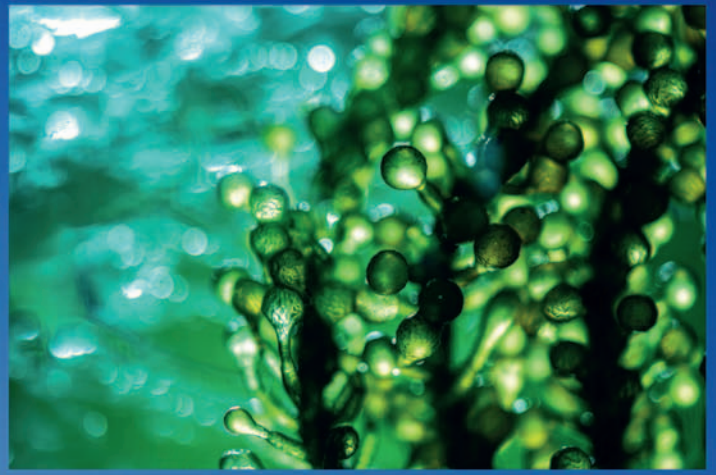




FRONTIERS IN AQUACULTURE BIOTECHNOLOGY

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Frontiers in Aquaculture Biotechnology

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Biotechnology in modern aquaculture: Innovations, advancements, and challenges

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1. Introduction

Aquaculture is one of the fastest growing sectors providing food and nutritional security and livelihood to the burgeoning world population. It continues to grow faster than other major food production sectors, although it no longer enjoys the high annual growth rates of the 1980s and 1990s (11.3 and 10.0% respectively, excluding aquatic plants). The annual growth in aquaculture declined to a moderate 5.8% during the period 2001–16, although double-digit growth still occurred in a small number of individual countries, particularly in Africa from 2006 to 2010 [1]. Biotechnology with basic and spin-off applications plays an important role in promoting productivity, boosting efficiency, and maintaining sustainability in aquaculture. The key issues in nutrition, health, and water quality management are being addressed with potential applications of biotechnology and high-throughput technologies. Enhancement of growth rate and quality fish production, health management (stress-tolerant variety in view of climate change, disease-resistant variety, rapid diagnostic techniques and testament, vaccination), nutritional management (formulated feed for brood stock and larvae), genetic manipulation for production of high yield variety and transgenesis, gene editing for improvement and management of genetic resources, quality enhancement, and assessment through genomics and proteomics techniques are the current biotechnological approaches enhancing production and productivity.

Biotechnology basically involves the use of molecules (synthetic or physiological), biological materials (tissues, cells, genes), or processes derived from living organisms to create new and practical products for the benefit of human beings. Aquaculture biotechnology facilitates application of genetic, cellular, and molecular technologies to enable fish farmers to produce a more abundant, resilient, and healthier supply of aquafood. Biotechnology provides powerful tools for the sustainable development of aquaculture, fisheries, and the food industry. Increased public demand for seafood and decreasing natural freshwater and marine habitats have necessitated to explore ways where biotechnology can increase the production of freshwater and marine food products and make aquaculture as a modern science based on technological advances. Biotechnology allows scientists to identify and combine traits in fish and shellfish to increase productivity and improve quality. The use of modern biotechnology to enhance production of aquaculture species holds great potential not only to meet demand but also to improve and maintain sustainable aquaculture system. Genetic modification through biotechnological tools and functional aspects through proteomics hold tremendous potential to improve the quality and quantity of fish reared in aquaculture system. The most remarkable intervention in recent times is of AquAdvantage salmon, a genetically modified Atlantic salmon. The goal is to increase the speed at which the fish grows without affecting its ultimate size or other qualities. Biotechnology helps in the production of sterile farmed animals to prevent the harm that unwanted gene escapees can do on biodiversity. Fish are also used as model organisms for researching gene functions and developing biotechnological methods for different purposes. As biotechnology continues to develop rapidly, its applications in aquaculture are increasing day by day. The major areas namely genetic modifications and improvement through gene editing, applications of cell cultures/stem cell cultures, transgenesis, vaccines, and the use of newer and hi-tech aquaculture system in promoting and maintaining sustainable aquaculture have been discussed in this chapter.

