction, which is measured photometrically. The secondary antibodies for ELISA are commonly conjugated with horseradish peroxidase or alkaline phosphatase. Each feed enzyme requires a specific primary antibody recognizing only the active enzyme and an appropriate secondary antibody. The ELISA is not only highly specific and sensitive, but it is also much faster compared to the enzyme reaction assays and allows high throughput in a short time. It requires no special equipment and can easily be transferred to other labs. ELISA can be set up in microtiter plates for routine analytics, but it is also suitable to set up quick tests to monitor the enzyme in place during feed manufacturing.

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CHAPTER

Registration of food and feed additives (enzymes) in the United States, Canada, and China

24

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24.1 INTRODUCTION

Food and/or feed additives are defined as substances added to food and feed to perform specific functions. Additives may be natural, nature identical, or artificial. The main groups of nutritional additives are enzymes, antioxidants, colors, flavor enhancers, sweeteners, emulsifiers, stabilizers, preservatives, and others (Tables 24.1 and 24.2). Generally, additives are thoroughly assessed for efficacy and safety before they are allowed to be used worldwide.

Natural additives are substances found naturally in a food- or feedstuff, and are extracted from one to be used in another. Nature identical additives are man-made copies of substances that occur naturally. For example, benzoic acid is a substance that is found in nature but is also made synthetically and used in a wide range of food (preservative) and feed (zootechnical enhancer for swine and in combination for poultry). Artificial additives are not naturally present in the food chain and are made synthetically. An example is azodicarbonamide, a flour improver that is used to help bread dough hold together.

Additives are thoroughly assessed for safety before they are permitted for use, and they are only then permitted to be used in a limited range of products and in certain amounts. These amounts are based on an acceptable daily intake calculated from the results of safety tests. Even when an additive has been approved, regular repeat testing is required to maintain its status as "approved." Food/feed labels give information about most additives present in the ingredients list, so that consumers can make informed choices.

Regarding the registrations or regulations of food and feed additives, the Joint FAO/WHO Expert Committee on Food Additives (JECFA), whose genesis established at the FAO/WHO Conference on Food Additives in 1955, continues to be

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Table 24.1 The Main Groups of Food Ingredients

Types of Ingredients	What They Do	Examples of Uses	Names Found on Product Labels
Enzymes	Modify proteins, polysaccharides and fats	Cheese, dairy products, meat	Enzymes, lactase, papain, rennet, chymosin
Antioxidants	Protect the body from damage caused by harmful molecules called free radicals and this damage is a factor in the development of blood vessel disease (atherosclerosis), cancer, and other conditions	All species or categories of animals	Ethoxyquin, Butylated Hydroxyanisole (BHA), Butylated Hydroxytoluene (BHT), Propyl Gallate, Tertiary Butyl Hydroquinone (TBHQ), Tea Polyphenol, alpha-Tocopherol (Vitamin E), 6-Palmityl-L-Ascorbic Acid
Color additives	Offset color loss due to exposure to light, air, temperature extremes, moisture and storage conditions; correct natural variations in color; enhance colors that occur naturally; provide color to colorless and "fun" foods	Many processed foods, (candies, snack foods margarine, cheese, soft drinks, jams/jellies, gelatins, pudding and pie fillings)	FD&C Blue Nos. 1 and 2, FD&C Green No. 3, FD&C Red Nos. 3 and 40, FD&C Yellow No. 5 (tartrazine) and No. 6, Orange B, Citrus Red No. 2, annatto extract, beta-carotene, grape skin extract, cochineal extract or carmine, paprika oleoresin, caramel color, fruit and vegetable juices, saffron (Note: Exempt color additives are not required to be declared by name on labels but may be declared simply as colorings or color added)
Flavor enhancers	Enhance flavors already present in foods (without providing their own separate flavor)	Many processed foods	Monosodium glutamate (MSG), hydrolyzed soy protein, autolyzed yeast extract, disodium guanylate or inosinate
Flavors and spices	Add specific flavors (natural and synthetic)	Pudding and pie fillings, gelatin dessert mixes, cake mixes, salad dressings, candies, soft drinks, ice cream, BBQ sauce	Natural flavoring, artificial flavor, and spices
Sweeteners	Add sweetness with or without the extra calories	Beverages, baked goods, confections, table-top sugar, substitutes, many processed foods	Sucrose (sugar), glucose, fructose, sorbitol, mannitol, corn syrup, high fructose corn syrup, saccharin, aspartame, sucralose, acesulfame potassium (acesulfame-K), neotame

Emulsifiers	Allow smooth mixing of ingredients, prevent separation, keep emulsified products stable, reduce stickiness, control crystallization, keep ingredients dispersed, and to help products dissolve more easily	Salad dressings, peanut butter, chocolate, margarine, frozen desserts	Soy lecithin, mono- and diglycerides, egg yolks, polysorbates, sorbitan monostearate
Stabilizers and thickeners, binders, texturizers	Produce uniform texture, improve "mouth-feel"	Frozen desserts, dairy products, cakes, pudding and gelatin mixes, dressings, jams and jellies, sauces	Gelatin, pectin, guar gum, carrageenan, xanthan gum, whey
Preservatives	Prevent food spoilage from bacteria, molds, fungi, or yeast (antimicrobials); slow or prevent changes in color, flavor, or texture and delay rancidity (antioxidants); maintain freshness	Fruit sauces and jellies, beverages, baked goods, cured meats, oils and margarines, cereals, dressings, snack foods, fruits and vegetables	Ascorbic acid, citric acid, sodium benzoate, calcium propionate, sodium erythorbate, sodium nitrite, calcium sorbate, potassium sorbate, BHA, BHT, EDTA, tocopherols (Vitamin E)
Fat Replacers (and components of formulations used to replace fats)	Provide expected texture and a creamy "mouth-feel" in reduced-fat foods	Baked goods, dressings, frozen desserts, confections, cake and dessert mixes, dairy products	Olestra, cellulose gel, carrageenan, polydextrose, modified food starch, microparticulated egg white protein, guar gum, xanthan gum, whey protein concentrate
Nutrients	Replace vitamins and minerals lost in processing (enrichment), add nutrients that may be lacking in the diet (fortification)	Flour, breads, cereals, rice, macaroni, margarine, salt, milk, fruit beverages, energy bars, instant breakfast drinks	Thiamine hydrochloride, riboflavin (Vitamin B_2), niacin, niacinamide, folate or folic acid, beta carotene, potassium iodide, iron or ferrous sulfate, alpha tocopherols, ascorbic acid, Vitamin D, amino acids (L-tryptophan, L-lysine, L-leucine, L-methionine)
Leavening agents	Promote rising of baked goods	Breads and other baked goods	Baking soda, monocalcium phosphate, calcium carbonate
Anticaking agents	Keep powdered foods free-flowing, prevent moisture absorption	Salt, baking powder, confectioner's sugar	Calcium silicate, iron ammonium citrate, silicon dioxide

(*Continued*)

Table 24.1 The Main Groups of Food Ingredients *Continued*

Types of Ingredients	What They Do	Examples of Uses	Names Found on Product Labels
Humectants	Retain moisture	Shredded coconut, marshmallows, soft candies, confections	Glycerin, sorbitol
Yeast nutrients	Promote growth of yeast	Breads and other baked goods	Calcium sulfate, ammonium phosphate
Dough strengtheners and conditioners	Produce more stable dough	Breads and other baked goods	Ammonium sulfate, azodicarbonamide, L-cysteine
Firming agents	Maintain crispness and firmness	Processed fruits and vegetables	Calcium chloride, calcium lactate
Gases	Serve as propellant, aerate, or create carbonation	Oil cooking spray, whipped cream, carbonated beverages	Carbon dioxide, nitrous oxide

Table 24.2 Feed and Feed Additive Categories Defined by AQSIQ and Status

Category	Market Access	Facility Registration	Trade
Processed aquatic animal protein (fat)	Yes	Yes	Yes
Processed terrestrial animal protein (fat)	Yes	Yes[b]	Yes
Processed plant protein	In progress[a]	Yes[c]	Rice bran and traditionally traded products
Pet food	Yes	Yes	Yes
Forage	Yes	Yes	Yes
Bait live animals	In progress	No	Traditionally traded products only
Compound feed (including premix feed additives)	In progress	No	Traditionally traded products only
Feed additives	In progress	No	Traditionally traded products only

[a]*In November 2015 AQSIQ conducted an audit for the market access request for sugar beet pulp. The market access process for rice bran is complete.*
[b]*Dairy feed product (whey) facility registration is in progress.*
[c]*Facility registration completed for rice bran only. Registration process for sugar beet pulp is near completion.*
Source: *AQSIQ Website and correspondence.*

of fundamental importance to the activities of the Codex Alimentarius Commission (CAC) and especially to the Codex Committee on Food and/or Feed Additives (CCFFA). While the outcome of JECFA's evaluations does not have any direct bearing on the regulatory approval of a food or feed additive in any specific country, JECFA's scientific evaluations and reassessments are widely recognized and may affect an application for approval for a new food and/or feed additive in a particular country [1]. Similar to JECFA, the CAC has no regulatory authority and its standards are not enforceable unless they have been adopted into the regulatory framework for a nation (e.g., the Food and Drug Administration (FDA) in the United States, The European Food Safety Authority (EFSA) in Europe, the Canadian Food Inspection Agency (CFIA) in Canada, the Administration of Quality Supervision, Inspection, and Quarantine (AQSIQ) in PR China, etc.); nevertheless, its standards for food and feed additives continue to serve as guidelines to many nations (further information is briefly presented below in Section 24.2).

In the 28 countries of the European Union (Austria, Belgium, Bulgaria, Croatia, Cyprus, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, The Netherlands, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Czech Republic, and the United Kingdom) the registration of food and feed enzymes as well as all additives are under the same regulatory legislation and authority—EFSA [3].

Generally, enzymes are substances or products extracted from animals, plants, or microbes that act by either promoting a desirable chemical reaction or are used in processing. The definition of an enzyme according to each target country is presented in Table 24.3. In the United States, Canada, and China enzymes are regulated as food additives. Similarly, the approval process for new enzymes also follows the same approval process as for direct food and/or feed additives. Nevertheless, depending on the context of use, Canada may consider enzymes to be processing aids [1].

24.2 REGULATORY MANAGEMENT FOR FEED INGREDIENTS AND ADDITIVES IN SOME BIG COUNTRIES

For feed ingredients there are comparisons of the regulatory management between, e.g., Brazil, Canada, China, European Union, Japan, South Africa, and the United States [1,4]. The report edited in 2013 covers the basic tenets of safety (target animal and human food/consumer safety), data to support intended use, and manufacturing information (which includes specifications, stability, and analytical methodology). Four of the jurisdictions (Canada, China, United States, and South Africa) are currently revising their feed ingredient regulation. Ingredients in Brazil, some ingredients in China (newly authorized feed additive within the first 5 years), South Africa, Canada, and the EU are proprietary or holder-specific listings, such that only persons receiving the authorization can market under that authorization. Authorization periods may be unlimited or limited. China, Japan, United States, and Canada have an unlimited authorization period. Once authorized, unless safety or other issues would retract the authorization period, the feed ingredients are permanently authorized. Brazil's authorization period is 5 years. Canada's and South Africa's authorization periods are 3 years. The EU feed additive authorization period is 10 years, and the feed material is unlimited. The regulations or laws of Brazil, China, EU, Japan, and South Africa have specific requirements for the regulation and/or labeling of feed ingredients manufactured using genetically modified organisms or plants. Canada and the United States have no specific laws or regulations for these products. They rely on laws/regulations that cover all feed ingredients. The regulation of companion animal versus livestock (animals intended for use as human food) also varies between jurisdictions. The definition of companion versus livestock also varies. For example, the FDA in the US considers horses to be companion animals, and not raised for food, while horses are considered livestock in Canada. China, EU, South Africa, and the United States do not have separate regulations that are specific for feed intended for companion animals and the regulation of companion animal and livestock ingredients are similar (some obvious data differences such as human/consumer safety are the exception). Brazil and Japan have a separate law that covers the regulation of companion animal feed. Canada does not

Table 24.3 Regulatory Framework of Food Additives (Enzymes) in the United States

Regulatory authority	*Name*: US Food and Drug Administration (USFDA) *Website*: http://www.fda.gov/Food/FoodIngredientsPackaging/default.htm *Historical overview*: • Began as the Bureau of Chemistry, as part of the US Department of Agriculture in 1862 • The Food and Drugs Act was passed in 1906 • Bureau of Chemistry is split into the Food Drug and Insecticide Administration and the Bureau of Chemistry and Soils in 1927 • Food Drug and Insecticide Administration is renamed the Food and Drug Administration in 1930 • The Federal Food Drug and Cosmetic Act was passed in 1938 • The Food Additives Amendment of 1958 was passed in 1958 • http://www.fda.gov/AboutFDA/WhatWeDo/History/Milestones/ucm081229.htm *Roles/responsibilities*: • Protect the public health by ensuring that foods are safe, wholesome, sanitary, and properly labeled • Promote the public health by promptly and efficiently reviewing clinical research and taking appropriate action on the marketing of regulated products in a timely manner • Participate through appropriate processes with representatives of other countries to reduce the burden of regulation, harmonize regulatory requirements, and achieve appropriate reciprocal arrangements • Where appropriate, carry out its mission in consultation with experts in science, medicine, and public health, and in cooperation with consumers, users, manufacturers, importers, packers, distributors, and retailers of regulated products
Advisory scientific body	*Name*: USFDA Science Board and USFDA Food Advisory Committee *Websites*: • Science Board to the US Food and Drug Administration (Science Board): http://www.fda.gov/AdvisoryCommittees/CommitteesMeetingMaterials/ScienceBoardtotheFoodandDrugAdministration/default.htm • Food Advisory Committee: http://www.fda.gov/AdvisoryCommittees/CommitteesMeetingMaterials/FoodAdvisoryCommittee/default.htm

(*Continued*)

Table 24.3 Regulatory Framework of Food Additives (Enzymes) in the United States *Continued*

	Historical overview:
	• Both the Science Board and the Food Advisory Committee were authorized by the Federal Advisory Committee Act (Pub. L. 92–463) passed in 1972
	• The Science Board was established on 26 June 1992
	• The Food Advisory Committee was established on 15 December 1991 (21 CFR §14.100)
	
	Roles/responsibilities:
	• Science Board: Provides advice to the Commissioner and other appropriate officials on a wide variety of food- and drug-related issues including:
	  ◦ specific complex scientific and technical issues important to the agency and its mission
	  ◦ emerging issues within the scientific community
	  ◦ keeping pace with technical and scientific developments, including in regulatory science
	  ◦ agency research
	  ◦ upgrading the agency's scientific and research facilities and training opportunities
	• As of 2012, the Board is made up of a committee with a core of 21 voting members knowledgeable in fields including food science, safety, and nutrition; chemistry; pharmacology; toxicology; public health and epidemiology; international health and regulation; and nanotechnology
	• Food Advisory Committee: provides advice to the Commissioner and other appropriate officials on emerging food safety, food science, nutrition, and other food-related health issues and may be tasked with making recommendations on matters including:
	  ◦ broad scientific and technical food- or cosmetic-related issues
	  ◦ the safety of new foods and food ingredients
	  ◦ labeling of foods
	  ◦ nutrient needs and nutritional adequacy
	  ◦ safe exposure limits for food contaminants
	As of 2012, the Committee consists of 17 standing members knowledgeable in the fields of physical sciences, biological and life sciences, food science, risk assessment, nutrition, food technology, molecular biology, and other relevant scientific and technical disciplines

Framework regulations	*Food and Drug Regulations*: 21 CFR, Chapter 1 (http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm)

(*Continued*)

Table 24.3 Regulatory Framework of Food Additives (Enzymes) in the United States *Continued*

Part of an overarching international organization	• The United States has been a member of the Codex Alimentarius Commission since it was established in 1963 (http://www.codexalimentarius.org/members-observers/members/en/?no_cache = 1) • The United States also has been a member of the World Trade Organization since 1995 (http://www.wto.org/english/thewto_e/whatis_e/tif_e/org6_e.htm) • http://www.fda.gov/InternationalPrograms/HarmonizationInitiatives/default.htm
Recent/pending changes	The Food Safety Modernization Act was signed into law on 4 January 2011. Changes include requiring food facilities to document in writing that all substances at the facility that are intended to be added to food are allowed by the food additive regulatory program (http://www.fda.gov/food/foodsafety/fsma/default.htm)
Regulatory overview of food additives	A substance added directly to food can fall into one of several categories: food additive, prior sanctioned substance, color additive, or the use of the substance is considered GRAS, depending on its intended use and the mechanism through which approval is sought. *Definition*: • A food additive is a substance "the intended use of which results or may reasonably be expected to result, directly or indirectly, in its becoming a component or otherwise affecting the characteristics of any food (including any substance intended for use in producing, manufacturing, packing, processing, preparing, treating, packaging, transporting or holding food; and including any source of radiation intended for any such use)"; if such substance is not GRAS or sanctioned prior to 1958 or otherwise excluded from the definition of food additives • Direct food additives are a subcategory of this larger category. They are substances intentionally added directly to food whose use has been expressly approved by the USFDA, usually in response to a food additive petition from a manufacturer or manufacturer's representative • Prior-sanctioned substances are chemicals that were government-approved for use in food prior to 1958 • GRAS substances are substances "generally recognized among experts qualified by scientific training and experience to evaluate their safety, as having been adequately shown through scientific procedures (or, in the case of a substance used in food prior to 1 January 1958, through either scientific procedures or experience based on common use in