2. Chromosome manipulations

Chromosome sex manipulation techniques to induce polyploidy (triploidy and tetraploidy) and uniparental chromosome inheritance (gynogenesis and androgenesis) have been applied extensively in cultured fish species [2,3]. These techniques are important in the improvement of fish breeding as they provide a rapid approach for gonadal sterilization, sex control, improvement of hybrid viability, and clonation. Most vertebrates are diploid, meaning that they possess two complete chromosome sets in their somatic cells. Polyploid individuals possess one or more additional chromosome sets, bringing the total to three in triploids, four in tetraploids, and so on. Induced triploidy is widely accepted as the most effective method for producing sterile fish for aquaculture and fisheries management. The methods used to induce triploids and other types of chromosome set manipulations in fishes and the applications of these biotechnologies to aquaculture and fisheries management are well described [3]. Tetraploid breeding lines are of potential benefit to aquaculture, by providing a convenient way to produce large numbers of sterile triploid fish through simple interploidy crosses between tetraploids and diploids [4,5]. Although tetraploidy has been induced in many finfish species, the viability of tetraploids was low in most instances [6]. Commercial production of triploid Pacific oysters has been practiced on the West Coast of North America for the past several years. Ref. [7] at Institute of Aquaculture, University of Stirling, Scotland, are involved in commercial triploid salmon production. Freshwater Fisheries Society of BC, Canada, is continually researching methods and techniques to improve the growth, survival, and quality of the freshwater fish stocked into British Columbia's lakes and rivers. The Freshwater Fisheries Society produces triploid rainbow trout, eastern brook trout, kokanee salmon, and cutthroat trout (<https://www.gofishbc.com/Blog/Science-and-Research/Maximizing-Fish-Performance.aspx>, 2016).

The production of gynogenetic individuals is of particular interest to fish breeders because a high level of inbreeding can be induced in a single generation. Gynogenesis may also be used to produce all-female populations in species with female homogamety and to reveal the sex determination mechanisms in fish. It is convenient to use all-female gynogenetic progenies (instead of normal bisexual progenies) for sex inversion experiments. Methodologies combining use of induced gynogenesis with hormonal sex inversion have been developed for several aquaculture species [8]. Induction of androgenesis can produce all-male population in fish, which would have commercial application in aquaculture. It can also be used in generating homozygous lines of fish and in the recovery of lost genotypes from the cryopreserved sperms. Androgenetic individuals have been produced in a few species of cyprinids, cichlids, and salmonids [9]. Meiotic gynogenesis was successfully induced in Indian major carps for the first time in *Labeo rohita* and *Catla catla* [10] at the Central Institute of Freshwater Aquaculture, Bhubaneswar, under the guidance of Dr. V. R. P. Sinha as the National Director. Artificial gynogenesis has been successfully induced in many species of fish including carps [11].

3. Genomics in aquaculture

A consortium of biologists and genome scientists launched a Genome 10K Project that targeted to sequence the complete genomes of 10,000 vertebrate species, including some 4000 fish species, to understand vertebrate evolution and save many endangered species [12–14]. The Earth BioGenome Project, a moonshot for biology, aims at sequencing, cataloging, and characterizing the genomes of all of the Earth's eukaryotic biodiversity over 10 years [15]. Regardless, all known species of fish (over 34,000 species recorded in FishBase, <http://fishbase.org>) will soon have their genomes sequenced. The subsequent challenges are how to make use of these genomic data and transform genomic knowledge into fisheries and aquaculture practices, such as genetic resources management and selective breeding. Full potential of genomic application in fisheries and aquaculture remains to be explored and reaped. The applications of genomic techniques such as genome editing and genomic selection, along with the use of emerging intelligence systems, in aquaculture and fisheries will contribute significantly to genetic improvements of farmed fish [16]. Since the first fish with its whole genome sequenced was Japanese puffer fish *Takifugu rubripes* [17], about 1386 whole genome sequences of fishes are now available on the National Center for Biotechnology Information repository as of December [18]. These genomic resources promote not only basic sciences such as comparative genomics, evolution, and systematics but also applied practices in aquaculture and fisheries [19–22]. A list of whole genome size of some important fish species is given in Table 1.3.

4. Proteomics

Advances in genomics and proteomics have opened the door to gene expression studies on an unprecedented scale, ranging from hundreds of gene products to global, genome-wide patterns of expression. Proteomics is a tool that can be of great value to the food scientist, giving valuable insight into the composition of the raw materials; quality involution within the product before, during, and after processing or storage; and the interactions of the proteins with one another, with other

food components, or with the human immune system after consumption. Proteomics research has enormous applications in human and animal research. After a successful completion of the Human Genome Project, deciphering the mystery surrounding the human proteome posed a major challenge. The Indian scientific community has contributed toward proteomic research along with the global community [33]. However, proteomic studies in fisheries science are quite scanty, especially in India where it requires more intense research programs.

Since most of the fishes are used for food, we should ensure that the fish grown on farms is the same as wild fish in their native ecosystem. At first glance, this seems an odd aspect to consider, but several factors might influence the physiology of the fish. The diet of farmed fish will be different to that of wild fish, so it is reasonable to assume that different metabolites are formed. It is easy to assume that the flesh of farmed fish is the same as free fish, but an integrated proteomic and chemical study has revealed significant differences. Comparative proteomics of farmed and wild *Sparus aurata* has helped in development of proteome database and the data obtained are being processed using “omic” tools for large-scale screening and toxicity evaluation of various seafood products [34]. A recent study by [35], performed a comprehensive organ-based protein profiling of one of the most important aquaculture species, *L. rohita*, which revealed 8498 proteins at 1% false discovery rate that make up about 26% of the total annotated protein-coding sequences in rohu.

5. Gene editing

Gene editing is a powerful functional genomics tool that has enormous applications in fish health, genetics, and biotechnological research. CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9 (CRISPR-associated protein 9) has revolutionized gene editing. This technique has been mostly used for genome editing in mammalian cell line, whereas use of gene editing for fish cell lines is in infancy stage. The use of CRISPR-Cas9—based gene editing method has been reported in fish [36,37].

Genome editing using CRISPR/Cas9 was recently successfully applied in vivo and/or in cell lines of several major aquaculture species of Salmonidae (Atlantic salmon, *Salmo salar*; and rainbow trout, *Oncorhynchus mykiss*), Cyprinidae (rohu, grass, and common carp, *L. rohita*, *Ctenopharyngodon idella*, and *Cyprinus carpio*, respectively), Siluridae (channel and southern catfish, *Ictalurus punctatus*), as well as Pacific oyster (*Crassostrea gigas*), Nile tilapia (*Oreochromis niloticus*), and gilt-head sea bream (*S. aurata*) [38]. One of the most important recent technological advances for zebrafish disease modeling has been in precision genome editing. CRISPR-Cas9 has enabled the rapid and efficient generation of genetic mutations in zebrafish, and, importantly, this can be used to generate site-specific mutations in a tissue-specific manner [39]. This method has also been used for gene editing in a fish cell line—Chinook salmon, *Oncorhynchus tshawytscha* cell line, CHSE-214—for the first time [40].

6. DNA barcoding

Prof. Paul Hebert from the University of Guelph, Canada, first initiated barcoding programs in Canada in 2003. DNA barcoding promises to revolutionize our capacity to know and monitor biodiversity and will exert broad impacts on all areas in which society interacts with biodiversity including pest and disease control, food safety, resource management, conservation, research, education, and recreation. Prof. Hebert emphasized the importance of India in joining the global Consortium for the Barcode of Life as India has the capacity to contribute immensely by virtue of its biodiversity richness and the scientific and technical strength. At national level, Prof. W. S. Lakra initiated the fish DNA barcoding in India in 2008. Eventually, DNA barcodes of over 500 fish species have been generated under his leadership [41,42]. A first **international training** program on “DNA Barcoding of Marine Life” was conducted at the National Bureau of Fish Genetic Resources (NBFGR) from April 16 to 21, 2007. Subsequently, an international workshop on DNA barcoding, first of its kind in India, was organized by NBFGR, Lucknow, on December 4, 2008, to strengthen the national capacity in DNA barcoding and to identify priority areas and funding sources for strengthening the ongoing and future barcoding activities in the country for plants and animals.

Species-specific molecular signatures of commercially important finfish and shellfish species have been generated to resolve taxonomic ambiguity and for accurate documentation of species diversity. In addition, legal disputes have been resolved based on the DNA barcoding, viz. forensic identification of pomfret (*Pampus chinensis*) and endangered and wildlife protected whale shark (*Rhincodon typus*). Government of Maharashtra and Government of Kerala have shown the potential application of barcoding technology in product identification and management of endangered species. DNA barcodes have been used to detect seafood mislabeling and authentication [43,44]. It is expected that the DNA barcoding technology would play a major role in identification of transboundary movement of animals, ornamental fish, and aquatic by-products.

7. Fish cell and stem cell culture

Fish cell lines offer many advantages over mammalian cell lines and have recently become the choice of researchers for in vitro biological research. Fish cell lines have been increasingly used in virus isolation and disease diagnosis as there are repeated reports on the occurrence of disease outbreaks in aquaculture. Fish cell lines have also been used as a valuable, fast, and economic in vitro tool for screening toxicity of chemicals and environmental samples. Hence, development and maintenance of cell lines from commercial and endangered fish species are of great importance for aquaculture as well as conservation of fish germplasm.

Since the first fish cell line reported in 1962, about 283 cell lines have been established from finfish around the world [45]. Most cell lines in the past were developed from temperate fish, and the recent reports from tropical species include those of *Epinephelus coioides* and *Chanos chanos* [46], *L. rohita* [45], *Puntius sophore* [41], and *Schizothorax richardsonii* [47]. A number of embryonic stem-like cell lines have also been established by various workers from fish species [48–50]. The crustacean cell culture has gained potential scope for the development of diagnostic reagents and probes for use in shrimp, crayfish, and lobster industries [51]. Despite numerous attempts, no established cell line from crustaceans has been reported to date. A list of fish cell line developed by our group is given in Table 1.1.

Fish cell lines serve as essential tools for pathological, toxicological, and immunological studies. Fish cell lines have enormous applications in biomedical research, toxicology, gene regulation, gene expressions, and gene transfer. The cultured animal cells produce a wide range of biological products of commercial interest including immunoregulators, antibodies, polypeptide growth factors, enzymes, and hormones. They are used in the development of viral vaccines, tissue plasminogen activator, interferon, monoclonal antibodies, tumor specific antigens, etc. The cultured cells would also be used as a reconstituent skin to facilitate wound healing. The main advantage of cell culture is that cell lines allow higher control of conditions of experiments and at the same time reduce the variability of the in vivo responses that arise due to the responses of fish to stress and environmental influences. Fish leukocyte cell lines with cells of specific lineage and function like T cells, B cells, and macrophages developed from catfish, *I. punctatus*, *O. mykiss*, etc., have been employed to generate immunological information useful for disease prevention in farmed fish. Cell lines of gut, skin, and gill origin have been used to study the local defense responses. The immunoresponse potential of DNA vaccines, recombinant protein, and synthetic peptide vaccines and immunostimulants can be studied with the aid of cell lines. Fish cell cultures have been used for studying carcinogenesis in vitro and for understanding immune response in fish for vaccine development and for study of comparative evolutionary aspects of immune response. Recently, fish muscle cell lines have tremendous potential for cultivated seafood meat production.