(*Continued*)

Table 24.3 Regulatory Framework of Food Additives (Enzymes) in the United States *Continued*

	food) to be safe under the conditions of its intended use." GRAS substances are distinguished from food additives by the type of information that supports the GRAS determination, that it is publicly available and generally accepted by the scientific community, but should be the same quantity and quality of information that would support the safety of a food additive. USFDA notification of the GRAS determination of a chemical's use is voluntary • Color additives are substances that are capable (alone or through reaction with other substances) of imparting color when added or applied to food. Substances intended to be used solely for purposes other than coloring, that may also impart color, do not fall within this category. The USFDA must approve all color additives, typically in response to a color additive petition. Color additives cannot be GRAS *Regulation*: • 21 CFR §§70–82 and §§170–189 (http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm) • GRAS Notifications: http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/default.htm *Guidance documents*: http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodIngredientsandPackaging/default.htm#food*Approval process for a new substances*: Same as described for food ingredients [1]. In this case, the petitions submitted would be food or color additive petitions. Regarding voluntary notifications, the notification submitted by a manufacturer would be known as a GRAS notification and if the agency's review of this notification raises no concerns, the USFDA would send a letter stating that it has "no questions" regarding the manufacturer's Decision
Enzymes	*Definition*: There is no specific regulation governing enzymes. They would be regulated as direct or secondary direct additives, or GRAS substances depending on their intended use and the method used to allow the substances in food*Regulation*: See "Direct food additives" (above) *Guidance document*: See "Direct food additives" (above)*Approval process for a new substances*: See "Direct food additives" (above)

Source*: Adapted from Magnuson B, Munro I, Abbot P, Baldwin N, Lopez-Garcia R, Ly K, et al. Review of the regulation and safety assessment of food substances in various countries and jurisdictions. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 2013;30 (7):1147–1220.*

authorize ingredients for companion animals (as their Feeds Act only covers specific livestock species). All jurisdictions reported that the claim or function of a feed ingredient could alter the regulatory category of the ingredient. South Africa and the United States have the most dramatic examples, as all products that are intended to increase animal production are regulated as drugs. All jurisdictions reported that ingredients intended to diagnose, prevent, mitigate or treat a disease were regulated as animal (veterinary) health products. However, the EU has special categories of feed additives for accordionists and histomonistats and Brazil also includes coccidiostats feed additives. In China, products must be registered via registration agent in China, the operator must be authorized by a competent authority in the country of origin, and products must be registered with the Ministry of Agriculture. In the EU, operators must be registered with their location and must be represented within the EU. Other requirements are the same for an EU or foreign operator. The regulations or laws of Brazil, China, EU, Japan, and South Africa have specific requirements for the regulation and/or labeling of feed ingredient manufactured using genetically modified organisms or plants.

24.3 UNITED STATES OF AMERICA

In the United States, domestic and foreign food, drug (human, veterinary, and homeopathic), and medical device establishments whose products are marketed in the United States should register with the FDA. For cosmetic establishments, FDA registration is not mandatory. Assistance in FDA regulations, FDA registration, US FDA agent requirements guidance can be provided by Liberty Management Group Ltd. (LMG). LMG is a globally recognized FDA, CE Marking, and ISO consulting firm headquartered in Greater Chicago, Illinois, USA, with Associates and offices around the globe. LMG provide a full range of consulting services in FDA regulations to domestic and foreign drug, medical device, cosmetic, and food manufacturing companies through the right guidance in FDA regulations, as well as providing the latest information about the changes in FDA regulations (Table 24.1).

All foreign food, medical device, drug, and blood establishments should identify a US agent while in the registration process. LMG provide US agent service to foreign establishments.

FDA registration or FDA registration number does not denote FDA certification of the facility or the products. Any representation of registration number on product label or labeling which implies FDA certification or FDA approval of a facility or product is misleading and may cause misbranding of the product. FDA will not issue any certificate after registration, also FDA will not recognize any third party certificate. Most Importers and Shipping companies always ask for a registration certificate or proof of FDA registration to the manufacturer. LMG will issue a registration certificate. All drug and medical device establishments as well as food facilities registered with FDA must renew their registration. FDA may consider the products of companies, which are not complying with renewal requirements as misbranded.

CE Marking, also referred to as CE Mark, is a legal requirement for medical devices and certain other product groups to market in Europe. CE Marking (CE Mark) is recognized worldwide as a symbol of quality. LMG provide complete solutions for medical device CE Marking including medical device software as well as assist medical device companies with ISO 13485 implementation, which is a mandatory requirement for CE Certification.

In the United States the use of food products is governed by the provisions of the Federal Food, Drug, and Cosmetic Act (FFDCA), and the regulations issued under its authority published in the Code of Federal Regulations (CFR). The FFDCA defines food as "articles used for food or drink for man or other animals..." and thus, any article that is intended to be used as an animal feed ingredient, to become part of an ingredient or feed, or added to an animal's drinking water is considered a "food" and thus, is subject to regulation. FDA's CFSAN is responsible for the regulation of human food products and CVM is responsible for the regulation of animal food (feed) products.

The FFDCA sets forth requirements for "foods" in Sections 402 and 403. Failure to meet these requirements can result in a product being deemed adulterated or misbranded.

The Dietary Supplement and Health Education Act (DSHEA) of 1994, has affected the way in which the FDA regulates "food for humans," i.e., among other things, it restricts substances from being food additives or drugs if the product meets the definition of a dietary supplement. However, the agency's assessment of the law is that it was not intended and does not apply to animal feed, including pet food. This assessment was published in the Federal Register on April 22, 1996 (61 FR 17706). Thus, products marketed as dietary supplements or "feed supplements" for animals still fall under the FFDCA prior to DSHEA, i.e., they are considered "foods" or "new animal drugs," depending on the intended use. The regulatory status of a product is determined by CVM on a case-by-case basis, using criteria provided in Guide 1240.36.05 in the Program Policy and Procedures Manual. The FDA carries out its responsibility for the regulation of animal feed in cooperation with state and local partners through a variety of mechanisms: cooperative agreements, contracts, grants, memoranda of understanding and partnerships. For instance, FDA cooperates with the Association of American Feed Control Officials (AAFCO) and the States for the implementation of uniform policies for regulating the use of animal feed products. This includes the establishment of uniform feed ingredient definitions and proper labeling to ensure the safe use of feeds. The ingredient definitions are important because animal feeds and feed ingredients must be correctly and truthfully labeled when they enter commerce. Although the FDA has the responsibility for regulating the use of animal feed products, the ultimate responsibility for the production of safe and effective animal feed products lies with the manufacturers and distributors of the products.

The FFDCA requires that any substance that is added to or is expected to become a component of animal food, either directly or indirectly, must be used in accordance with a food additive regulation unless it is generally recognized as

safe (GRAS) for that intended use. Typical feed ingredients such as forages, grains, and most minerals and vitamins are GRAS as sources of nutrients. The legal definition for a food additive is found in Section 201(s) of the FFDCA. In Section 409 of the FFDCA, a food additive is unsafe unless its use conforms to an existing food additive regulation. Further, a substance that does not become a component of a final animal food product but that is used, e.g., in preparing an ingredient of the animal food to give it a different flavor, texture, or other characteristic may also be a food additive.

One of the charges of the Center for Food Safety and Applied Nutrition (CFSAN) from the FDA is to ensure that the use of food ingredients and color additives is safe. The authority for this mission is derived from the 1958 food additive and 1960 color additive amendments to the FFDCA. Towards this end, CFSAN evaluates new petitions to determine whether substances are considered safe for addition to the food supply. When a petition for a food ingredient or color additive is approved, a regulation identifying the conditions of use is published in the Federal Register. Information about additional food ingredient review programs in the Office of Food Additive Safety, can be obtained in the Inventory of Effective Food Contact Substance (FCS) Notifications and the Summary of all GRAS Notices. Last issue of these documents took place in May 2017. The basis for a food additive regulation is an approved food additive petition. Use of a food ingredient that is neither GRAS nor an approved food additive can cause a "food" to be adulterated, which cannot be legally marketed in the United States. Section 409 of the FFDCA sets forth the statutory requirements for approval of a food additive.

Under Section 409(c)(3) of the FFDCA, the FDA is not to approve a food additive petition if a fair evaluation of the data fails to establish that the proposed use of the food additive, under the conditions of use to be specified in the regulation, will be safe. Only if the petitioner meets this burden can the food additive be approved. Regulations, which apply specifically to food additives in feeds, are published in Title 21, Part 570 of the CFR. Part 571 prescribes the kinds of data that should be submitted by the petitioner and the required format for the petition itself. While the actual content may vary from petition to petition, depending primarily on the composition of the food additive and its intended use, each of the following subject areas should be addressed: human food safety, target animal safety, environmental impact, utility (intended physical, nutritional or other technical effect), manufacturing chemistry, labeling (in addition to information required under Title 21, Part 501 of the CFR, labeling may include: cautions, warnings, shelf life, and directions for use) and proposed regulation.

The United States have applied the concept of GRAS for several years. Under sections 201(s) and 409 of the FFDCA any substance that is intentionally added to food is a food additive, i.e., subject to premarket review and approval by FDA, unless the substance is generally recognized, among qualified experts, as having been adequately shown to be safe under the conditions of its intended use, or unless the use of the substance is otherwise excepted from the definition of a

food additive. There are three forms of GRAS designation: "self-affirmed,"—the producer has performed all investigations demonstrating the product safety; "FDA has no questions"—FDA has reviewed the dossier and has no supplementary questions; and "pending"—FDA is evaluating the application dossier [5]. The list of approved food additives for use in animal feed can be found in Part 573 and a partial list of GRAS substances for use in animal feed is found in section 582 of Title 21 of the CFR. Substances affirmed as GRAS for use in animal feeds are listed under 21 CFR 584. Irradiation is considered a food additive and approvals for the use of irradiation for animal feed are found in 21 CFR 579. Copies of the CFR may be obtained by fax: +1-202-512-2233 or by mail: Superintendent of Documents, Government Printing Office, Washington DC, DC 20402, USA. However, the GRAS concept is generating some concerns. Neltner et al. [6] examined recently 451 voluntary GRAS notifications submitted to the FDA between 1997 and 2012, and found that none of the safety assessments were executed by an independent third party without financial interest in the study outcome. Specifically, 22.4% of the safety assessments were made by an actual employee of the food additive manufacturer, 13.3% by an employee of a consulting firm hired by the manufacturer, and 64.3% by a panel of experts selected by either the hired consulting firm or the food additives manufacturer itself. Based on these data, the study researchers concluded that financial conflicts of interest are pervasive in this GRAS designation process, especially considering that this study did not review GRAS food additives not submitted to the FDA.

A feed label should contain information describing the feed product and any details necessary for the safe and effective use of the feed. The federal regulations concerning the labeling of animal feeds are published in Part 501 of the 21 CFR. The FFDCA defines "labeling" as all labels and other written, printed, or graphic matter on any article or any of its containers or wrappers, or accompanying such articles. In addition to meeting the federal labeling requirements, animal feed products are also subject to individual state laws. Additional information concerning State registration and labeling requirements may be obtained from the individual States where the products will be distributed or from the Official Publication of AAFCO. Copies of this publication, which is updated yearly, can be obtained from: . AAFCO@aafco.orgCVM has incorporated the philosophy of the Nutrition La belling Education Act (NLEA) in its policies to permit meaningful "health" information on the label of some animal food products. For example, the use of "urinary tract health" and "dental health" claims on cat food diets falls under this policy. Biotechnology products with Intentionally Altered Genomic DNA in Animals and Plants are a growing proportion of the feed components regulated by the Center for Veterinary Medicine (CVM). The spectrum of products being presented to the CVM for regulation includes biotechnology products from plants, microbes, and animals.

The FDA has specific legislation for most of the food and feed additives to be imported or offered for import into the United States and verifies/enforces at the time they are imported. An example is that of animal and veterinary products.

The CVM is the FDA center responsible for overseeing the animal and veterinary program. The CVM regulates animal and veterinary products offered for importation into the United States. These products include animal food and feed, pet food, animal drugs, medicated feed, and veterinary devices [7].

24.4 CANADA

In Canada, all new food/feed additives or changes to the permitted uses of already approved food additives under Division 16 of the Food and Drug Regulations must undergo a premarket assessment focused on safety [1]. Canadian legislation for the registration of food and feed additives is relatively similar to that of the United States.

It is under the authority of the Canadian Food Inspection Agency (CFIA), which verifies that foods and feeds manufactured and sold in Canada or imported are safe, effective, and are labeled appropriately. The Canadian regulation is codified in Regulatory Guidance edited by CFIA [8].

24.5 PEOPLE'S REPUBLIC OF CHINA

The aims of the China legislation on feed and feed additives are to strengthen the administration to improve their quality, increase the development of the feed industry and animal production, and enhance public health safety. For instance, food scandals like that of the inclusion of melamine in baby foods obliged the Country authorities to strengthen the legislation as well as the punishment to infractions [9,10].

The categories of feed additives are registered and ordained by the Ministry of Agriculture of the State Council (MASC). The MASC is responsible for the national administration of feed and feed additives; the feed and feed additives administrative department of the County government is responsible for local feed and feed additives administration. The clear meaning of the terms is presented in the legislation. Before the manufacture of new feeds and feed additives, the researcher or manufacturer (hereafter called the "applicant") must put forward an application for evaluation of the new product to the MASC. Feed analysis and feeding trials are conducted by an organization assigned by the Ministry. The National Feed Evaluation Council evaluates the results of the feed analysis and feeding. Veterinary drugs or other prohibited drugs should not be added directly to feed or feed additives; the permitted drugs can only be added after diluting with suitable materials in "drug containing feed additives." No feed should contain hormone material (Table 24.4).

Feed or feed additives without the quality standard, quality certification, production, clearance, or production registration number are prohibited from sale.

Table 24.4 Product Category Approved for Export to China (Updated November 24, 2015)

Country/ Region	Product	Status
USA	Processed aquatic animal protein (fat)	Approved imports from the registered facility (list)
	Dairy feed product (Milk and Milk Products)	Approved. Complete facility registration gradually
	Pet food	Approved imports from the registered facility (list)
	Forage alfalfa	Approved imports from the registered facility (list)
	Brine shrimp eggs and larva	Approved. Complete facility registration gradually
	Non-ruminant feed ingredients	Approved imports from the registered facility (list)
	DDGS (Dried Distillers Grains)	Approved. Complete facility registration gradually
	Rice bran	Approved imports from the registered facility (list)

The administration of drug feed additives is conducted according to "the Legislation on Veterinary Medicine Administration." Imported feed or feed additives must be registered by the MASC. Changes to animal feed and feed additives regulations is the second large category of developments that happened the China's food and feed regulatory system in recent years (2009). In addition to the change in quality standards for most grains and oilseeds, China published a host of feed quarantine regulations throughout 2009, and the draft regulation appears to collect many of the various themes of the 2009 regulations and repackage them through the State Council. The current situation with feed and feed additive standards is presented in the 2009 China Export Certification Report and the supporting documents that can be found in various FAS GAIN reports.