8. Vaccines in aquaculture

The effective control and treatment of disease of aquatic animal require access to diagnostic tests that are rapid, reliable, and highly sensitive. Direct culture of pathogens is also widely used. However, these methods are time consuming and costly, and for shrimps/crustaceans cell lines suitable for virus culture, these methods have not been established. Efforts to overcome these problems have led to the development of immunoassay and DNA-based diagnostic methods and polymerase chain reaction amplification techniques.

Modern technology allow preventive measures to be taken to combat disease through vaccination or immunity enhancement. Fish vaccines developed during the last 2 to 3 decades have also become an established, proven, and cost-effective method for controlling certain infectious diseases in cultured animals worldwide. There are now many commercially available vaccines for finfish diseases, for example, furunculosis (*Aeromonas salmonicida*), as well as many more are under development, for example, viral hemorrhagic septicemia (VHS). In addition to reducing the severity of disease losses, vaccines also reduce the need for antibiotics, leave no residues in the product or environment, and do not induce pathogen resistance [65]. The vaccines and immunostimulants can be administered via additives in feeds, immersion, or, in the case of the larger culture animals like fish, by injection. Genetically engineered vaccines are also being developed to protect fish against pathogens. Genetically applied immunization of rainbow trout with a glycoprotein gene from the virus causing VHS has recently been shown to induce high levels of protection against the virus.

Sahoo et al. [66] have reviewed significant viral pathogens of finfish in the Asia–Pacific including both those with a long history in Asian aquaculture and emerging pathogens including betanodaviruses and koi herpes virus that have caused massive losses in the freshwater aquaculture and ornamental fish industries. Indian Council of Agricultural Research (ICAR)—Central Institute of Fisheries Education, Mumbai, has reported some of the research breakthroughs including development of vaccine for shrimp against white spot syndrome virus (WSSV) and a bicistronic DNA vaccine against *Edwardsiella tarda* infection in carps, which are in the eld trial stage (CIFE, Annual Report, 2017–18). Rajesh et al. [67]

TABLE 1.1 List of fish cell culture/cell lines developed by our research group.

Sl. No.	Name of cell line	Organ/Tissue of origin	Species	Reference
1.	TP1	Fry	<i>Tor putitora</i>	[52]
2.	LCE and LCF	Fry	<i>Lates calcarifer</i>	[53]
3.	RH	Heart	<i>Labeo rohita</i>	[45]
4.	RF	Fin	<i>L. rohita</i>	[45]
5.	RSB	Swim bladder	<i>L. rohita</i> (Hamilton)	[45]
6.	Embryo	Embryonic stem cell line	<i>Clarias batrachus</i>	[54]
7.	CCF	Fin	<i>Cyprinus carpio</i>	[54]
8.	CCH	Heart	<i>C. carpio</i>	[54]
9.	PDF	Fin	<i>Puntius denisonii</i>	[54]
10.	PDH	Heart	<i>P. denisonii</i>	[54]
11.	PSCF	Caudal fin	<i>Puntius sophore</i>	[41]
12.	RESC	Embryo	<i>L. rohita</i>	[49]
13.	MRH	Heart	<i>Macrobrachium</i>	[55]
14.	PCF	Caudal fin	<i>Puntius chelynoides</i>	[56]
15.	PCE	Eye	<i>P. chelynoides</i>	[56]
16.	SRCF	Caudal fin	<i>Schizothorax richardsonii</i>	[47]
17.	TTCF	Caudal fin	<i>Tor tor</i>	[57]
18.	WAF	Caudal fin	<i>Wallago attu</i>	[58]
19.	WAG	Gill	<i>W. attu</i>	[59]
20.	WAM	Muscle	<i>W. attu</i>	[59]
21.	CPG	Gill	<i>Channa punctatus</i>	NRFC049
22.	DRM	Muscle	<i>Danio rerio</i>	[60]
23.	DRCF	Caudal fin	<i>D. rerio</i>	[61]
24.	DGH	Gill	<i>Pangasianodon hypophthalmus</i>	[62]
25.	ADF	Caudal fin	<i>Amphiprion ocellaris</i>	[63]
26.	AOG	Gill	<i>A. ocellaris</i>	[63]
27.	CMM	Muscle	<i>Clarias magur</i>	[64]

in the past studied the immunological responses of *Penaeus monodon* to DNA vaccine and its efficacy to protect shrimp against WSSV. Kulkarni et al. [68] have investigated truncated VP28 as oral vaccine candidate against WSSV infection in shrimp: an uptake and processing study in the midgut of *Penaeus monodon*. The nearly identical uptake pattern of the different WSSV antigens suggests that receptors on the apical membrane of shrimp enterocytes recognize rec-VP28 efficiently. Hence, the truncated VP28 can be considered suitable for oral vaccination. In spite of these reports, Indian scenario in commercial vaccine for aquaculture is far from realization.

9. Biofloc/bioremediation

Biofloc technology is a technique of enhancing water quality through the addition of extra carbon to the aquaculture system, through an external carbon source or elevated carbon content of the feed. This promoted nitrogen uptake by bacterial growth, decreasing the ammonium concentration more rapidly than nitrification. Immobilization of ammonium by heterotrophic bacteria occurs much more rapidly because the growth rate and microbial biomass yield per unit substrate of heterotrophs are a factor 10 higher than that of nitrifying bacteria. Biofloc technology makes it possible to minimize water

exchange and water usage in aquaculture systems through maintaining adequate water quality within the culture unit, while producing low-cost bioflocs rich in protein, which in turn can serve as a feed for aquatic organisms [69]. Compared to conventional water treatment technologies used in aquaculture, biofloc technology provides a more economical alternative (decrease of water treatment expenses in the order of 30%), and additionally, a potential gain on feed expenses (the efficiency of protein utilization is twice as high in biofloc technology systems when compared to conventional ponds), making it a low-cost sustainable constituent to future aquaculture development [70,71]. Biofloc technology in comparison to the conventional technologies to manage and remove nitrogen compound is robust, economical, and easy in operation. The key to minimizing possible negative impacts of climate change on aquaculture and maximizing opportunities will be through understanding and promoting a wide range of inventive adaptive new technologies, such as the biofloc technology combined with greenhouse ponds.

Farmed aquatic animals are much more sensitive to their immediate environment than land animals. The water in which they depend for oxygen and a range of other important chemicals also takes up their waste products and may carry pollution from the nearby environment [72]. The process of disease in aquaculture species is thus much more strongly connected to environmental factors than would be the case say, with cattle. A further biotechnology field that has developed in aquaculture, because of the nature of this relationship, is that of bioremediation. This refers to the use of friendly bacteria or “probiotics” to treat water or feeds and, by natural processes, discourages the development of “unfriendly” bacteria that potentially would cause disease [73].

10. Biobank, cell line repository, and state fish

A significant increasing trend of development of fish cell lines has been observed. This has raised the urgent need of conserving these cell lines in cell line repositories and biobanks. Fish cell lines are not only the source material for in vitro research but also they are utmost essential for conservation of fish germplasm. The authors for the first time contributed significantly in the development of the state-of-the-art facility for development and storage of cell lines at NBFGR, Lucknow, India. Department of Biotechnology (DBT), Government of India, New Delhi, has been instrumental in supporting research for development, characterization, and conservation of fish cell lines. As the number of fish cell lines has been increasing, DBT, Government of India, has realized the need of conserving the fish cell lines in cell line repository. DBT initially funded a project entitled Establishment of a National Repository at NBFGR, Lucknow, for Conservation and Characterization of Fish Cell Lines. **National Repository of Fish Cell Lines (NRFC)** for maintenance and conservation of fish cell lines is in operation at NBFGR, Lucknow, under the leadership of the authors. Later DBT, Government of India, has funded another fish cell line repository at t C. Abdul Hakeem College, Melvisharam, Tamil Nadu.

The facility provides services for deposition, characterization, cryopreservation, and distribution of fish cell lines to the research communities in India. The repository is serving as a national referral center of Indian fish cell lines in the country. At present, more than 50 fish cell lines from different freshwater and marine fish species are being maintained and cryopreserved in the NRFC. These cell lines were deposited by various research groups in India including the researchers at NBFGR, Lucknow (Table 1.2). All the cell lines have been characterized using molecular and cytogenetic markers and

TABLE 1.2 List of fish cell lines available in National Repository of Fish Cell lines (NRFC).

Name of cell line	Fish species	Organ	Cell type	NRFC code	Depositor/Reference
PCF	<i>Puntius chelynoides</i>	Fin	Fibroblast	NRFC001	[56]
SRF	<i>Schizothorax richardsonii</i>	Fin	Fibroblast	NRFC002	[47]
TTCF	<i>Tor tor</i>	Fin	Fibroblast	NRFC003	[74]
CCF	<i>Cyprinus carpio</i>	Fin	Epithelial	NRFC004	[54]
WAF	<i>Wallago attu</i>	Fin	Fibroblast	NRFC005	[58]
RF	<i>Labeo rohita</i>	Fin	Fibroblast	NRFC006	[75]
WAM	<i>W. attu</i>	Muscle	Fibroblast	NRFC047	[59]
WAG	<i>W. attu</i>	Gill	Epithelial	NRFC048	[76]

TABLE 1.2 List of fish cell lines available in National Repository of Fish Cell lines (NRFC).—cont'd