The two main changes in 2009 appeared in AQSIQ Feed Quarantine Regulation 118 (CH9071) and AQSIQ Feed Quarantine Implementing Announcement 372 (CH9074). Much of the information in Regulation 118 and Announcement 372 is itself not new; it is a consolidation of older measures and incorporates feed and feed additives within the general reorganization of measures resulting from the Food Safety Law. Exporters should read and evaluate the measures to understand how they might be affected. One point of clarification relates to the requirement for exporter registration that is set out in Regulation 118 Chapter III Import Inspection and Quarantine, Section 1 Registration, Articles 10 and 11. The statement in I Import Inspection and Quarantine, (1) Registration of Announcement 372 adds some clarity, "AQSIQ will publish a list of countries/

regions which are eligible to export their feed products to China, as well as a list of the permitted products, and will carry out this registration of foreign feed producers in an orderly manner. For those countries/regions who have completed registration, the imported feeds shall only be sourced from the registered producers or processors." China has noted that this registration process is for quarantine, product safety, and trace back reasons. As of November 2009, there is a quarantine facility registration process for exporting of nonruminant animal-derived feed with China's AQSIQ, such as pet food. However, there currently is no mandatory registration requirement for other US feed and feed additive exporters. According to preliminary explanations provided by AQSIQ to USDA representatives, AQSIQ plans to complete feed registration as a multistage process. The first stage, as noted in the quote above, is a process that many countries go through to assess whether the general laws, regulations, and oversight of a supplier country are "equivalent" to their own. Thus, China intends to send a detailed questionnaire out to trading partners to gather information about the equivalency of an exporter's regulatory structure versus their own. Part of this examination will involve detailing how the supplier generally regulates feed and feed additives domestically and for export and how exports are handled in terms of regulation, monitoring, and certification. This questionnaire will be sent to the US Government. Assuming that China agrees that an exporter has an equivalent system, it would request that the exporting country create and maintain a list of companies that are eligible and want to produce feed and feed additive products for export to China. This list of possible exporters would be submitted to AQSIQ authorities. China then intends to maintain some type of monitoring as described in Chapter III of Regulation 118. At the current time, AQSIQ has not submitted the questionnaire to the United States and has not stated when it is likely to do so. According to AQSIQ, registration for plant- and chemical-based feed and feed additives will not begin until the equivalency process has run its course and the two countries have negotiated an understanding of these new registration requirements. As noted above, one class of feed and feed additives, nonruminant-derived animal feed, currently undergo a quarantine registration process. For these products, they must request inclusion onto a potential exporter list by USDA's Animal and Plant Health Inspection Service (APHIS). Once these products gain APHIS's concurrence that they meet China's quarantine requirements, APHIS places them on a list that is submitted to AQSIQ. Generally speaking, AQSIQ accepts the list several times a year and updates their internal list for distribution to their provincial affiliate offices (CIQs) in a timely fashion. At this point, a product is eligible for a "Health Certificate/Export Certificate for Animal Products" for export to China issued by APHIS whose provisions are governed by the Protocol for Veterinary Health Requirements for Nonruminant Derived Animal Feed and Tallow to be imported from the United States into the People's Republic of China. As of November 2009, 143 US facilities producing nonruminant-derived animal feed are currently registered with AQSIQ for export to China [11]. The recent history and breadth of this issue is presented in GAIN

Reports CH9033 (2008 Catalogue of Feed Additives), CH9071 (AQSIQ Feed Quarantine Regulation 118), CH9074 (Implementation of Feed Quarantine Regulation 118), CH9075 (Risk Levels and Inspection and Quarantine Supervision Methods for Import and Export of Feed and Feed Additives), CH9057 (Corn Quality Standards), and CH9083 (Feed Labeling). Additional attention has been paid to the existing registration for animal feed and feed additives currently conducted by China's Ministry of Agriculture. Basically speaking, all nongrain or nontraditional feed and feed additives need to be registered with the Ministry of Agriculture. This definition is not described clearly by MOA, but appears to mean any feed except grains/oilseeds and their most basic/standard processed derivatives (e.g., soybeans/soy meal/soy oil or corn/corn meal/starch). Details on feed registration process and history can be found in GAIN Reports CH6083, CH6084, CH6080, CH6099, CH6101, and CH6091. This process involves extensive submissions of information to MOA and the registration process can take a significant amount of time. Many in the US export industry complain that the current MOA registration process requests product production information, which might be the intellectual property of the company. They note that MOA's process does not provide additional value to Chinese regulators and that the proposed AQSIQ registration system makes it clearly duplicative and trade inhibiting. Not surprisingly, the shifting situation and unclear understanding by many in the industry and Chinese officials outside the central government has caused a number of contradictions to persist in the system as it relates to imports. An example is the status of imported processed feed grains, which includes dried distillers grains with solubles (DDGS). China currently produces a limited amount of ethanol from corn and has DDGS as a by-product. This Chinese-produced DDGS is marketed, but is not recognized in MOA regulations in any feed component category and does not have a national quality standard. Imported DDGS have never been approved by MOA through its official feed registration process, though some facilities are currently making their way through the process. However, provincial CIQs regularly grant imported DDGS the official right to enter the country for Chinese domestic distribution. Moreover, DDGS officially appears in AQSIQ feed quarantine standards (risk classifications) for the United States. This contradictory situation persists despite the fact that the regulation of imported DDGS has not been subject to any of the regulatory changes mentioned in the paragraphs above. At this time, USDA China recommends that companies adhere to quarantine requirements necessary to obtain quarantine import permits and strive to complete the MOA feed registration process (Table 24.5).

Nowadays, in China, enterprises contravening the legislation by manufacturing or handling feed or feed additives against the legal constraints are severely punished by the authorities.

The FAO has recently published a synthesis of the Chinese legislation on feed and feed additives [12].

Recently (in 2015), China was in the process of implementing a revised regulatory and registration system for imported feed and feed additives. Under

Table 24.5 Regulatory Framework of Food and Feed Additives (Including Enzymes) in China

Regulatory authority	*Name*: Ministry of Agriculture (MOA) *Website*: For Catalogue of Feed Materials (http://www.moa.gov.cn/zwllm/tzgg/gg/201206/P020120614568242256328.doc and http://www.moa.gov.cn/govpublic/XMYS/201304/t20130408_3426383.htm) or for the Catalogue of Feed Additives (http://www.moa.gov.cn/zwllm/tzgg/gg/201401/t20140103_3730193.htm, bilingual version available for Appendix I) issued by the Ministry of Agriculture of China *Historical overview*: Founded September 1954 (The Ministry of Agriculture of China) *Roles/responsibilities*: • To draft agricultural laws, regulations and policies; to propose agricultural development programs and strategic goals; to formulate technical protocols, agricultural standards, and to supervise their enforcement • To propose regional agricultural programs, to conduct overall planning, and to coordinate the nationwide allocation of agricultural resources • To strengthen the administration to improve their quality, increase the development of the food/feed industry and animal production, and enhance product safety • To coordinate safety and agricultural exchanges and collaborations between China and the international organizations • To undertake other assignments entrusted by the State Council
Advisory scientific body	*Name*: Ministry of Agriculture of the State Council (MASC), and Administration of Quality Supervision, Inspection and Quarantine (AQSIQ) *Website*: Same as Regulatory authority (above) *Historical overview*: Same as Regulatory authority (above) *Roles/responsibilities*: • The MASC is responsible for the national administration of feed and feed additives; the feed and feed additives administrative department of the County government is responsible for local feed and feed additives administration. The clear meaning of the terms is presented in the legislation. Before the manufacture of new feeds and feed additives, the researcher or manufacturer (hereafter called the "applicant") must put forward an application for evaluation of the new product to the MASC. Feed analysis and feeding trials are conducted by an organization assigned by the Ministry

(*Continued*)

Table 24.5 Regulatory Framework of Food and Feed Additives (Including Enzymes) in China *Continued*

	• AQSIQ is required to conduct risk analysis and to review the feed safety regulatory systems of countries and regions exporting feed ingredients and additives to China for the first time, and to perform retrospective inspections on countries and regions that have exported or are exporting these products to China. Based on the results of the risk analysis or the retrospective inspection, AQSIQ is required to formulate, adjust and make public a list of approved countries and facilities for categories of feed ingredients and additives • The risk analysis and food safety system regulatory review are conducted by product category. AQSIQ then conducts market access reviews and facility registration by category, subcategory, or product. It is unclear what process AQSIQ uses to determine how it will handle facility registration for different categories. Processed aquatic animal protein (fishmeal) was registered together as one category. AQSIQ is currently considering registering feed additives by subcategories. AQSIQ has typically required audits as part of the market access and registration processes. The exporters are required to pay all of AQSIQ's audit expenses • Products with a record of export to China considered to be "traditionally traded" by AQSIQ can continue to be exported to China while facilities complete the registration process. AQSIQ may require a "traditionally traded" product to also undergo a second market access review if it determines that the existing protocol does not meet current regulations, although trade can continue while this is completed. Products that have had intermittent or low volume trade with China have sometimes had trouble getting recognized as "traditionally traded" • The current market access and registration process is relatively new and is still evolving. So far, it has taken applicants roughly 2 years to complete both the market access and registration process. The US Government and other exporting countries are engaging AQSIQ on ways to make this process more streamlined and less duplicative of MOA's review process
Framework regulations	• Administrative Measures for Feed and Feed Additives, Decree 609 of State Council, enforced on May 1, 2012 • Regulation on Feed and Feed Additive Import Registration, MOA Decree No.2, 2014, enforced on July 1, 2014

(*Continued*)

Table 24.5 Regulatory Framework of Food and Feed Additives (Including Enzymes) in China *Continued*

	• Feed Ingredient Catalogue, MOA Decree No. 1773 (Enforced on January 1, 2013) and No. 2038 (Enforced on December 29, 2013) • Feed Additive Catalogue, MOA Decree No. 2045 (Enforced on February 1, 2014) • Medical Feed Additive Catalogue, MOA Notice No. 168
Part of an overarching international organization	• China has been a member of the World Trade Organization since 2001 • China is a member of the Codex Alimentarius Commission • China has engaged with USA (AQSIQ with USDA), particularly in the status of US Feed and Feed Additive Market Access and Facility Registration [2]
Recent or pending changes	China is in the process of implementing a revised regulatory and registration system for imported feed and feed additives. Under this system, companies need to complete the following three steps before they can export feed ingredients or additives to China: (1) obtain an import registration license from the Ministry of Agriculture (MOA); (2) apply for market access with the General Administration for Quality Supervision, Inspection and Quarantine (AQSIQ); and (3) have their manufacturing facility registered by AQSIQ. This process can include an audit, which China requires the exporter to pay for. Products considered to be "traditionally traded" by AQSIQ can continue to be exported to China while they complete steps two and three. MOA's registration process typically takes several months, while the market access and registration process with AQSIQ can take 2 or more years (GAIN Report Number: CH15062, Date: 12/7/2015).
Regulatory overview of feed and feed additives	• See "Recent or pending changes" (above) • Exporters should take care to ensure they have: (1) an import registration license from MOA, and (2) confirmation that the specific manufacturing facility is listed on AQSIQ's website (or that the product is considered "traditionally traded") *before* shipping feed ingredients or feed additives to China. Failure to do so will likely result in shipments being refused entry at the Chinese port of inspection *Regulation*: • Specifically, the Department of Animal Husbandry and National Feed Management Office under MOA and the Department for Supervision of Animal and Plant Quarantine under AQSIQ are charged with regulating the importation and marketing of feed and feed additives in China

(*Continued*)

Table 24.5 Regulatory Framework of Food and Feed Additives (Including Enzymes) in China *Continued*

	• See "Framework regulations" (above) • Complementary measures to the regulations: (1) Administrative Measures of Production License for Animal Feed and Feed Additives, MOA Decree No. 3; (2) Administrative Measures of New Feed and New Feed Additives, MOA Decree No. 4; (3) Administrative Measures of Product Approval Number for Feed Additives and Premixture; MOA Decree No. 5; (4) Practice for Safety Usage of Feed Additive, MOA Notice No.1224
Enzymes	*Definition*: "Biological products directly extracted from edible or nonedible parts of a plant or animal or fermented and extracted from traditional or genetically modified microorganisms (including but not limited to bacteria, actinomycetes, and fungi) that are used in food processing and have a special catalytic function" [1] *Regulation*: Hygienic Standard for Enzyme Preparations Used in Food Processing (Notified to the WTO as G/SPS/N/CHN/112 on 5 January 2009) *Guidance document*: See "Regulatory overview of feed and feed additives" (above) *Approval process for a new substances*: See "Regulatory overview of feed and feed additives" (above)

this system, companies need to complete the following three steps before they can export feed ingredients or additives to China: (1) obtain an import registration license from the Ministry of Agriculture (MOA); (2) apply for market access with the General Administration for Quality Supervision, Inspection and Quarantine (AQSIQ); and (3) have their manufacturing facility registered by AQSIQ. This process can include an audit, which China requires the exporter to pay for. Products considered to be "traditionally traded" by AQSIQ can continue to be exported to China while they complete steps two and three. MOA's registration process typically takes several months, while the market access and registration with process AQSIQ can take 2 or more years [2] (Table 24.2).

24.6 CONCLUSION AND FUTURE PERSPECTIVES

In all the target countries, the main purpose of each regulatory authority is to establish a framework and maintain/enforce regulations to ensure the safety of food and/or feed additives consumed and sold within its respective countries. Although the path for approval of different categories of food and/or feed additives varies from jurisdiction to jurisdiction of these countries, there are many commonalities in terms of the data requirements and considerations for

assessment of the safety of use of substances/additives (including enzymes) added to food and/or feed, including the use of positive lists of approved substances/additives, premarket approval, and a separation between science and policy decisions. International collaboration is taking place to address the challenge of developing regulatory guidance and safety assessment for use of food and feed additives, including enzymes. Harmonization of global food and feed additives regulations is envisioned to promote the use of all available food and feed additives including enzymes through free trade for developing sustainable agriculture (including aquaculture), and ultimately, for minimizing production costs and reducing hunger and poverty globally (Table 24.6).

Table 24.6 Comparison of the Regulations on Enzymes Among the Target Countries

	United States	Canada	China
Definition	Not defined	Not specifically defined in the Food and Drug Regulations	Biological products directly extracted from edible or nonedible parts of a plant or animal or fermented and extracted from traditional or genetically modified microorganisms (including but not limited to bacteria, actinomycetes, and fungi) that are used in food processing and have a special catalytic function
Regulation	Enzymes are regulated as substances added directly to food, specifically as direct food additives, secondary direct food additives, or GRAS substances	Enzymes are regulated as food additives or may be considered as processing aids, depending on their context of use	Enzymes are regulated as food additives
Approval process	Same process as substances added directly to food. See "direct food additives" (above)	Same process as direct food additives and processing aids	Same process as direct food additives

Source: *Adapted from Magnuson B, Munro I, Abbot P, Baldwin N, Lopez-Garcia R, Ly K, et al. Review of the regulation and safety assessment of food substances in various countries and jurisdictions. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 2013;30 (7):1147–1220.*

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CHAPTER

Evaluation of Enzymes for Animal Nutrition by the EFSA in the European Union

25

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25.1 INTRODUCTION

The European Food Safety Authority (EFSA) provides scientific advice and communicates on existing and emerging risks associated with the food chain. EFSA issues advice on existing and emerging food risks. This advice informs European laws, rules and policymaking—and so helps protect consumers from risks in the food chain. Its remit covers food and feed safety, nutrition, animal health and welfare, plant protection, plant health. EFSA's scientific work is led by its scientific committee and its 10 panels, including the panel on Additives and Products or Substances used in Animal Feed (FEEDAP) (https://www.efsa.europa.eu/).

The FEEDAP panel provides scientific advice on the safety and/or efficacy of additives and products or substances used in animal feed. This panel evaluates their safety and efficacy for the target species, the user, the consumer of products of animal origin and the environment. Safe animal feed is important for the health of animals, the environment,t and for the safety of foods of animal origin. There are many examples of the close link between the safety of animal feed and the foods we eat. For instance, mammalian meat and bone meal (MBM) was banned from all farm animal feed in the European Union (EU) in 2001 because it was linked to the spread of bovine spongiform encephalopathy (BSE) in cattle and BSE-infected meat was associated with the variant Creutzfeldt–Jakob Disease (vCJD) in humans.

What farmers feed to livestock depends on a range of factors including the species and age of animals, the type of food produced—such as meat, milk, or eggs—the price, availability, and nutritive value of different feedstuffs, and geographical factors including soil type and climate. Types of feed include fodder—such as hay, straw, silage, oils, and grains—and manufactured products which are typically compound mixtures of feed materials that may contain additives.

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25.2 FEED ADDITIVES

Feed additives play an important role in agriculture and are a focus of the EU regulatory framework. They are products used in animal nutrition to improve the characteristics of feed, for instance to enhance flavor or to make feed materials more readily digestible. They are often used in intensive farming production on a large scale. A company wishing to put a feed additive on the EU market must obtain prior authorization. As part of this process, EFSA evaluates the safety and efficacy of each additive and checks for adverse effects on human and animal health and on the environment (https://www.efsa.europa.eu/). Feed additives include:

- technological additives, e.g., preservatives, antioxidants, emulsifiers, stabilising agents, acidity regulators, silage additives;
- sensory additives, e.g., flavors, colorants;
- nutritional additives, e.g., vitamins, aminoacids, trace elements
- zootechnical additives, e.g., digestibility enhancers, gut flora stabilizers; and
- coccidiostats and histomonostats.

The European legislation on animal feed provides a framework for ensuring that feedstuffs do not present any danger to human or animal health or to the environment. It includes rules on the circulation and use of feed materials, requirements for feed hygiene, rules on undesirable substances in animal feed, legislation on genetically modified food and feed, and conditions for the use of additives in animal nutrition.

25.3 EFSA'S ROLE IN THE EVALUATION OF FEED ADDITIVES

EFSA has a key role in providing independent scientific advice to support the authorization process for feed additives. This work is carried out by FEEDAP. Cooperating with the European Commission, the Panel carries out an evaluation of each new additive submitted for authorization. Companies wishing to market a specific additive in the EU need to submit an application with information on the identity of the additive, its conditions of use, control methods, and data demonstrating its efficacy and safety.

The FEEDAP Panel reviews this information and examines the efficacy and safety of the additive in terms of animal and human health as well as the environment, including residues in soil, ground water and surface water. In parallel, the Community Reference Laboratory for feed additives evaluates the analytical methods used to determine the presence of the additive in feed and its possible residues in food.

If EFSA's Opinion is favorable, the European Commission prepares a draft Regulation to authorize the additive, in a procedure involving Member States

represented in the Standing Committee on the Food Chain and Animal Health—Animal Nutrition. Authorizations are granted for specific animal species and/or conditions of use and are limited to 10-year periods which may be renewed (http://ec.europa.eu/food/safety/animal-feed/feed-additives/eu-register/index_en.htm). The evaluation of the safety of food additives and feed additives is carried out by different Scientific Panels at EFSA, with different data requirements due to the separate regulatory frameworks in the two areas. However, EFSA's Panels coordinate their scientific work to ensure consistency in their risk assessment approaches and consideration of the available scientific information within these fields.

The application procedure for authorizations is described in Regulation EC 1831/2003 (http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32003R1831+). Applicants must send: (1) an application to the European Commission, (2) a technical dossier directly to EFSA, (3) three reference samples of the feed additive to the European Union Reference Laboratory. The European Commission website provides an overview of the authorization process for feed additives. EFSA carries out the risk assessment whilst the European Commission decides whether or not to authorize the feed additive.

25.4 EVALUATION OF ENZYMES FOR ANIMAL NUTRITION

Enzymes are widely used as additives in animal nutrition. They are intended to favorably affect animal production by improving the digestibility of feedstuffs (EU Regulation 1831/2003, article 5). The feed enzymes are classified in the category "digestibility enhancers" which designs substances which, when fed to animals, increase the digestibility of the diet, through action on target feed materials (EU Regulation 1831/2003, Annex 1).

Tables 25.1 and 25.2 give an overview of the enzyme products (commercial names, enzymes composition, target species) which have been assessed by the EFSA FEEDAP panel since 2003. More than 40 different enzyme preparations intended to be used as feed additives have been evaluated. All full reports concerning the safety and efficacy assessments of these additives can be found on the EFSA website (https://www.efsa.europa.eu/). The target species are mainly poultry and porcine species, and in particular young animals for which the digestive functions are not fully mature. Two enzyme products have been proposed for salmonids and only one enzyme preparation has been proposed for ruminant species. Indeed, the particular stomach compartment of ruminants displays major hydrolytic functions which explain the more or less absence of enzyme preparations for these species.

Two main categories of enzyme preparations can be distinguished: (1) enzymes aiming at hydrolyzing phytic acid to improve phosphorus utilization and (2) enzymes aiming at hydrolyzing complex polysaccharides which are present in

Table 25.1 Phytase Additives Evaluated by the FEEDAP Panel of EFSA

Commercial Names	Enzymes	Target Animal Species
	Phytases	
Natuphos	3-Phytase	Poultry species
Phytase SP	3-Phytase	Poultry and porcine
Rovabio PHY	3-Phytase	Poultry and porcine
Axtra PHY	6-Phytase	Poultry and porcine
Bio-Feed Phytase	6-Phytase	Salmonids
Enzy Phostar	6-Phytase	Poultry and porcine
Finase	6-Phytase	Poultry and porcine
Optiphos	6-Phytase	Poultry and porcine
Phyzyme XP	6- Phytase	Poultry and porcine
Quantum	6-Phytase	Poultry and porcine
Ronozyme HiPhos/P	6-Phytase	Poultry, porcine, and salmonids

Table 25.2 Polysaccharide Enzymes Additives Evaluated by the FEEDAP Panel of EFSA

Commercial Names	Enzymes	Target Animal Species
	Xylanases and Glucanases	
Belfeed	Endo-1,4-β xylanase	Poultry
Bio-Feed Wheat	Endo-1,4-β-xylanase	Ducks and pigs for fattening
Danisco Xylanase	Endo-1,4,-β-xylanase	Poultry and porcine
Econase XT P/L	Endo-1,4-β-xylanase	Poultry and porcine
Hostazym X	Endo-1,4-β-xylanase	Poultry and porcine
Natugrain Wheat	Endo-1,4-β-xylanase	Poultry
Feedlyve AXC	Endo-1,4-β-xylanase	Turkeys
Ronozyme WX	Endo-1,4-β-xylanase	Poultry and porcine
Safizym X	Endo-1,4-β-xylanase	Poultry species and piglets
Hostazym C	Endo-1,4-β-glucanase	Poultry for fattening and piglets
AveMix	Endo-1,4-β-xylanase and endo-1,3(4)-β-glucanase	Poultry and porcine
Axtra XB	Endo-1,4-β-xylanase and endo-1,3(4)-β-glucanase	Lactating sows
Bio-Feed Combi	Endo-1,4-β-xylanase and endo−1,3(4)-β-glucanase	Chickens for fattening and piglets
Danisco Glycosidase	Endo-1,4-β-xylanase and endo-1,3(4)-β-glucanase	Poultry and porcine

(*Continued*)

Table 25.2 Polysaccharide Enzymes Additives Evaluated by the FEEDAP Panel of EFSA *Continued*

Commercial Names	Enzymes	Target Animal Species
Endofeed	Endo-1,3(4)-β-glucanase and endo-1,4-β-xylanase	Poultry and porcine
Econase Wheat Plus	Endo-1,4-β-xylanase and endo-1,3(4)-β-glucanase	Chickens for fattening
Grindazym GP	Endo-1,4-β-xylanase and endo-1,4-β-glucanase	Ducks
Natugrain TS	Endo-1,4-β-xylanase and endo-1,4-β-glucanase	Poultry and porcine
Rovabio Excel	Endo-1,3(4)- β-glucanase and endo-1,4-β-xylanase	Poultry and porcine
Rovabio Spiky	Endo-1,4-β-xylanase and endo-1,3(4)-β-glucanase	Poultry
Porzyme 9100	Endo-1,3(4)- β-glucanase and endo-1,4-beta-xylanase	Piglets
Roxazyme G2	Endo-1,4-β-xylanase, endo-1,4-β-glucanase and endo-1,(3)4-β-glucanase	Poultry species and piglets
Mannanases/Amylases		
Hemicell	β-D-Mannanase	Chickens for fattening
Ronozyme Rumistar	α-Amylase	Dairy cows
Galactosidases and Glucanases		
AGal-Pro	α-Galactosidase and endo-1,4-β-glucanase	Poultry species
Biogalactosidase	α-Galactosidase and endo-1,4-β-glucanase	Chickens for fattening
Multiple Enzyme Combinations		
Amylofeed	Endo-1,3(4)- β-glucanase, endo-1,4-β-xylanase and α-amylase	Piglets
Avizyme	Endo-1,4-β-xylanase, subtilisin and α-amylase	Poultry
Kemzyme	Endo-1,3(4)-β-glucanase, endo-1,4-β-glucanase, α-amylase, bacillolysin and endo-1,4-β-xylanase	Poultry and porcine

high amounts in animal feed. The first category (Table 25.1) includes 3-phytases and 6-phytases. These phosphatase enzymes catalyze the hydrolysis of phytic acid (myo-inositol hexakisphosphate)—an indigestible, organic form of phosphorus that is found in grains and oil seeds—and release a usable form of inorganic phosphorus. Thus, phytases increase the availability of phosphorus in diets, lowering

the amount of supplemental phosphorus required in the diet. The second category (Table 25.2) mainly concerns endo-1,4-β xylanases and endo-1,3(4)-β-glucanases. Endo-1,4-β xylanases play an important role by degrading the linear polysaccharide beta-1,4-xylan into xylose, thus breaking down hemicellulose, one of the major components of plant cell walls. Endo-1,3(4)-β-glucanases break down glucans (polysaccharides made of several glucose subunits). To a lesser extent, this category also includes α-amylase, α-galactosidase, or b-mannosidase, which hydrolyze alpha-linked polysaccharides, such as starch and glycogen, the terminal alpha-galactosyl moieties from glycolipids and glycoproteins or terminal, nonreducing beta-D-mannose residues in beta-D-mannosides.

All these enzyme preparations have been assessed in terms of safety for the target animals, the consumers, the users and the environment. Furthermore, an assessment of efficacy was performed. These assessments were performed on the basis of the EU Regulation 429/2008 (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri = OJ:L:2008:133:0001:0065:en:PDF) and the relevant Technical Guidances available at the EFSA website (https://www.efsa.europa.eu/). These enzyme preparations whose modes of action are well characterized were found to have a real potential to improve the zootechnical parameters of the target species when included in the diet at adequate doses (at least three positive trials expected for each enzyme product): the details of the positive effects can be seen in the full reports of each enzyme preparations (https://www.efsa.europa.eu/). For each enzyme additive, the details of the FEEDAP report can be found on the EFSA web site (https://www.efsa.europa.eu/). Regarding the safety assessment, the enzyme preparations were generally considered safe for the target species, the consumer and the environment (for details, see the individual reports, https://www.efsa.europa.eu/). Owing to the proteinaceous nature of their active substances, the enzymes preparations may represent potential hazards as respiratory sensitizers or skin sentitizer, and they are potentially harmfull if inhaled. Furthermore they may be dermal and eye irritants. When appropriate protective measures are employed to avoid dermal and inhalation exposure there is also no concern for the safety of users.

On the basis of the EFSA scientific assessments, the European Commission provides or not an authorization of enzymes preparations to be placed on the market. The list of current authorized additives are listed in the European Union Register of Feed Additives pursuant to Regulation (EC) No. 1831/2003 (http://ec.europa.eu/food/safety/animal-feed/feed-additives/eu-register/index_en.htm).

CHAPTER

Economics of food and feed enzymes: Status and prospectives

26

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26.1 INDUSTRIAL ENZYMES: A GLOBAL MARKET OVERVIEW

The global industrial enzymes market can be analyzed from different perspectives: by application, by key enzyme category (functionality), or by geographic segmentation.

The global market is analyzed by presenting commercial value of products rather than quantities (volume of enzymes sold by application or category). Such values are estimated and consolidated from various open business sources.

26.1.1 GLOBAL ENZYME MARKET, BY APPLICATION

The following application markets can be considered:

- cleaning agents, including laundry and dishwasher detergents, household care and personal care products;
- food and beverages, including dairy, baking, fruit and vegetable processing, brewing;
- animal feed;
- agrofuel, including both first and second generation agrofuels
- others, including textile and leather processing, waste treatment, and paper processing, to name a few.

The global market for industrial enzymes is estimated at a global value of between US $4500 million and US $5000 million in 2015. Food and beverages is the largest application for industrial enzymes. The global demand for processed food combined with a tremendous increase in the per capita income of the population in emerging economies are driving the demand for food and beverages, leading the food industry to optimize their production. In many food processing applications, the use of enzymes significantly improves yield and quality.

Altogether, food and feed applications account for 55%–60% of the global enzymes market (Table 26.1), and the market will continue growing at an estimated 6%–8% annual growth in the period 2016–20.

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Table 26.1 Global Enzymes' Market Share by Application (2015)

Application Market	Market Share (%)
Food and beverages	35
Cleaning agents	25
Animal feed	20
Agrofuel	10
Others	10

26.1.2 GLOBAL ENZYME MARKET, BY GEOGRAPHY

North America is by far the largest market for industrial enzymes with an estimated 40% market share, followed by Europe with a 30% market share. In both regions, the industrial enzymes market is expected to growing at 5%−6% annual rate (CAGR) in the period 2016−20.

The Asia-Pacific region is expected to be the fastest growing enzyme market at a CAGR of 8%−9% driven by an increased demand for food and feed applications.

26.1.3 GLOBAL ENZYME MARKET, BY ENZYME FUNCTIONALITY

Carbohydrases, proteases, and lipases are the key enzyme functionalities for industrial applications. The carbohydrases segment accounts for almost 50% of the industrial enzymes market, reaching 70% for the food and beverages application. Proteases represent between 25% and 30% of the global market, followed by lipases with less than 10% of the market share.

Other categories include oxidases (e.g., glucose oxidase), phytase, transglutaminase, which are most of the time associated with specific applications and markets. For instance, phytase is almost exclusively used in the animal feed sector.

The carbohydrases is a very broad category of enzymes comprising enzymes such as amylase, cellulase, pectinase, lactase, invertase, and various other enzymes. Carbohydrases are the key enzymes for processing plant-derived substrates, making them the most important category of enzymes for food, feed, and agrofuel applications.

26.1.4 INDUSTRIAL ENZYMES PRODUCTION

Industrial enzymes can be obtained from microbial, plant, and animal sources. Most of the enzymes currently developed for industrial applications are obtained from microorganisms (bacteria, yeasts, or fungi).

The main plant-sourced enzymes are proteases: papain from papaya and bromelain from pineapple.

Some enzymes are still from animal sources, such as cheese coagulants from calf rennet or lipases from pig pancreas. Both plant- and animal-derived enzymes are limited in their development by the scarcity of sourcing, the lack of batch-to-batch consistency and, for the animal tissues enzymes, by the sanitary issues.

Microbial enzymes are produced by microorganisms grown in industrial-scale fermentors and their quality is not impaired by season/climate. Their quality is globally speaking more constant from one batch to another and protein expression has been optimized to reach high active protein yields.

Modern biotechnology tools are massively used to optimize the performance of microorganisms used for producing industrial enzymes. Genetically modified microorganisms (GMM) such as bacteria or filamentous fungi are used in all application segments with only a few exceptions (e.g, wine).

Microbial enzymes account for 90% of the global market and their producers are located mainly in Europe and Asia. The three dominant global enzyme producers (Novozymes, Dupont, DSM) account for more than 75% of the global enzymes business. Novozymes accounts for 45%–50% of the market and is the sole company active in all enzyme markets and being almost fully dedicated to the enzymes business. At DuPont and DSM, the enzyme business is a minor portion of their global activity.

Other active players are AB Enzymes, Chr Hansen, Kerry, and Soufflet Biotechnologies in Europe.

Japan also has enzyme suppliers that have been established for a long time such as Ajinomoto, Amano, Nagase, and Shin Nihon.

There are numerous enzyme producers also in China (Leveking, Pangbo, Sunson, etc.) and India (Advanced Enzymes, Aumgene, Lumis, etc.) serving both local and global markets.

Microbial enzyme producers can also be categorized by the fermentation system they use, with two leading technologies:

- Submerged (liquid) fermentation, where the microorganisms are cultivated on a liquid substrate in large stainless steel vessels. This is the dominant technology in terms of quantity of enzymes produced worldwide and is the technology producers mostly use in Europe.
- Solid state fermentation (SSF), where the microorganisms grow on a solid substrate (rice, wheat bran, beet pulp). This technology is derived from traditional Asian fermentation technologies (Koji) and not surprisingly is still the dominant fermentation process for enzymes production in Japan. It is worth noting that Soufflet Biotechnologies, established in France, relies on SSF for their enzymes production.

Both technologies have their advantages and inconveniences and without entering into complex technical considerations, it is widely recognized that submerged fermentation is more flexible and allows large-scale fermentors (e.g., 300–350 m^3). Typically, enzymes produced by the mean of GMM are obtained using liquid submerged fermentation.

SSF technology is mainly used for the production of enzyme formulations with multiple activities suitable for the hydrolysis of complex polymers, such as cellulose and pectin, typically found in the food and feed sectors. SSF technology involves smaller but more complex fermentation vessels.

26.2 ECONOMICS OF FOOD PROCESSING ENZYMES, BY APPLICATION

26.2.1 SUGAR AND STARCH

The market for sugar and starch enzymes includes all the enzymes used for the production of potable alcohol, sugar and sweeteners, and starch for food applications, as well as smaller markets such as oligosaccharides and prebiotic fibers. Alcohol production as an alternative to fossil fuel (first-generation fuel ethanol) and starch for industrial applications are not included in this review.

The global market size for the in-scope sugar- and starch-processing enzymes is estimated at US $300 million. Amylases are the largest category in the baking segment, with 65% of the total sugar and starch category. Other enzymes are invertase, glucose isomerase, proteases, and phytase (Table 26.2). The enzymes market for the production of distilled beverages represents one-third of the total sugar and starch enzymes.

From a geographical perspective the starch and sugar enzymes markets is structured as follows:

- North America: 45% market share;
- Asia: 25%–30% market share;
- Europe: 15% market share;
- ROW: 10% market share.

The North American market is driven by the production of high fructose corn syrups (HFCS) for the soft drinks industry. More than 60% of corn starch is processed into HFCS, with the application of immobilized glucose isomerase. The European sugar market is driven by beet sugar and HFCS capacities are limited.

Distilled alcohol for human consumption is produced mainly in Europe (40% of global production) and North Asia (at least 30% of global volume), followed by North America with 10%–15% market share. The bacterial alpha amylases and glucoamylases prices range from US $1.5 to US $3 per kilogram. Specific, thermostable amylases can cost up to US $6–8 kg^{-1}. Based on average dosage recommendations, it can be estimated that the cost of enzymes to produce 1 hL (100 L) of distilled spirit is around US $1.

Invertase is sold either in liquid or microgranulate form, and under a broad concentration range (anything from 1000 to 200,000 invertase units per gram) and the invertase selling price ranges from US $5 to US $200 kg^{-1}. Addition rate is

Table 26.2 Starch and Sugar Enzymes' Key Benefits and Market by Functionality (2015)

Enzyme Functionality	Key Benefits	Market Share (%)
Amylases (fungal amylase, glucoamylase)	Starch hydrolysis, from native starch to oligomers and monomeric sugars (fermentable sugars for the production of ethanol, sweeteners, functional food ingredients...)	60–65
Glucose isomerase (immobilized)	Glucose to fructose conversion to produce high fructose corn syrups (HFCS)	30–35
Invertase	Sucrose hydrolysis to glucose and fructose, to minimize crystallization in confectionery applications, and to increase the sweetening power of sucrose, as an alternative to acid hydrolysis	<5
Others (dextranase, pullulanase, glycosyl transferases, etc.)	Dextranase used in cane sugar production in case of microbial spoilage to decrease the juice high viscosity due to microbial dextrans	<5

calculated taking into account the initial sugar concentration, the reaction temperature, and the conversion (hydrolysis) degree desired. Invertase is produced by the fermentation of baker's yeast *Saccharomyces cerevisiae*.

Glucose isomerase cost-in-use is more difficult to evaluate as the enzyme is being used immobilized, allowing multiple cycles of use in the process.

26.2.1.1 Market structure and value chain

Starch- and sugar-processing enzymes are in large majority directly supplied to the processing industry from the enzymes manufacturers, in large containers (e.g., 1000-L containers). Some niche markets such as invertase for confectionery are served through regional distribution channels.

Both Novozymes and DuPont are dominant in the amylase and glucose isomerase markets, with an estimated total 80%–90% market share. In China, local enzyme producers supply local industry players and they have also started exporting to North America and Europe.

As this is a large volume/commodity business, Novozymes and Dupont benefit from large scale capacity and multiple production sites, as well as long-established relationships with sugar and starch global players.

26.2.1.2 Key trends in starch and sugar enzymes

Amylases are a commodity market, with a few exceptions (thermostable amylases) and generally speaking the production of enzymes by GMM is not an issue in this

sector. Amylase prices are constantly under pressure because of the strong buying power of large accounts, and also because of new lower-cost players from Asia (China and India).