Name of cell line	Fish species	Organ	Cell type	NRFC code	Depositor/Reference
CPG	<i>Channa punctatus</i>	Gill	Epithelial	NRFC049	M Goswami
DRM	<i>Danio rerio</i>	Muscle	Fibroblast	NRFC050	[60]
CTM	<i>Catla catla</i>	Thymus (macrophage)	Fibroblast	NRFC028	[77]
CTE	<i>C. catla</i>	Thymus (epithelial)	Epithelial	NRFC029	[78]
CCM	<i>C. catla</i>	Blood (lymphocytes)	Epithelial	NRFC034	[79]
KCF	Koi carp	Fin	Epithelial	NRFC007	[80]
HBF	<i>Horabagrus brachysoma</i>	Fin	Epithelial	NRFC008	[81]
PDF	<i>Puntius denisonii</i>	Fin	Epithelial	NRFC009	[82]
CFF	<i>Pristolepis fasciata</i>	Fin	Fibroblast	NRFC039	[83]
CFFN2	<i>Amphiprion sebae</i>	Fin	Epithelial	NRFC010	K. Riji John, Fisheries College and Research Institute (FCRI), Tuticorin, Tamil Nadu, Tamil Nadu Veterinary and Animal Sciences University
CFBR		Brain	Epithelial	NRFC011	
CFSP		Spleen	Epithelial	NRFC012	
CFCP1		Caudal peduncle	Epithelial	NRFC013	
SISK	<i>Lates calcarifer</i>	Kidney	Epithelial	NRFC014	[84]
SISS		Spleen	Epithelial	NRFC015	[85]
SIGE	<i>Epinephelus coioides</i>	Eye muscle	Epithelial	NRFC016	A. S. Sahul Hameed
IGK		Kidney	Epithelial	NRFC017	
SICE	<i>C. catla</i>	Eye muscle	Epithelial	NRFC018	[86]
SICH		Heart	Epithelial	NRFC019	[87]
CB		Brain	Epithelial	NRFC020	[87]
ICG		Gill tissue	Epithelial	NRFC021	[88]
ICF	<i>Clarius batrachus</i>	Fin	Epithelial	NRFC022	[89]
LRG	<i>L. rohita</i>	Gill	Epithelial	NRFC023	[90]
IEE	<i>Etroplus suratensis</i>	Eye	Epithelial	NRFC040	[91]
IEK		Kidney	Epithelial	NRFC041	
IEG		Gill	Epithelial	NRFC042	
IEB		Brain	Fibroblast	NRFC043	
RE	<i>L. rohita</i>	Eye	Epithelial	NRFC044	[87]
CSK	<i>Channa striata</i>	Kidney	Fibroblast	NRFC045	[90]
CSG		Gill	Epithelial	NRFC046	[92]
DT1CPEx	<i>Dascyllus trimaculatus</i>	Caudal peduncle	Fibroblast	NRFC024	K S Sobhana, Central Marine Fisheries Research Institute, Kochi
DT1F4Ex		Fin	Fibroblast	NRFC025	
DT1CPTr		Caudal peduncle	Fibroblast	NRFC026	
RC4H1Tr	<i>Rachycentron canadum</i>	Heart	Fibroblast	NRFC027	

Continued

TABLE 1.2 List of fish cell lines available in National Repository of Fish Cell lines (NRFC).—cont'd

Name of cell line	Fish species	Organ	Cell type	NRFC code	Depositor/Reference
EM2HTr	<i>Epinephelus malabaricus</i>	Heart	Fibroblast	NRFC030	[93]
EM2GEx		Gill	Fibroblast	NRFC031	
EM3GEx		Gill	Epithelial	NRFC032	
EM4SPEx		Spleen	Epithelial	NRFC033	
PC1CpTr	<i>Pomacentrus caeruleus</i>	Caudal peduncle	Fibroblast	NRFC035	K S Sobhana
PC1F1Ex		Fin	Fibroblast	NRFC036	
PC1L1Tr		Liver	Fibroblast	NRFC037	
HC2SpEx	<i>Epinephelus merra</i>	Spleen	Epithelial	NRFC038	

TABLE 1.3 Details of whole genomics of important fish species.

Sl. No.	Name of the species	Genome size	Reference
1.	<i>Protopterus aethiopicus</i>	129,907.74 Mb (largest fish genome)	
2.	<i>Tetraodon nigroviridis</i>	342.30 Mb (smallest fish genome)	[23]
3.	<i>Labeo rohita</i>	1480 Mb	[24]
4.	<i>Labeo catla</i>	1019.78 Mb	https://www.ncbi.nlm.nih.gov/genome
5.	<i>Clarias batrachus</i>	900 Mb	[25]
6.	<i>Clarias magur</i>	929 Mb	[26]
7.	Yap hadal snailfish (YHS)	731.75 Mb	[27]
8.	<i>Pangasianodon hypophthalmus</i>	~ 700 Mb	[28]
9.	<i>Ictalurus punctatus</i>	1.0 Gb/1000 Mb	[29]
10.	<i>Tenualosa ilisha</i>	816 MB	[30]
11.	<i>Clupea harengus</i> (Atlantic herring)	807 Mb	[31]
12.	<i>Hypophthalmichthys molitrix</i>	1104.68 Mb	https://www.ncbi.nlm.nih.gov/genome
13.	<i>Cyprinus carpio</i>	1800 Mb/1.8 Gb	[32]
14.	Grass carp	1076.2 Mb	[18]
15.	Asian sea bass	668.5 Mb	https://www.ncbi.nlm.nih.gov/genome
16.	Atlantic cod	824.3 Mb	https://www.ncbi.nlm.nih.gov/genome
17.	Atlantic salmon	2966.9 Mb	https://www.ncbi.nlm.nih.gov/genome
18.	Nile tilapia, <i>Oreochromis niloticus</i>	1005.68 Mb	https://www.ncbi.nlm.nih.gov/genome
19.	Pacific bluefin tuna	684.5 Mb	https://www.ncbi.nlm.nih.gov/genome
20.	Rainbow trout	2179.0 Mb	https://www.ncbi.nlm.nih.gov/genome
21.	Turbot	568.0 Mb	https://www.ncbi.nlm.nih.gov/genome
22.	Tongue sole	470.2 Mb	https://www.ncbi.nlm.nih.gov/genome
23.	<i>Chanos chanos</i>	656.93 Mb	https://www.ncbi.nlm.nih.gov/genome

cryopreserved and maintained. Many cell lines have been supplied to the researchers for their various research works. Researchers from Canada, Germany, and the Netherlands have requested for cell lines from this repository. Certain guidelines have to be followed for sending fish cell lines outside India, and researchers from abroad have to contact the National Biodiversity Authority, Chennai, in this regard (<http://www.nbaindia.org/>).

11. Concept and declaration of State Fish

Prof. W. S. Lakra introduced an innovative concept of State Fish while working at ICAR-NBFGR, Lucknow, in 2006. The concept involves identification of one important fish species as a State Fish, which facilitates integration of key stakeholders from the state governments in its conservation plan. The objective of the State Fish is to let the State adopt a fish and conserve its biodiversity and promote aquaculture as the case may be. This is a very pragmatic approach for conservation of endangered fish species. Different state governments have started working in collaboration with NBFGR in developing strategies for conservation and enhancement of their selected State Fish to achieve success in real-time conservation. Seventeen Indian States and one Union Territory have adopted State Fish (Table 1.4). Interestingly, Lakshadweep has chosen butterflyfish as its State Animal.

TABLE 1.4 Fish species declared as State Fish: R&D opportunities.

State	Scientific name	Common name	Image
Uttarakhand	<i>Tor putitora</i>	Golden mahseer	
Arunachal Pradesh			
Himachal Pradesh			
Jammu and Kashmir (UT)			
Haryana	<i>Labeo calbasu</i>	Kalbasu	
Andhra Pradesh	<i>Channa striata</i>	Striped murrel	
Telangana			
Kerala	<i>Etroplus suratensis</i>	Pearl spot	
Karnataka	<i>Puntius carnaticus</i>	Carnatic carp	
Odisha	<i>Tor mahanadicus</i>	Mahanadi mahseer	
Manipur	<i>Osteobrama belangeri</i>	Pengba	
Mizoram	<i>Semiplotus modestus</i>	Nghavang	

Continued

TABLE 1.4 Fish species declared as State Fish: R&D opportunities.—cont'd

State	Scientific name	Common name	Image
Nagaland	<i>Neolissochilus hexagonolepis</i>	Chocolate mahseer	
West Bengal	<i>Tenualosa ilisha</i>	Hilsa	
Bihar	<i>Clarias magur</i>	Magur	
Uttar Pradesh	<i>Chitala chitala</i>	Chital	
Tripura	<i>Ompok bimaculatus</i>	Pabda	
Lakshadweep	<i>Chaetodon decussatus</i>	Butterflyfish	

12. Conclusion

The technological innovations in aquaculture are showing visible impacts globally in relation to species and systems diversification, disease diagnosis and management, improved strains of aquatic animals, and intensive aquaculture. This development has to be upscaled in partnership with technology, scientist, and industry and policy support whenever necessary along with capacity building, new infrastructure creation, and continuous interaction between the producers and the researchers. The sustainability of aquaculture is the most challenging aspect of this fast-growing food sector. The future technology infusion and innovation warrants a judicious application of new technology and scientific knowledge with sustainability and profitability of the farming community at the center of the modernization.

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Opportunities and challenges in aquaculture biotechnology

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1. Introduction

Although half of the food fish is coming from capture fisheries sector, it has come to a stagnation and one third of exploited fish stocks is already overfished for commercial use. This development has led to a rapid upturn in global aquaculture, which has recently accounted for contributing to more than half of the production around the world. However, aquaculture uses mostly intensive mono- or polyculture systems in which individual species are reared in limited closed environment with high densities, often leading to problems with diseases and pesticide and chemical use, and causing a concern to ecological balance. The culture of aquatic organisms (fish, crustaceans, mollusks, aquatic plants, and algae) in a closed or controlled environment refers to aquaculture, and it is practiced throughout the globe except in extreme polar regions. The current trends in aquaculture aim at large for increasing productivity from unit area and total production of quality fish with better economic return at a limited time through the use of novel technologies in feed development, alternate feed sources, feeding technologies, health-care measures, breeding technologies, genetic improvement to transgenics, harvest and postharvest technologies, etc.