New developments are expected in the field of oligosaccharides with prebiotic properties (which can be produced with enzymatic steps from simple sugars) and other functional food ingredients. The recent development in rare sugars, such as human milk oligosaccharides, may also open the door to new enzymatic developments but these are not expected to reach the market size of established starch and sugar positions.

The market for invertase as an alternative to acid hydrolysis of saccharose should increase because of its clear benefits (more "green" technology, better end-product quality) but it is somehow still limited because of cost-in-use and process modifications. It is worth noting that often the enzyme technology adoption can be very slow when new processes/equipments have to be implemented at the same time.

26.2.2 BAKERY

Bread in its simplest version is made from wheat flour, water, salt, and yeast. Industrial breadmaking processes include other ingredients and additives such as nonwheat flours, emulsifiers, preservatives, and other chemical additives. The addition of enzymes to the dough facilitates production operations (dough machinability, shorter proofing, etc.) and improves the quality of the bread (crust color and flavor, longer shelf life, etc.). Enzymes in bread making also act as alternatives to chemical additives, which is a global trend in the food-processing arena ("clean label" trend).

The global market size for baking enzymes is estimated at US $250 million. Carbohydrases (amylases and hemicellulases) are the largest category in the baking segment, with 75% of the total baking enzymes business. Other enzymes are proteases, glucose oxidase, lipases, and asparaginase (Table 26.3).

From a geographical perspective the baking enzymes markets is structured as follows:

- North America: 40% market share;
- Europe: 35% market share;
- Asia: 15% market share.

The European market is driven by two countries, France and Germany. Germany is still the largest baking enzymes market, accounting for 20%–25% of the European business.

The Asia-Pacific is projected to be the fastest growing region for the bakery enzymes market, growing at a CAGR of at least 9%, driven by population growth and food habits shift with an increased demand for industrial processed food.

There are strong regional differences in the usage of enzymes, in terms of end-user (milling, bread improvers manufacturers, industrial baking, artisanal baking) and in terms of bread (or biscuits) style.

Table 26.3 Baking Enzymes' Key Benefits and Market by Functionality (2015)

Enzyme Functionality	Key Benefits	Market Share (%)
Amylases (fungal amylase, autogenic amylase, glucoamylase)	Dough machinability, shorter proofing, improved CO_2 release, crust color and flavor, improved loaf volume, and longer shelf life	60
Hemicellulases (xylanase, arabinofuranosidase)	Dough handling, loaf volume, and structure	15
Others (lipases, glucose oxidase, proteases, asparaginase, etc.)	Proteases: dough structure and strength Glucose oxidase: stronger doughs Lipases: dough stability, loaf volume, reduction or replacement of chemical emulsifiers Asparaginase: prevention of the formation of acrylamide	25

Three factors do have a major impact on regional baking enzymes dynamics:

- enzymes supply market structure;
- global bread production (quantity and quality);
- industrial vs. artisanal bakeries ratio.

To better match with regional trends and market structure, most of the leading baking enzymes suppliers have established regional hubs including enzyme formulation and application facilities ("baking laboratories").

26.2.2.1 Baking enzymes pricing and cost-in-use

Most enzymes applied in baking are in dry form (powders, microgranulates) and blended with a flour carrier.

As in many other industrial applications, it is very difficult to get a clear-cut picture of enzyme cost-in-use in baking. There are major differences in concentration for the same enzyme category and enzyme suppliers often sell baking enzymes as complex formulations made from different enzyme functionalities. Moreover, in many cases the enzyme activity is defined by nonharmonized/standardized methods making the comparison almost impossible.

Cost-in-use is the enzyme price per quantity of substrate, typically expressed in enzymes cost in \$ per 100 kg of flour. The calculation is based on the recommended dosage of enzymes (expressed either in grams of enzyme mixture per 100 kg of flour or in ppm units), given by the enzyme supplier.

The average price of amylases is US \$5 kg^{-1} but can cost US \$50 kg^{-1} and above for highly concentrated/highly specific formulations, and their application rate can be anything between 1 g and 50 g per 100 kg of flour. Calculated cost-in-use is in a range from US \$0.05 per 100 kg to US \$0.25 per 100 kg.

The same reasoning applies to other baking enzymescategories with the following price and dosage indications:

- hemicellulases: concentrated, specific formulations at US $25–50 per kg/ 0.5–2 g per 100 kg dosage. Diluted, commodity products at US$5–10 per kg/ 5–10 g per 100 kg dosage;
- the price for lipases and proteases is even more heterogenous as those categories include enzymes from very distinct origins and functionalities. Some products can reach prices of US $100 per kg and above.

26.2.2.2 Market structure and value chain

The baking enzymes value chain is a complex picture: enzyme producers can sell their products via different channels:

- enzyme blenders and traders;
- bread improvers companies;
- millers;
- industrial bakers.

Typically, the artisan bakers are not directly served by enzyme producers. The enzymes are supplied to artisan bakers as part of the bread premix they source from millers and bread improver companies. The proportion of enzymes going from the enzyme primary producer to one or the other actor of the value chain (blender, miller, etc.) is extremely variable from one region to another. On average it can be estimated that less than 10% of the enzymes are directly supplied to bread makers by the enzyme primary producer. The key suppliers of baking enzymes are Novozymes, DSM, DuPont, and AB Enzymes. In France, Soufflet Group has a unique position being at the same time a wheat collector, a milling company, a bread structure improver with their structure AIT, and a supplier of enzymes (Soufflet Biotechnologies).

26.2.2.3 Key trends in baking enzymes

The baking enzymes market is strongly driven by the total bread and other cereal-based products consumption (biscuits, crackers, etc.) and regional shifts (e.g., Asian countries moving more and more to bread consumption). The consumer-driven trends can also have an impact on baking enzymes development:

- clean label—enzymes as alternatives to chemical ingredients (emulsifiers, preservatives);
- health—growth is expected in the asparaginase category, to prevent acrylamide formation in baked products;
- convenience—longer shelf life is expected by both retailers and consumers and enzymes can significantly contribute to shelf life extension;
- consumer experience—impact of enzymes on taste, color, and texture.

26.2.3 DAIRY

The market for dairy enzymes includes animal-derived enzymes and microbial enzymes: coagulants (animal rennet and also microbial proteases), lipases, and lactase. Dairy enzymes are applied in cheese production to help the process and also to enhance the flavor of cheese during the ripening stage. In recent years, lactase application has grown significantly for the production of lactose-free dairy products.

The global market size for dairy enzymes is estimated at US $250 million. Milk-clotting enzymes (proteases) are the largest category in the dairy segment, with 60% of the total dairy enzymes business. Lactase is a rapidly growing market and is estimated to represent 25% of the global dairy enzymes sector. Other categories include antimicrobial enzymes (lysozyme), bleaching enzyme (peroxidase), and protein cross-linking transglutaminase (Table 26.4).

From a geographical perspective the dairy enzymes markets is structured as follows:

- Europe: 55% market share;
- North America: 30% market share;
- Asia and ROW: 15% market share.

Europe and North America are still the largest dairy-producing and -consuming regions. In North America, the trend for lactose-free products is confirmed and drives the market growth for lactase. In Europe, the application of enzymes in cheese processing is impacted by regulations. Some enzymes are not approved for use in controlled origin labels. Asia-Pacific is a fast growing market for dairy enzymes, driven by the increase in the consumption of processed dairy products, mainly yogurts. Cheese is also growing, but not at the same rate as fresh dairy goods. Also in Asia lactose-free is a confirmed trend in the industry.

26.2.3.1 Dairy enzymes pricing and cost-in-use

Most enzymes used in dairy applications are in liquid form. Solid forms (powders and microgranulates) are sometimes used, especially at the level of small, artisan

Table 26.4 Dairy Enzymes' Key Benefits and Market by Functionality (2015)

Enzyme Functionality	Key Benefits	Market Share (%)
Chymosin, milk clotting enzymes (microbial and animal rennet)	Milk coagulation during cheese-making process	60
Lactase	Lactose removal for the lactose-intolerant population	25
Others (proteases, lipases, lysozyme, nisin, transglutaminase, peroxidase)	Flavor development (lipases and proteases), bioprotection (lysozyme and nisin), bleaching (peroxidase), protein cross-linking—transglutaminase	15

cheese producers (easier handling and storage of the enzyme). As for all other food sectors, it is very complex to establish a clear picture of dairy enzymes prices (large differences in concentration, lack of harmonized activity measurement, etc.). For chymosin, standards do exist but the product sold to the end user is often blended with other activities (e.g., pepsin). Animal rennet and microbial clotting enzymes cost around US $\$4-6\ L^{-1}$. On average, the addition rate of milk-clotting enzymes is 0.1%–0.2% (100–200 mL of enzyme solution for 100 L of milk). For the production of 1 kg of a cheese requiring 10 L of milk, the cost-in-use of the coagulant is very low, not more than US $0.2. Lactase is sold at a higher price per kg, in the US $\$40-50\ kg^{-1}$ range. Cost-in-use is therefore relatively high and dosages need to be adapted to achieve a complete lactose hydrolysis. The cost of lactase treatment has a significant impact on lactose-free dairy products. As a consequence, lactose-free milk can cost 30%–50% more than regular milk at the retailer level.

26.2.3.2 Market structure and value chain

Enzymes for the dairy industry are sold direct to end-users and via distributors. Enzymes can be sold as a single offer or in commercial combinations with other dairy ingredients (starter cultures, emulsifiers, colorants, etc.). Novozymes only supplies enzymes while Chr Hansen and DuPont can offer a broader range of ingredients. Chr Hansen from Denmark holds an estimated 35% market share, DSM is second in the market with 25%–30% market share followed by Dupont, Novozymes, and Amano. DSM has a robust positions in the lactase enzyme market. Ajinomoto from Japan supplies transglutaminase, notably for yogurt production. The dairy industry is a highly consolidated sector, with large global players (Friesland Campina, Danone, Savencia (ex Bongrain), Fonterra, Glanbia, Lactalis, etc.). Most of the key global players have European roots, mainly in France, Ireland, and The Netherlands. Besides large multinational players, there is a significant fragmented dairy enzymes market, especially for European cheese production. Smaller operations will buy enzymes and other ingredients from national distributors or regional laboratories. The distribution networks are supplied by the large enzyme producers and the enzymes are sold either under the manufacturer's brand or under distributor private label brands.

26.2.3.3 Key trends in dairy enzymes

Lactose intolerance (LI) is due to the lack of lactase in the small intestine to hydrolyze lactose into glucose and galactose. The LI population is very heterogenous between human ethnic groups as well as from one country to another. Thus, about 75% of Africans and Amerindians are LI, whilst only less than 10% of North Europeans are suffering. There are four types of LI. Primary LI is observed when the amount of lactase declines as people age. Secondary LI is due to injury to the small intestine such as from infection, celiac disease, inflammatory bowel disease, etc. Developmental LI may occur in premature babies and usually improves over a short period of time. Congenital LI is an extremely rare genetic

disorder in which little or no lactase is produced from birth. LI individuals suffer from diarrhea, bloating, and other gastrointestinal discomfort symptoms when they consume dairy products, especially whole milk. Another category of individuals which is not lactase deficient also suffer from similar symptoms after consuming dairy products. Such individuals represent up to 30% of the adult population in regions like North America. Therefore, the global demand for lactose-free dairy consumer goods has increased tremendously, leading to high demand for lactase solution. In addition to the health benefits, either clinically proven or consumer perception based, the application of lactase to milk slightly modifies its taste, because the hydrolysis of lactose produces sugars with higher sweetening power, and is leading the food industry to launch new products. In 2016, Novozymes launched a new lactase from a *Bifidobacterium* strain (current market reference lactases are produced by yeasts) called Saphera and presented to be more efficient at lower pH and higher temperatures as compared to market standards. Other trends include a continuous shift from animal enzymes (rennet) to microbial clotting enzymes and dairy market expansion in Asia and other nontraditionnal dairy areas.

26.2.4 BREWING

The market for brewing enzymes includes all the enzymes added during the brewing process, from mashing to conditioning. Malt can also be viewed as a source of enzymes during the process, but malt-market dynamics will not be discussed in this review. Enzymes used as cleaning additives—filter cleaning enzymes—are also not reviewed here. The global market size for brewing enzymes is estimated to be at US $95 million. Enzyme categories in brewing are amylases (alpha-amylases and amyloglucosidases), betaglucanase, hemicellulases (xylanases, etc.), proteases, and alpha acetolactate decarboxylase (ALDC). The most important categories in terms of market share are amylases and betaglucanase based formulations (Table 26.5).

Table 26.5 Brewing Enzymes' Key Benefits and Market by Functionality (2015)

Enzyme Functionality	Key Benefits	Market Share (%)
Amylases (alpha-amylase, fungal amylase, amyloglucosidase)	Improve fermentation yield by releasing fermentable sugars from polysaccharides (starch)	30–35
Glucanase and hemicellulases	Fermentation yield improvement, viscosity reduction, beer clarity	25–30
Proteases	Fermentation yield improvement (releasing free amino acids from proteins); beer stabilization; gluten-free beer	20–25
Acetolactate decarboxylase (ALDC) and others	Beer maturation, preventing diacetyl formation	<10

From a geographical perspective the brewing enzymes markets is structured as follows:

- Europe: 20%–25% market share;
- North America: 25%–30% market share;
- Asia (China): 20% market share;
- ROW: >25% market share.

Brewing enzymes dynamics are extremely different from one region to another. First of all, the more malt is used in the process, the less enzymes (amylases, glucanases) are added. In regions with lower access to malt (Africa, Asia), enzymes are massively used to convert nonmalted sources of carbohydrates into fermentable sugars. In Europe, there are two distinct situations: Germany (and to some extent Austria and Czech Republic) do follow the beer purity law (also known as Reinheitsgebot) where enzyme addition to the brewing process is not permitted, and the other countries where enzymes can be used. Also in European countries there remains a GMO public concern and some breweries decided to ban any GMM-derived enzyme. China is the largest beer-producing country, accounting for at least 25% of the world beer production and enzymes are massively used. Africa is a significant beer-producing area, and a key enzymes market such as African beer does not include as much malt as in other regions. Local crops such as cassava are used, making the use of enzymes a determinant technology in the process. In North America there is a growing demand for gluten-free beer, stimulating the demand for some specific proteases (such as the prolin-specific Brewers Clarex formulation from DSM).

26.2.4.1 Brewing enzymes pricing and cost-in-use

Brewing enzymes are sold either in microgranulated or liquid form. Dosages are determined based on the step at which the enzyme is added and are typically expressed in grams of enzymes per hectoliter (hL) to be treated, or in kg per metric ton of substrate. Amylases can cost US \$3–5 per kg, with higher prices for specific amyloglucosidases. Their addition rate is around 0.5 g to 1 kg/ton. Pure glucanases formulations can also fall into the same cost figures as amylases but very often glucanases are part of complex formulations including hemicellulases (xylanase and other activities). Formulated glucanase-based products can cost up to US \$20–25 per kg. Dosages are very much dependent on the nature of substrate to be treated (malt addition percentage) and concentration of the enzyme mixture, but average dosages are 200–400 g/T. Proteases range from US \$10 to US \$50 kg^{-1}, depending on concentration and specificity of action. Broad-spectrum plant proteases (papain) cost less than US \$10 kg^{-1} while amino-acid specific proteases can be sold at \$50 kg^{-1}.

ALDC is available from only a limited number of producers and is still not massively employed by the industry; its cost is therefore on the high-end, greater than US \$100 kg^{-1} with a recommended dosage of 1–2 g/hL.

26.2.4.2 Market structure and value chain

The brewing industry is now dominated by multinational groups and during the period 2000–15, massive mergers and acquisitions have been finalized. The top five brewing companies are Anheuser-Busch InBev, SABMiller, Heineken, Carlsberg, and China Resources. Ongoing in 2016 is the planned acquisition of SABMiller by AB InBev for a transaction greater than US $100 billion. Together the top five brewing groups account for more than 60% of the global beer production.

In North America there is still a growing number of craft breweries and large beer conglomerates regularly acquire some of the most successful newcomers.

Large breweries purchase their ingredients, enzymes included, directly from the producers while artisan/craft breweries are served through local distributors who often sell other goods (equipment, bottling, etc.), and services (beer analysis, brewing consulting, etc.).

The leading enzyme suppliers to the brewing industry are Novozymes (40% market share), DuPont, and DSM (each with an estimated 20% market share). Kerry from Ireland is also a significant brewing enzymes producer, and the Chinese market is supplied by a growing number of local producers.

26.2.4.3 Key trends in brewing enzymes

As the industry is under a strong consolidation, enzyme purchase is driven by corporate purchase management teams rather than by production engineers or brewmasters. The increased buying power of large multinationals leads to a price erosion of brewing enzymes, especially for the commodity functionalities (amylases and pure glucanases). At the same time, raw material and energy prices are rising, malt is often replaced with cheaper sources of starch (crude grain, rice, cassava, sorghum, etc.), stimulating the demand for complex enzyme combinations. Such enzyme blends are more difficult to compare from one supplier to another and high prices are somehow maintained. Technologies like proteases to reduce the stabilization time of the beer are also being developed to save on energy and product immobilization costs. The fierce competition between beer brands is also driving the industry to innovate with new beer styles (new flavors, different alcohol percentages, etc.), thus opening the door to enzyme-based technologies. In North America, and also in other regions, the global trend on gluten free food has forced the industry to launch low-gluten/gluten-free beers. This can be achieved by a complete exclusion of wheat and barley from the process or by using traditional grains in the process and hydrolyzing the gluten using specific enzymes.

26.2.5 WINEMAKING

The wine enzymes market comprises enzymes applied for all wine styles (white, red, rosés, sparkling). Enzymes in winemaking are used for the clarification, maceration, filtration, and aging steps of the process. The global market for

Table 26.6 Wine Enzymes' Key Benefits and Market by Functionality (2015)

Enzyme Functionality	Key Benefits	Market Share (%)
Pectinases	Juice clarification, improved color extraction, filtration	95
Betaglucanase, Betaglucosidase	Filtration, flavor enhancement	<5
Lysozyme	Microbial stabilization	<1

winemaking enzymes is estimated at US \$15–20 million, and Europe has the largest share of this sector. Pectinases are by far the dominant category of enzymes for winemaking. Other enzymes are betaglucanase and betaglucosidase, and also lysozyme (Table 26.6).

Within the pectinases functionality, there are two subcategories:

- clarification pectinases: these are mainly used in white wine production after pressing and prior to fermentation, to remove pectins and other colloids and obtain a clear juice
- maceration (or extraction) pectinases: these are mostly used in red wine production to facilitate and improve the extraction of color and tanins from grape skins. They directly impact the quality of the final product. Betaglucosidase-based formulations are added towards the end of alcoholic fermentation to release free volatile aromas from glycosylated (bound, nonvolatile) chemical structures. They directly improve the quality perception of wine. Betaglucanases have two purposes: they facilitate the filtration operations and they also improve the quality of wine through the release of polysaccharides (better "mouthfeel").

From a geographical perspective the wine-making enzymes market is structured as follows:

- Europe: 60% market share;
- North America: 20% market share;
- Southern hemisphere: 15% market share;
- ROW: 5% market share.

Europe is the largest wine-producing region; France, Italy, and Spain produce 50% of the world's wine volume. Enzymes have been used in wine production in Europe since the mid-1970s and volumes remain stable, following wine production figures. European prices are still rather high compared to the Southern Hemisphere mainly because of the preference for concentrated, microgranulated forms of enzymes. Globally speaking, the market for winemaking enzymes is rather stable.

26.2.5.1 Wine enzymes pricing and cost-in-use

Enzymes for winemaking are sold either in liquid or in solid (microgranulated) forms, and there are strong, historic regional preferences. Most of the European

market is dominated by granulated enzymes while the Southern Hemisphere and North American markets are supplied with liquid enzymes. As a general rule, smaller wineries select solid-form formulations (easier handling and storage) while larger wineries will go for the liquid enzymes.

The cost for pectinases in winemaking ranges from US \$20 to US \$30 kg^{-1} for the liquid formulations and from US \$40 to US \$70 kg^{-1} for the microgranulated products. Wine-specific betaglucanase and betaglucosidases can cost up to US \$150 kg^{-1}. Dosages are usually expressed in grams or milliliters of enzyme per hectoliter of juice or wine, and can range from 1−2 g/hL for the most concentrated microgranulated preparations to 10−20 mL/hL for liquid pectinases. Recently, the use of glycerol as a carrier for wine liquid enzymes has been approved in Europe, opening the door to cheaper fruit-processing pectinases (where glycerol has always been approved) and price erosion in wine-processing pectinases is expected.

26.2.5.2 Market structure and value chain

The wine industry is still a very fragmented sector, especially in the European countries. It is very different from the brewing sector, which indeed is an industry while wine making remains very much in the hands of small- to medium-sized producers.

For these reasons, European wineries mainly source their enzymes (and the other winemaking supplies) from distributors and subdistributors, very often located in the region of wine production.

Only a few consolidated large wineries in California, South America, and Australia purchase enzymes directly from their manufacturers.

Leading suppliers (manufacturers) of enzymes for the wine industry are Novozymes, DSM, AB Enzymes, and Soufflet Biotechnologies. Key global distributors for winemaking enzymes are Laffort, Lallemand, Enartis, Erbsloeh, Oenobrands and Scott Laboratories.

26.2.5.3 Key trends in wine enzymes

The wine enzymes market very much follows the wine production volume, which has been pretty much stable in the last decade.

There has been no major innovation in the winemaking enzymes in the last 20 years and only new blends of existing enzymes are introduced to the market.

The market is conservative and enzymes produced by means of GMM are not yet viewed favorably, which can be seen as a limitation to radical innovation in this sector.

26.2.6 FRUIT AND VEGETABLE PROCESSING

Fruit processing has been one the first food industries to routinely use enzymes, back to the 1930s. All fruits and vegetables contain pectin, a major component of plant cell walls acting as a “glue” and leading to difficulties during processing,

due to its viscosity. The addition of pectinases to the processing of fruits, mainly in the juice industry, leads to a drastic improvement in yield and the obtention of clear, stable juices.

The market is driven by two industry segments: the apple juice and the juices made from berries (grapes, cranberries, blackcurrant, etc.). Other markets of significance are the fruit-firming market (strawberries, tomatoes) and the lemon /citrus market. Orange juice production does not involve enzymes as it is sold in cloudy form. Orange juice by-products (pulp and skin) can be enzymatically processed for the recovery of other products such as essential oils.

The fruit (and vegetable) processing enzymes are estimated at a global value of US \$40–\$50 million, and it mainly consists of pectinases used to increase the juice yield after pressing. Other applications are pectin methyl esterase (PME) for fruit firming and cellulases/hemicellulases for filtration. Cellulases and hemicellulases are often sold in combination with pectinases and it is therefore difficult to give an accurate estimation of their market importance (Table 26.7).

From a geographical perspective the fruit processing enzymes market is structured as follows:

- Europe: 35% market share;
- North America: 30% market share;
- China: 25% market share;
- ROW: 10% market share.

Europe is still the largest fruit pectinases market, from a value perspective. The European market is driven by the berry-processing industry in Germany, Poland, and the United Kingdom, and to a lower extent, apple juice in Poland and Germany. In North America, the market is driven by berry-processing applications (mainly cranberries and grapes). China is the largest producer of apple juice concentrate (AJC) and uses pectinases from GMM to boost the yield of juice extraction. The market for pectinases in China fluctuates according to AJC production versus AJC demand ratios. Other markets are very fragmented, with the application of pectinases to process a large variety of fruits and vegetables in all countries of the world. The apple and pear processing industry is the largest

Table 26.7 Fruit-Processing Enzymes' Key Benefits and Market by Functionality (2015)

Enzyme Functionality	Key Benefits	Market Share (%)
Pectinases	Juice yield increase, color extraction, filtration and clarification improvement	85
Pectin methyl esterase (PME)	Fruit and vegetable structure reinforcement (fruit firming)	<10
Cellulases and hemicellulases	Filtration, yield increase	<10

market for fruit enzymes, with 35%–40% of the total market, followed by berries with 25% market share. Vegetable processing (carrot juice, beet juice, etc.) is still a small portion of the global fruit and vegetable sector, with less than 10% market share.

26.2.6.1 Fruit-processing enzymes pricing and cost-in-use

Most fruit and vegetable processing enzymes are sold in liquid form, stabilized with 45%–50% glycerol and supplied in small (20 L) to very large (1000 L) containers.

The price structure for fruit-processing enzymes is very heterogeneous due to the very large range of products available. Enzyme formulations have been developed for the different process technologies and for the different fruits to be processed.

On average, liquid pectinases obtained from non-GM microbial strains cost US \$15–20 L^{-1} while liquid pectinases produced with GMM can cost as low as US \$8 L^{-1}. Complex formulations containing pectinases, cellulase, and hemicellulase are in the cost range US \$15–25 L^{-1}.

Dosage recommendations are very much dependent on the fruit characteristics and the processing technology, and can be anything from 50 to 200 g of pectinase preparation per ton of fruit.

26.2.6.2 Market structure and value chain

The fruit-processing market is a rather industrialized sector, with strong regional differences.

In Asia and in China mainly there are only a few apple juice producers, purchasing the enzymes they need directly from the manufacturers, either from Europe or locally. In the last decade, more enzymes have been produced in China, either in factories owned by European companies (Novozymes, DSM) or by Chinese biotechnology companies.

In North America, fruit-processing pectinases are imported from Europe and are sometimes formulated locally to match with local needs. Large fruit-processing companies such as Ocean Spray (cranberry juice) or Welch's (grape juice) are directly supplied by enzyme manufacturers.

The European market is more fragmented with both large companies (Dohler, Agrana, etc.) sourcing enzymes from producers and small/local players purchasing enzymes and other ingredients from specialized enzyme formulators and distributors (Erbsloeh, Biocatalysts, Weissbiotech, etc.).

26.2.6.3 Key trends in fruit and vegetable processing enzymes

There has not been any significant innovation in enzymes for fruit processing since the market introduction of GM-produced/single-activity pectinases in the 1990s. Apple juice processing pectinases sales do follow the AJC market, which is very fluctuant. The berry juice segment is the fastest growing one as it benefits from a positive, health-benefits image due to their content in vitamins and

antioxidants. The market of pectinases for berry processing is therefore expected to grow, with new fruits processed to juice ("superfruits" such as acai, goji, etc.). There are also some real opportunities for high performance pectinases delivering benefits that the consumers can perceive and value (color, flavor, nutritional value). While the AJC market is dominated by commodity pectinases, berry processing requires more technology (pectinases adapted to low pH and/or resistant to higher processing temperatures). It is therefore expected that technology leaders will maintain their position in this sector and might also introduce new products in the medium term. The fruit firming application, developed in the early 1990s and based on PME enzyme could gain an increased interest. It has been initially deployed for the strawberry-processing industry, to produce fruit preparations further incorporated in yogurts and ice creams. The same technology can be applied to a large variety of fruits and vegetables (mangoes, kiwis, carrots, tomatoes). The current market trends in clean-label (fruit firming is typically achieved using chemical additives) and government health campaigns ("5-a-day") should stimulate the enzymes-based fruit-firming technology in the near future.

26.2.7 PROTEINS PROCESSING WITH ENZYMES

The market for protein-processing enzymes in this review includes all applications in the transformation of plant- and animal-derived proteins for the food and pet-food industries. It is a proteases market, with a few other enzymes used in combination with proteases for optimized performance. Within these in-scope applications, proteases are used as processing aids to improve protein product yields and qualities while reducing costs and processing times. Global demand for in-scope protease enzymes applications is estimated at US $100 million, constituting a small portion of the overall protease market, the largest application for industrial proteases being the detergent industry. It is a market with a growth projection of 8%–9% annually, driven by the increasing demand for low-cost proteins as global meat consumption rises and by the development of highly specific protein-derived ingredients, such as bioactive peptides. Meat coproduct valorization and meat processing are the leading in-scope protease applications, collectively accounting for 60% of total demand. In meat coproduct valorization, proteases are used by rendering companies to hydrolyze bone, meat, blood, and feather coproducts into meal, oils, flavors, and also fertilizers. Meat-processing applications primarily use proteases as meat tenderizers, sometimes in combination with other enzymes. In fish-waste valorization and fish sauce production, proteases are used to break down waste fish components and increase the hydrolysis process. Proteases are also used as palatability enhancers and digestive aids in the pet-food sector (Table 26.8).

From a geographical perspective the food protein enzymes market is structured as follows:

- North America: 45%–50% market share;
- Europe: 30% market share;

Table 26.8 Proteases' Key Benefits and Market Applications in the Protein-Processing Sector

Application	Key Benefits	Market Share (%)
Meat coproducts	Protein extraction and functionalization (savor enhancers products)	30
Meat processing	Meat tenderization, processed meat based products	30
Fish by-products and fish sauces	Protein extraction, functional hydrolyzates	10–15
Pet food	Hypoallergenic proteins, palatants, high digestibility and functional peptides	5–10
Hydrolyzed plant proteins (HVP)	Higher digestibility, savor enhancers	10–15

- Asia: 15% market share;
- ROW: 5%–10% market share.

North America is the leading regional market due to the traditional and widespread use of protease in the meat-processing and meat coproduct valorization segments. Protease demand in Europe is still restrained due to regulatory restrictions in some countries, particularly with products intended for human consumption. Although protease utilization in South America and the Asia/Pacific region is less intensive than in Europe and North America, these two smaller markets are expected to grow significantly as local protein demand expands. European, South American, and Asian protease markets will also benefit from the rapid expansion in the aquaculture sector. In fish and shrimp processing, by-products represent up to 50% of the body mass and can greatly benefit from protease processing to generate high-added-value products such as savory ingredients, pet food palatants, and bioactive peptides.

26.2.7.1 Protein processing enzymes pricing and cost-in-use

Global protease demand is evenly split between microbial and plant-based (papain from papaya) forms in value terms.

Depending on the usage rate of proteases and the application (meat coproducts, fish coproducts) and the targeted functionalities, the cost of the enzyme will vary. Prices may range from US \$10–30 kg^{-1} for microbial proteases. Plant proteases (bromelain and papain) prices range from US \$10 to US \$15 kg^{-1}. Typical application rates range from 1 to 5 kg of enzyme preparations per metric ton of substrate, for an average enzyme cost-in-use of less than US \$0.05 kg^{-1} of hydrolyzed substrate. Considering the added value carried by the enzyme hydrolysis (improved digestibility, better taste, optimized functionalities) the protease cost is considered as a minor cost in the overall process expenses.

26.2.7.2 Market structure and value chain

Enzyme manufacturers generally supply proteases directly to the processing industry (rendering companies, tenderizer and flavor blend producers, and pet food ingredient suppliers). As in almost all other food enzymes markets, dedicated distributors and blenders are active in the industry to serve smaller accounts, but they are not the main protease sales channel. Two leading companies, Novozymes and Dupont, account for 50%–60% of global demand. The Japanese enzyme producer Amano has well-established positions in this sector. Other active players are DSM and AB Enzymes. The leading protease suppliers are generally focused on microbial proteases and operate on a global level, while much of the remaining market consists of smaller, regional firms that are focused on specific plant proteases such as papain or bromelain. Papain suppliers are either located in India (Patel, Senthil) or in Belgium (Enzybel, importing raw material from India and Africa).

26.2.7.3 Key trends in protein enzymes

Plant-derived proteases, papain mainly, are expected to be more and more substituted by microbial proteases. The supply and price of papain is highly fluctuant due to seasonal variations and political issues in some of the sourcing areas. Moreover, papain protease is not a single-activity enzyme and some industry users find it very unpredictable in its hydrolysis pattern.

Protein producers are looking for financial performance optimization and they are constantly innovating in the valorization of coproducts. New enzyme technologies allowing a better valorization of by-products such as poultry feathers, blood, or collagen tissues will be very attractive for the industry. New protein sources are also being explored and will be requiring proteases for process optimization. The recent development of the insect-farming industry is a good illustration of those new potential applications. Already for more than a decade, milk ingredients companies have developed sophisticated enzyme-based processes to generate pure, characterized bioactive peptides. Some of those peptides may possess drug-like effects such as, e.g., anxiolytic bioactive peptides. The fish-processing industry is also exploring the field of bioactive peptides, and such functional hydrolyzates are already on the marketplace in Europe and in North America. There should be more developments in this sector, driving the demand for highly specific proteases.

26.2.8 OILS AND FATS AND OTHER FOOD ENZYMES APPLICATIONS

The oils and fats industry enzymes are mainly lipases used for oil degumming during the process. Crude vegetal oils (soybean, rapeseed, sunflower, etc.) can be refined using phospholipases as an alternative to chemical processing. Three main forms of phospholipases (PL) are used by the industry: PLA1, PLA2, and PLC. PL used in the oil industry are of microbial origin (*Trichoderma*,

Aspergillus, *Pichia*, etc.) or still from porcine pancreas. Leading suppliers for the PL range of enzymes are Novozymes, DSM, Dupont, AB Enzymes, and Verenium (now BASF).

The benefits of using PL in oil refining vs. acid extraction or high-temperature extraction are:

- energy saving, working at lower temperatures (50−60°C instead of 85°C);
- yield improvement, up to +2%;
- easier pumping and separation operations.

Oil processors are very large companies (ADM, Bunge, Cargill, etc.) purchasing enzymes directly from suppliers and typically codeveloping the enzyme processes with suppliers. The global market is difficult to estimate. Enzyme dosage for oil degumming is in the range 30−50 g of PL solution per metric ton of oil, and one can estimate the cost of microbial PL to be US \$30−50 kg^{-1}.

26.2.9 OTHER FOOD APPLICATIONS OF ENZYMES NOT REVIEWED IN DETAIL

Other food applications of enzymes not reviewed in detail include:

- Lipases to improve the emulsification properties of egg yolk.
- Glucose oxidase to remove glucose from egg yolk prior to its industrial processing.
- Betaglucosidases and cellulases to improve the quality of vanilla during the vanilla bean curing process.
- Lipases and other enzymes application in cocoa processing, for better machinability, texture and flavor.
- Transglutaminase from meat and fish protein cross-linking
- etc.

26.3 ECONOMICS OF FEED ENZYMES

Animal feed enzymes are added to feed for various purposes, mainly to enhance feed digestibility and promote growth, as well as for environmental reasons. As many animals cannot digest 15%−20% of the feed that they consume due to hard-to-digest feed components, enzymes are added to ensure an optimized release of energy and micronutrients from the ingested materials. Environmental concerns such as reduction of phosphorous contents in manure are also in favor of using feed enzymes: specific enzymes can unlock phosphorous from feed, thus preventing its loss. The global animal feed enzyme market is still growing and offers a promising future due to increasing demand for meat and meat products, especially within the fast economy-growth regions. Europe is still the largest market for animal feed enzyme.

Table 26.9 Feed Enzymes' Key Benefits and Market Share per Category

Enzyme Category	Key Benefits	Market Share (%)
Phytase	Enhance nutritional value of feed, reduce the amount of phosphorous in manure (environmental benefits)	35–40
Xylanases	Improve the uptake of nutrients by decreasing the viscosity of feed. Increase feed energy value	15
Cellulases		15
Glucanase		10
Other NSP-ases (pectinases, mannanase, etc.)		<10
Proteases	Improves the uptake of nutrients by breaking-down proteins; supplements the impact of endogenous proteases	<10

The global feed enzymes market is estimated at US $850 million. Swine and poultry are the dominant animal categories in this sector. Other species with a significant enzyme market include ruminants and aquacultured species such as fish and shrimps. The market for pet food is not included in this review. Annual growth for the global feed enzymes is 6%–8% and is very much region dependent. The global feed additives market is valued at US $15 billion and therefore enzymes account for less than 10% of the feed additives sector. Other additives of greater market importance are vitamins, synthetic amino acids, and minerals.

Enzymes categories supplied to the feed industry are mainly phytase, non-starch polysaccharidases (NSP), other carbohydrases—a category including cellulases, xylanases, glucanases, mannanase, pectinases—and proteases (Table 26.9).

26.3.1 GEOGRAPHICALLY THE FEED ENZYMES MARKET IS STRUCTURED AS FOLLOWS

- Europe: 45% market share;
- Asia: 25% market share;
- North America: 20% market share;
- ROW: 10% market share.

Europe is the largest feed enzymes market with US $380 million in 2015 and is expected to grow at CAGR of 6%–7% during the period 2015–20. The feed enzymes market in Asia is valued at US $210 million and has become the second-largest feed enzymes market over the last decade. The North American market is now in the third position for feed enzymes, valued at US $170 million. Fastest growth is expected in the Asian market, driven by China and India at a CAGR greater than 8%.

26.3.2 ECONOMICS OF FEED ENZYMES, BY ENZYME CATEGORY

26.3.2.1 Phytase

Phytase is essential for enhancing nutritional value of feed and improving animal growth performance and health. It hydrolyzes phytate substrates to release phosphorous in a free form which animals can absorb efficiently, resulting in a lower demand for supplemental inorganic phosphorus. Moreover, the addition of phytase to feed results in a lower excretion of the phosphorous content in manure, contributing to a lower environmental impact of livestock farming. Phytase for feed application is produced by fermentation of microbial strains and is mostly used in swine and poultry feeds. Phytase accounts for over 40% of the total feed enzyme market, and is valued at US \$300−350 million. There are significant differences in the market price for phytase and the product is sold under different concentrations. Phytase activity is expressed in FTU units and industrial phytase formulations are typically in a range from 5000 to 100,000 FTU/g (microgranulated form only). For a product standardized at 5000 FTU/g, market price can be anything from US \$1 to US \$5 kg^{-1}. Phytase products manufactured in China for the local market are in the lowest price range. However, comparison from one product to another cannot be made on a simple FTU activity basis as other elements will affect the enzyme performance. Without entering into complex technical considerations, four key factors differentiate one phytase from another: mode of action (3-phytase vs 6-phytase), stability (to temperature, pH, and proteolysis), optimum pH, and optimum temperature. Moreover, phytases are produced from encoding genes of very distinct microorganisms (*Aspergillus niger*, *Peniophora lycii*, *Escherichia coli*, *Citrobacter braaki*, etc.) making the global market picture very complex (Table 26.10).

Table 26.10 Overview of Commercial Phytases, by Organism, Phytase Class, and Producing Company

Donor Organism	Production Organism (host)	Phytase Class	Company
Aspergillus ficuum	*Aspergillus niger*	3-phytase	BASF
Aspergillus niger	*Trichoderma reesei*	3-phytase	AB Vista
Penicillium funiculosum	*Penicillium funiculosum*	3-phytase	Adisseo
Peniophora lycii	*Aspergillus oryzae*	6-phytase	Novozymes/ DSM
Escherichia coli	*Schizosaccharomyces pombe*	6-phytase	DuPont
Escherichia coli	*Pichia pastoris*	6-phytase	AB Vista
Escherichia coli	*Trichoderma reesei*	6-phytase	AB Vista
Citrobacter braakii	*Aspergillus oryzae*	6-phytase	Novozymes/ DSM
Buttiauxella sp.	*Trichoderma reesei*	6-phytase	DuPont

Source: Adapted from J. Vehmaanperä, IPS2 Congress; 2012.12.13, Rome.

26.3.2.2 Nonstarch polysaccharides enzymes

NSP are carbohydrates defined for not being starch. They are a broad category of complex carbohydrates such as glucans, xylans, mannans, etc. In animal feed they originate from wheat, rice, barley, rye, etc. They are often insoluble and show antinutritive activity in animals. Because of their insolubility, they increase the viscosity of the feed in the animal digestive tracts and reduce its efficiency, typically expressed as Feed conversion ratio (FCR).

NSP enzymes are added mainly to poultry and swine feed, to increase their digestive processes. Use of some NSP enzymes increases the nutritional value of the feed ingredient and metabolized energy content increases.

NSP enzymes play a significant role in maintaining the environment of the farm, as it decreases the humidity of droppings, which also contributes to animal health by reducing the risk of microbial contamination. In addition, the emission rate of ammonia in the production facility is reduced. Altogether, NSP enzymes contribute to a better feed performance, a more healthy environment for the animals, and a cleaner environment. Total share of NSP enzymes in the total feed enzymes market is around 50%, valued at US $425 million.

Within the NSP enzymes category, four subcategories can be defined and valued:

- xylanases, with an estimated value of US $150 million;
- cellulases, with an estimated value of US $130 million;
- glucanases, with an estimated value of US $90 million;
- mannanase and pectinases, with an estimated value of $55 million.

Market price and cost-in-use for NSP enzymes is very fragmented, as products are often blends of different enzymes at different concentrations. Average market price for NSP formulations can be estimated at US $8–10 kg^{-1} of enzyme.

26.3.2.3 Proteases

Proteases are added to improve the uptake of feed nutrients, mainly in poultry application. They improve the protein digestibility and contribute to a cleaner environment by decreasing the nitrogen emission from livestock production. Global proteases market for feed applications is valued at US $8–10 million.

26.3.3 ECONOMICS OF FEED ENZYMES, BY ANIMAL CATEGORY

Poultry and swine are the two most important animal categories when it comes to the application of enzymes in animal feed. They account for close to 70% of the global feed enzymes market. Within the poultry and swine subcategory, the poultry segment shows the fastest increase, correlated with the global demand for chicken meat. Other animal categories include ruminants (10% of the feed enzymes market), aquaculture, and other small categories such as horses, birds, etc.

26.3.3.1 Poultry

Cereal-based diets developed for poultry nutrition contain high levels of NSP, such as arabinoxylans resulting in increased viscosity of the digesta. This increased viscosity reduces the passage rate of the feed, leading to overall reductions in consumption and decreased performance, as well as sticky droppings and dirty eggs. The addition of enzymes to the diet to address NSP viscosity can optimize feed efficiency, improve manure quality, and increase the use of lower-cost feed ingredients. Grains are also high in phytate compounds, and the attached phosphorous molecules need to be released for both feed performance and environmental considerations. Thus, key poultry-feed enzymes are NSP enzymes (xylanases, cellulases, and glucanases) and phytase. Globally, the feed enzymes market for poultry is estimated at US $300 million. Europe dominates the poultry-feed enzyme market, valued at US $120 million and accounting for 40% of the global market, with a calculated CAGR of 7%. Asia is the second-largest market in poultry, and China is the main contributor to the regional market (at least 50% of the Asian market for poultry-feed enzymes). In South America, the market is driven by the Brazilian industry where a strong growth, greater than 8% of CAGR, is expected.

26.3.3.2 Swine

Swine are, like poultry, monogastric animals. Enzymes in swine nutrition uplift the feed digestibility and thus the available energy is increased. Two enzyme categories dominate the swine application: phytases and NSP enzymes. The use of phytases is very widespread and its their use is a common practice to break down the phytate molecule and release the attached phosphorus molecules, benefiting both the animal (improved phosphorus uptake) and the environment (less phosphorous released in the manure). Within the NSP enzymes category, xylanases and beta-glucanases are the most used. Xylanases and beta-glucanases improve the digestibility of the feed by acting on the more or less indigestible fiber-rich raw materials (wheat, barley, and others). Globally, the swine feed enzymes market is valued at US $280−300 million. Europe has the largest share of this market, accounting for 45% of the global market. In the Asia-Pacific region, valued at US $60 million, China is the market leader with close to 70% of the market and a growth expected at a CAGR of minimum 8%. Other regions (North America and South America) represent 35% of the swine-feed enzymes market with an average growth estimated at 7%.

26.3.3.3 Ruminants

Ruminants are polygastric mammals and the application of enzymes to their diet must be considered from a different angle as compared to monogastric animals (swine, poultry). Several studies have evaluated the potential for improving diet utilization (improved digestibility of the fibrous feed components) and dairy cow performance (milk production) with exogenous enzymes, mainly cellulases and

other NSP enzymes (xylanases and pectinases). The application of enzymes in ruminants feed is more controversial than their application to swine and poultry nutrition programs. The timing of enzyme addition is determinant in their performance: they can be added just prior to feeding the animal or they can be applied at ensilage plant material (grass, corn, etc.). When applied at or just prior to feeding, the products of plant cell wall hydrolysis can be directly fermented by ruminal bacteria, thereby releasing energy for the host animal. However, the efficacy of this approach is limited and extra care is needed to ensure a proper homogenization of the small quantity of enzyme added to the feed. When applied at ensilage, fibrolytic enzymes can be evenly distributed to the forage; moreover, enzymes added to the silage will hydrolyze plant cell walls and release fermentable substrates (mainly simple sugars) that will improve silage fermentation, and consequently optimize its digestibility. The global ruminants feed enzymes market is more complex to value as compared to swine and poultry, mainly because enzymes are applied either to ensilage forage or directly prior to feeding the cattle (making the identification and valuation of enzyme users more difficult). The ruminant application is estimated to be a small share of the global feed enzymes market, no more than 15% of the total, and is thus valued at US $90–110 million. It is estimated that Europe represents 40%–50% of the enzymes market for ruminant feed, mainly enzymes applied when ensilaging the forage.

26.3.3.4 Aquaculture and others

26.3.3.4.1 Aquaculture

Within the animal nutrition sector, aquaculture (fish, shrimp) is one of the fastest growing markets, and enzymes are increasingly used in that sector. One of the drivers for an increased application of enzyme to aquaculture is the growing substitution of animal proteins (mainly fish meal) by plant-derived ingredients. Plant feeds contain fibers and antinutritional factors such as phytate and enzymes can therefore improve their digestibility and boost their performance. Phytase is the main enzyme for the aquaculture market. The global market for aquaculture feed enzymes can be estimated at US $50 million, with 75% of total sales being realized in Europe and Asia. Considering the fast growth in aquaculture, the enzyme market is also expected to develop in the coming years. The development of new enzymes or new enzymes combinations developed for the aquaculture application should also contribute to the expansion of this relatively new category.

26.3.3.4.2 Others: Horse food, bird food, etc.

Equine nutrition is very specific and horses' diets are usually more complex. Enzymes are also considered for better digestibility and absorption of critical nutrients in maintaining horses' health and improving their performance in the specific case of race horses. The global enzymes market for miscellaneous applications is no more than 5% of the global market, with a value estimated at US $40 million.

26.3.3.5 Market structure and value chain

The feed additives industry is dominated by large multinational groups such BASF, DSM, CARGILL, and EVONIK, to name a few. Distribution to the end-user (the farmers) is usually ensured by companies formulating feed raw materials (e.g., cereals) with additives to produce ready-to-use complex feed recipes adapted to each animal specie, life stage, and production objectives. The top-three enzyme suppliers to the feed industry are the Alliance DSM-Novozymes, AB VISTA, and DUPONT, with a total estimated 80% market share. The phytase market is dominated by DSM-Novozymes and DUPONT with the respective leading brands Ronozyme and Phyzyme contributing to 70% of the phytase market. AB VISTA Quantum and Finase phytase brands also have robust market positions. Other players of importance in feed enzymes are ADISSEO, BASF, ALLTECH, as well as numerous companies located in India and in China.

26.3.4 KEY TRENDS IN FEED ENZYMES

26.3.4.1 Geography

Europe is the largest feed enzyme market and Asia-Pacific is the second-largest market of the segment, with a 25% share. China will grow at an expected minimum 8% CAGR and should reach a value of US $150 million by 2020. The Chinese market will be supplied by both global players (DUPONT, NOVOZYMES, etc.) and local enzyme producers. At the same time, local Chinese feed enzymes suppliers (Sunson, Leveking, etc.) should also explore export markets and supply to the other leading feed countries. The continuous expansion of the poultry industry in Brazil should drive the strong growth rate for feed enzymes, with a market growing at a CAGR minimum of 9%.

26.3.4.2 Animal categories

Both swine and poultry categories are expected to grow at a 7% annual rate, but the rise in demand for animal protein in emerging countries is very much in favor of faster and stronger developments in poultry production (low cost in production and global multiethnic acceptance for poultry meat).

26.3.4.3 Regulation and consumer awareness

The global rise in meat consumption is forcing the industry to continuously optimize its production costs, and enzymes will benefit from this pressure, for all animal species. Consumer awareness of the negative environmental impact of intense farming activities (such as the excessive release of nitrogen and phosphorus from livestock farms) are also in favor of enzymes application. The phytase and protease categories are expected to benefit from this trend. Global expansion of the antibiotics ban is also increasing the demand for feed enzymes.

26.3.4.4 Research and development

The intense R&D efforts from enzyme producers in the direction of cellulolytic and noncellulolytic fibers for the second-generation alcohol production should also benefit the feed enzyme industry, as some enzyme functionalities could fit both markets. On phytase, developments are also expected with new phytase generations capable of resisting higher temperatures for possible use in feed pelleting, work in lower-pH environments, and show optimized specific activity. Specific enzymes and enzyme formulations for targeted aquaculture applications are also expected to be on the horizon in 2020.

26.4 CONCLUSION

The food and feed enzymes market, accounting for more than half of the global industrial enzymes market is a highly complex business.

Conditions of application and expected benefits of enzyme application are totally different in food and feed. In food, enzyme application addresses both quantitative aspects ("more from less") and qualitative concerns (better taste, better texture). In feed, the key driver for using enzymes is to increase the feed-conversion ratio, in other words to produce more meat from less animal feed.

This complexity and market fragmentation is a real challenge for the leading enzyme global companies (Novozymes, Dupont, DSM). Just as an example, the fruit-processing market is worth less than US $50 million and involves enzymes specific for almost each fruit category to be sold to almost all countries on the planet.

It is therefore no surprise that the recent strongest R&D efforts from the industry did focus on large, homogenous applications in the nonfood/feed areas, such as the conversion of biomass to fuel alcohol.

On the other hand, the continuous global demand for processed food and increases in meat consumption are both very strong drivers for the global food and feed enzymes economy, which is expected to grow in the coming years at a comfortable 6%−8% CAGR.

The challenge for the food and feed enzymes industry will be to find a way to continuously invest in R&D while finding optimized channels for ensuring an efficient distribution of the enzyme solution from the large enzyme producers to fragmented applications and end-users.

The emergence of smaller enzyme producers, located in the regions where the demand is high (China, India) should also be taken into consideration.

CHAPTER

27 General perspectives of enzymes, environment preservation, and scarce natural resources—conclusions

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27.1 INTRODUCTION

Enzymes are biocatalyzers responsible for numerous metabolic processes authorizing life. They are nowadays used in several industries as well as in human and animal nutrition [1]. Some enzyme constructions are appearing as catalyzers of nonnatural reactions. An important function of enzymes take place in the digestive tract of humans and animals. Enzymes such as α-amylases, lipases, and proteases hydrolyze complex molecules into absorbable small molecules. Cellulosomes are examples of multicomponent microbial enzyme factors and lipase−colipase is an example of an inactive catalyzer complex in the absence of the cofactor.

Enzymes extracted from plants, as well as those produced by microorganisms and fungi, have been used for centuries in the production of food. The use of enzymes as food and feed additives is relatively recent. Large-scale applications of enzymes began during the second part of the last century.

27.2 ECONOMICS OF INDUSTRIAL ENZYMES

Enzymes for industrial applications have a market value estimated between 3.5 and 4 billion €. Principal utilizations of enzymes are in food production (dairy products, bakery, fruit juices, beer, wine, etc.), cleaning agents, agrofuels, feed, manufacturing industries (textiles, leather, paper), and the decontamination of several polluters. All together, human and animal applications represent about 60% of the whole enzymes sales, and this is growing by 6%−8% annually. In animal nutrition, poultry and swine are the main applications. North America is the biggest market for industrial enzymes (40%), followed by Europe (30%). It is

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foreseen that the market in Asia-Pacific will grow by 8%–9% annually, driven by the demand in the sectors related to nutrition.

Modern monogastric animals feeding is based on cereals and legume seeds which are also the basis of human diets in the majority of the countries. Progressively, such a situation resulted in direct competition between poultry and swine husbandry and human nutrition. Also, in recent decades, a spectacular increase in the use cereals, essentially of corn, was observed in the United States for the production of agroethanol. The immediate consequence was an enormous increase in the corn price in Latin American and African countries, where the cereal is the basis of human nutrition, bringing a permanent food crisis. US agroethanol production represents 40% of the world production.

In Europe, the cereals used for the production of agroethanol are wheat, barley, and rye, and in Brazil it is sugar cane. According to the FAO, the International Food Policy Research Institute and the French Institut National de la Recherche Agronomique (INRA), the agroethanol is responsible for 25% of the cereal's price increase. In any case the carbon balance of agroethanol is clearly negative. Thus, the reasonable option for second-generation agroethanol production would be to utilize as a basis the lignocellulolytic biomass, since that on the one hand will eliminate the competition between humans and animals for the same resource, and on the other hand the basis resource is practically unlimited. Enzymatic techniques will have a strong role to play. Besides the cellulases that are already being used, particular attention is expected to be directed to laccases alone and most probably in association with complementary enzymes.

27.3 CLASSIFICATION OF FEED ENZYMES

It is assumed that exogenous enzymes are used in animal feeding for four fundamental reasons: (1) to destroy or reduce antinutritional factors present in several feedstuffs that have deleterious effects on digestion and health of the animals; (2) to increase the accessibility of feed components to the endogenous digestive enzymes and consequently to improve the biodisponibilization of nutrients for absorption; (3) to supply the absence of endogenous enzymes able to hydrolyze special molecular links such as, e.g., the carbohydrate β links; (4) to overcome the immaturity of digestive endogenous secretions in the young animal [2]. There is now also a supplementary reason, which is progressively acquiring more and more importance: the reduction of the environmental impact of phosphorus (P), nitrogen (N), and zinc (Zn) pollution by excess of such elements present in animal excreta. These elements are the main causes of the eutrophication of continental waters from surface and groundwater tables origin, as well as from the maritime coastal regions; sometimes the latter have dramatic consequences. Generally, the enzymatic preparations used in animal feeding respond to more than one criterion.

From a practical point of view, i.e., taking into account the efficacy of exogenous enzymes in vitro as well as on physiology and zootechnical performances, the following classifications can be suggested: (1) enzymes with proven efficacy under both criteria, e.g., phytases, hemicellulases, and glucanases; (2) enzymes having in vitro and physiological effects without clear zootechnical effects, such as glalactosidases and ferulic-esterases; (3) enzymes with either in vitro and physiological positive or negative effects such as chitinases; (4) enzymes without clear effects under the three criteria, e.g., lipases, amylases, and individualized cellulases; (5) enzymes with high potential to be evaluated such as tyrosinases and lignolytic catalyzers [3].

27.4 EXTREMOPHILE ORGANISMS—POTENTIAL SOURCES FOR NEW INDUSTRIALS ENZYMES

Extremophile microorganisms—hyper-thermophiles, psycophiles, halophiles, barophiles, etc.—are a potential source of new hydrolases taking into account the characteristics of their enzymes [4]. Furthermore, extremophiles have specific zigzag proteins (chaperones) in the cellular surface giving them a resistance to denaturation and proteolysis, participating also in the restructuring of the molecular native ordinate and the functions after denaturation (refolding). Marine archaebacteria (archaea) also have an enzymatic equipment that is susceptible to valorization in the future [5]. The most known application of a product obtained from extremophile microorganisms is a DNA-polymerase utilized in the diagnostics of several pathogens [6].

Most probably, extremezymes and their derivatives will have applications in the domains of human and animal nutrition, human and veterinary medicines, and industries producing in extreme environmental conditions [7]. For example, a phytase has recently been isolated in Antarctic deep sea sediments from the yeast *Rhodotorula mucilaginosa*. The hydrolase has a higher activity than that of the classical phytases, and is also very resistant to pepsin and trypsin [8]. Such enzymes remain active when others need formulation which increases the cost of the application [9,10].

Barobacteria live in extreme conditions in marine abysses. Their enzymes perform at very high temperatures under a pressure which can reach 100 times atmospheric pressure, such as those isolated between two tectonic plaques in the marine mountain of Juan de Fuca close to Vancouver [11]. The oceanic lithosphere represents 60% of the earth and is the biggest surface ecosystem. Thus, it is not surprising that it has begun to be considered as a source of enzymes for industrial processes requiring high temperatures and pressures [12]. Nevertheless, it should be remembered that the knowledge of the physiology, metabolism, enzymology, and genetics of extremophiles is still quite limited. The research to be

pursued will lead to the development of new catalyzers, the extremezymes, able to improve industrial enzymatic processes [13].

27.5 ENZYMES WITH PROVEN PERFORMANCE IN VITRO, PHYSIOLOGICAL AND IN VIVO POSITIVE EFFECTS—PHYTASES

The cost of feed represents about 70% of the whole cost of the production in monogastric animals husbandry. Supply of energy (E), N, and P, in decreasing order, are the more expensive factors. Cereals and leguminous seeds are excellent sources of E and of most of the essential amino acids. However, they are a poor source of available P, even if they contain a high concentration of the mineral, because 60%–80% is in form of P-phytic which is known for its antinutritional effects and marginal availability [14,15]. Thus, swine and poultry feed is supplemented with inorganic-P leading to an increase on in environmental pollution by P, as well as increased production costs. Digestive utilization of P-phytic is clearly improved by phytases. These enzymes have multiple effects.

The first phytase to be introduced into the market, in 1991, was obtained from *Aspergillus niger*. In 1999 it was demonstrated that some phytases of bacterial origin, such as that of *Escherichia coli*, are more active than those produced by fungi [16,17].

Phytases are sensitive to high temperatures, gastric acidity, and gastrointestinal proteolysis. Under these criteria, *E. coli* phytases perform better in both swine and poultry than those of *A. niger* and *Peniophora lycii* [18].

The first phytase obtained from a full synthetic gene expressed in *Hansenula polymorpha*, named "Consensus," was developed in 1997 [19]. The enzyme is resistant to temperatures up to 90°C and has an activity equivalent to that of the *A. niger* phytase [20–22].

The addition of adequate dosages of phytase to growing-fattening pig diets containing enough total P, mostly in the form of phytate, make the supplementation of inorganic-P unnecessary [23,24]. This concept is being progressively introduced into practice [25].

The initial dose of phytase—500 U/kg—for swine and poultry diets was fixed in the early 1990s and remained the same until the end of 2010, and had become a kind of "dogma." The reality was that when the first phytase (from *A. niger*, Gist Brocardes, The Netherlands) was developed [26], the dosage of 500 U/kg was the highest dosage that was economically acceptable. Later on, it was shown that higher dosages induced better positive effects than those obtained with the classical 500 U/kg [27–29].

The beneficial effects attributed to phytases include the bioavailability of E and amino acids [30]. The effects on E are under controversy because, in the better cases, what is observed is a very marginal positive tendency and questions

arise on the proposed E "matrices" for phytase. On the contrary, the improvements in the amino acid are systematic and clear in swine, while they can be variable in poultry [31].

In humans, Fe and Zn deficiencies are serious health problems in several regions of the world, particularly in children and pregnant women, even if both minerals are present in sufficient quantities in the plant foodstuffs consumed in less-developed countries. It is known that the bioavailability of Fe and Zn is inhibited by the phytates. It has been demonstrated that phytates also inhibit Ca, Mg, and Mn [32]. The hydrolysis of phytates increase the availability of several cations and accordingly improve the nutritive value of food [33].

Several methods have been proposed to improve the nutritive value of plants, such as genetic engineering, fermentation, immersion, germination, and treatment with phytases. Phytase can be used in pretreatment or as a food additive. A significant reduction in phytates can be achieved in cereals and leguminous seeds, thus improving the Fe absorption [34]. In a synthesis of 12 essays performed in several countries [35] it was clearly evidenced that phytase had beneficial effects on the Fe and Zn absorption in foodstuffs with high level of phytates. With the aim of a more coherent application of phytases in human and animal nutrition, including the cost, the development of new phytases or formulations is needed. In human nutrition, the determination of the recommended dosages must be performed [32].

Besides the food and feed applications of phytases, two potential applications of the enzyme should be mentioned. On the one hand, the addition of phytase to sandy soils originates the formation of cements starting from crystals of Ca phosphate, and can be a form of stabilization of sandy areas [36]. One the other hand, the evaluation of the possible utilization of phytases in industrial biocatalysis should be pursued [37].

27.6 ENZYMES SHARING IN VITRO AND IN VIVO POSITIVE AND NEGATIVE EFFECTS—CHITINASES

Chitanases are most probably the sole enzymes with dual profile. Most latex-allergic persons have cross-reactive antibodies to the antigens from some vegetable foods [38]. Increasing numbers of plant-derived foods are suspected to cross-react with latex antigens and several fruits have cross-reactivity, known as "latex–fruit syndrome" [39–41]. The defense-related proteins of the rubber tree and of cross-reactive plants act as pan-allergens that are responsible for the large cross-reactivity.

Chattiness have one or two chitin-binding domains more or less related to herein. In natural rubber latex, besides hevein there are two minor allergens with chitinase activities: hevamine, a bifunctional lysozyme/chitinase enzyme homologous to class III chitinases, and a class II enzyme [42]. The chitinase of numerous fruits (banana, avocado, potato, tomato, kiwi, chestnut, passion fruit, pear,

grapefruit, mango, pineapple, papaya, peach, orange, strawberry, watermelon, coconut, apricot, cherry, nectarine, etc.) is a pathogenesis-related protein (PR-3), which is an important cross-reactive allergen for latex-sensitized people. The establishment of latex−fruit syndrome is comparable with that of pollen−food allergy syndrome.

Only special food proteins that are stable to heat treatments and resistant to digestive enzymes can become food allergens. This concept is called "class 1 food allergy" (or type I food allergy). In addition, food proteins responsible for such food allergies are now designated as "complete food allergens or class 1 food allergens." A food allergy based on the cross-reactivity between the sensitizing antigen and the symptom-eliciting antigen is called a "class 2 food allergy" or "type II food allergy."

Chitin is a major component in a variety of allergy-triggering environmental components, including house-dust mites or fungal spores, and fungal asthma is increasingly appreciated as an under-diagnosed disease entity. Understanding the complex immunological and pathophysiological implications of chitin−chitinase interactions in the human body is of high relevance for identifying new biomarkers and therapeutic targets for fungal diseases and other conditions, where chitin-coated microbial derivatives play a critical role.

An elicited expression of acidic mammalian chitinase (AMCase) may be involved in the pathogenesis of asthma. The specific suppression of elevated AMCase leads to reduced eosinophilia and Th2-mediated immune responses in a mouse model of allergic asthma. The exposure of chitin and the fundamental component of the major allergen mite and several of the inflammatory mediators, show significant enhancement of AMCase expression. These observations are the basis of developing a promising therapeutic strategy for asthma by silencing AMCase expression [43].

Chitotriosidase enzyme (EC: 3.2.1.14) is the major active chitinase in the human body. It is produced mainly by activated macrophages, in which its expression is regulated by multiple intrinsic and extrinsic signals. Chitotriosidase is an essential element in the innate immunity against chitin-containing organisms such as fungi and protozoa. Its immunomodulatory effects extend far beyond innate immunity [44].

27.7 LIGNOCELLULOLYTIC ENZYMES—CATALYZERS WITH A STRONG POTENTIAL OF APPLICATIONS

Cellulose is the most abundant polysaccharide on Earth, and is composed of glucose monomers linked in chain by β-1-4 bonds. β links are not hydrolyzed by animals, with the exception of some species of termites [45]. Cellulose has two essential characteristics, first it is nontoxic, and second it is resistant to degradation. Lignin is the most abundant renewable aromatic polymer on Earth and is

composed of nonphenolic (80%–90%) and phenolic structures. It is essentially a polymerized aromatic substance, very resistant to hydrolysis. In wood, lignin represents 20–30% of the plant [46]. Cellulose and lignin are present together with hemicellulose in the complex lignocellulose.

Selective lignin degradation has potential applications in biotechnology when lignin elimination is fundamental for obtaining cellulose. It is not possible to define the precise structure of lignin since all show a certain variation in their chemical composition. However, the definition common to all is a dendritic network polymer of phenyl propene basic units. There are two principal categories of lignin: those that are sulfur-bearing and those that are sulfur-free. The sulfur-bearing lignins are those which have to date been commercialized. These include lignosulfonates (world annual production of 500,000 tons) and kraft lignins (under 100,000 tons p.a.).

Cellulases are responsible for cellulose depolymerization allowing the liberation of cellobiose and glucose. Cellulose hydrolysis already plays an important role in the conversion of lignocellulolytic biomass into agroethanol. It is foreseen that in the future it will be one of the most interesting natural and sustainable resource materials. It can be assumed that there is a clear need for valorization, not only for feed, but also for food, particularly in regions where chronic misery and undernutrition conditions prevail.

Cellulases play a fundamental role in food biotechnology. Complexes of macerating enzymes (cellulases, xylanases, and pectinases) are currently utilized in several agroindustries, including that of olive oil extraction [47]. Cellulases are also used as human therapeutic factors in the treatment of phytobezoars [48]. These enzymes are used in detergents, textiles, paper, waste treatment and agroindustries, and in a small range as nutritional additives. The combination of cellulolytic, hemicellulolytic, and pectinolytic activities works synergistically on the recalcitrant lignocellulose complex which is difficult to degrade [49].

Direct-fed cellulases as monocomponent feed additives are generally not very succesful in improving monogastric animals' (pig and poultry) performance in terms of digestibility, feed-conversion ratio, and daily weight gain [50,51].

A combination of cellulase, hemicellulose, and pectinase has been shown to improve the in vitro digestibility of a corn-soybean meal broiler diet as well as in vivo the birds' zoo technical performance by effectively degrading indigestible cell constituents and thus enabling the protein of the broiler feed to become more digestible [52].

Extrusion technology has been used successfully to improve the nutritive value of full-fat oilseeds by exposing more peptide bonds to enzymatic hydrolysis. However, the fibrous portion of the seeds is largely intact after extrusion. Therefore, application of carbohydrase mixtures targeting nonstarch polysaccharides might further improve the nutritive value of extruded full-fat oilseeds [53].

It should be underlined that mono-component cellulase addition to pig and poultry diets remains without effects on performance or on the availability of nutrients from diets containing high levels of nonstarch polysaccharides [54,55].

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are multicopper oxidases, together with different proteins such as plant ascorbic oxidase, mammalian ceruloplasmin, or Fet3p ferroxidase. They catalyze the oxidation of various substrates with the simultaneous reduction of molecular oxygen to water. Most biotechnologically useful laccases are of fungus origin. The ability of laccase to attack and degrade lignin in conjunction with laccase mediators is currently viewed as one of the fundamental potential applications for the increased utilization of lignin. Lignin resists microorganisms but some basidomycetes, as well as some bacteria, are able to degrade it efficiently by employing different agents and catabolic pathways in a combination with lignocellulosic enzymes, organic acid mediators, and accessory enzymes. Selective lignin degradation has potential applications in biotechnology when lignin elimination is fundamental for obtaining cellulose. It is not possible to define the precise structure of lignin because lignins all show a certain variation in their chemical composition.

Laccases have demonstrated potential for degradation of drugs such as antimicrobials, phenols, pesticides, industrial colorants, waste residues from the paper industry, some mycotoxins such as fumonisine. An extremely important field for laccases application is the valorization of plant biomass for nutrition [3]. In the last 10 years, more than 100 laccases, with high lignin degradation activities, have been isolated, characterized, and a large number have been expressed in production strains.

In the food industry, laccase is already used for the elimination of undesirable phenolic compounds in baking, juice processing, wine stabilization, and bioremediation of waste water. In the beer industry, laccase not only provides stability but also increases the shelf life of beer. Phenol compounds and their oxidative products present naturally in the fruit juice give color and taste to the juice. Color and aroma change when polymerization and oxidation of phenolics and polyphenols take place. These changes are due to the high concentration of polyphenol and are referred to as enzymatic darkening. Laccase treatment removes phenol as well as substrate−enzyme complex with the help of membrane filtration, and color stability is achieved, although turbidity is present. In order to improve the organoleptic characteristics of bread, a wide range of enzymes is used. When laccase is added to the dough, it strengthens the gluten structures in the dough and the baked products are improved: product volume increases, crumb structure improves, and softening of the baked products takes place. At crushing and pressing stage, the high concentrations of phenolic and polyphenolic compounds play an important role in wine production. The high concentration of polyphenol obtained from the stems, seeds, and skins, which depends on the grape variety and vinification conditions, contributes to the color and astringency. Due to this complex event, polyphenol oxidation occurs in musts and wines resulting in an increase in color and a change in flavor, which is referred to as maderization. Catalytic factors, polyphenol removal, clarification, polyvinylpolypyrrolidone (PVPP), and high doses of sulfur dioxide are utilized to prevent maderization. Polyphenol removal is selective and results in undesirable organoleptic

characteristics. Laccase treatment is feasible, increasing storability and reducing processing costs.

Since laccases catalyze the electron transfer reactions without additional cofactors, they can also be used as biosensors to detect various phenolic compounds, oxygen, and azide. As a biosensor, laccase can detect morphine, codeine, catecholamine, estimate phenol or other enzymes in fruit juice, and plant flavonoids.

Up until now, in the food and feed industries, laccases have almost never been used in practice. Thus, the final conclusion should be to strongly suggest the intensification of studies in this field.

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ENZYMES IN HUMAN AND ANIMAL NUTRITION
Principles and Perspectives

Edited by
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Enzymes in Human and Animal Nutrition: Principles and Perspectives is a detailed reference on enzymes covering information on all relevant aspects fundamental for final use of enzymes in human and animal nutrition. Topics explored include depolymerization of enzymes, both from a food and feed perspective, different enzymes and some direct-fed microbials, and the important technological issues related to enzyme use and production. Readers will develop new insights for applications of enzymes in foods and feeds with the wealth of knowledge brought forth by the authors of the various chapters.

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