Biotechnology, the use of living organisms or biological systems in the production process, has many applications in aquaculture for augmenting quality food production. Over the years, our knowledge in biotechnology has led to improved variety of food fish production with higher growth, disease resistance, and meat quality; production of ornamental fish with high market value; beneficial microbes as or for fish feed development; maintaining water quality; marker-assisted selection; quality control in fish as safe food; year-round breeding and seed production; conservation of fish stocks; health management of fish, etc. These have been the key factors that have facilitated the application of advanced biotechnological tools in aquaculture.

The earlier researchers in China have tried to generate ornamental fish of various shapes, sizes, and colors from a single species through centuries of breeding. The recent developments in molecular biology, particularly genetic engineering, have directed microbes as the drug factories, insulin for diabetics through genetically altered bacteria using recombinant DNA technology, high-yielding food crops, biopesticides, biofertilizers, etc. It has also equally contributed to the “Blue Revolution” in fisheries and aquaculture to meet the ever-increasing demand of growing population and polluted environment in the event of reduced land area and poor availability of quality water for fish farming. The success of aquaculture depends on controlled breeding and sustainable quality fish production with high economic return. To this end, the sector has shown tremendous contribution in terms of selective breeding for higher growth and disease resistance in salmonids and carps, inducing agents in the form of hormones of breeding of large variety of fish in captivity, biofortification of feeds, larval diet development, new generations of vaccines and diagnostics along with pro- and prebiotics for environment and health management, increasing shelf life of processed meat, and above all, increasing productivity from unit area in terms of biofloc, integrated multitrophic aquaculture, and recirculatory aquaculture systems technologies. Large consortia of database on important food and ornamental fish species have been generated to target novel gene pools for their wider application. In the aspect of ornamental aquaculture, the biotechnological tools have shown transgenics for color development, indicator fish of pollution, genetically engineered vaccines and molecular diagnostics, etc.

Advances in biotechnology are paving the way for new opportunities that may benefit aquaculture, making it more efficient, environmentally friendly, and sustainable. However, all biotechnological tools do not have wide acceptability in the public domain. Some of the genetic engineering tools or products are being rejected by the consumers outrightly. However, genetic engineering is only one of the many opportunities that biotechnology offers us. Genetic engineering has been remaining as a focus in the public controversy about the benefits, risks, advantages, and disadvantages of biotechnology. When the genetically modified AquaAdvantage salmon from AquaBounty Technologies that grows to market size in 1.5 years instead of 3 years got approval from the US Food and Drug Administration (FDA), the consumers revealed their fears confirming that the last inhibition thresholds have now fallen and genetically modified organisms (GMOs) will soon dominate the markets. Criticizers of aquaculture shade a slanted picture of this industry, accusing it of purely profit-oriented decision-making and too willing acceptance of possible risks to the environment and consumer health.

2. Genetic engineering using gene editing tools

In the recent past, ever-evolving genetic engineering tools have revolutionized biotechnological research worldwide. Empirically, conventional phenotypic selective breeding method has been followed by several researchers to develop or modify an animal with maximized desirable traits. With the progress of modern biotechnological tools, it becomes possible to transfer genetic material from one organism to another organism that belongs to the same or different taxonomical clade. But the method of gene transfer has several public acceptance and ethical issues which pave way for different gene editing technology. Gene editing technology interferes the genome of the organism, leading to modification of genome in a species-specific manner. Several techniques are in use for addition or deletion of a particular section of gene, i.e., zinc finger nucleases [1,2], followed by transcription activator-like effector nucleases, meganucleases, and most recently, the CRISPR/Cas system [3]. Among different gene editing technologies available, the CRISPR/Cas9 method is the most widely used one in aquaculture. Using the CRISPR/Cas9 procedure, specific target gene of the fertilized fish egg can be cut at a specific place. Subsequently, the cell recognizes the damage and repairs the injury independently. Hence, after the repair, the present form of DNA differs from the original sequence. In several economically important aquaculture species, i.e., rainbow trout, Atlantic salmon, catfishes, tilapia, and carp, the CRISPR/Cas9 technology has already been applied successfully. Empirically, few genes have been identified, i.e., by modifying the myostatin gene (*mstn*), muscle growth can be increased [4] or elimination of the dead-end gene (*dnd*) makes the fish sterile [5] or fatty acid elongase gene (*elovl2*) modification can enhance the content of omega-3 fatty acid EPA in the fillet [6]. However, underlying mechanisms of these modifications are still not completely understood, and the need for research in this line remains high. The complex interaction of different molecules also needs to be studied as it has been observed that in fish whose *mstn* gene has been switched off, the immune system gets impaired, which makes the fish more susceptible to different diseases. Despite these drawbacks, genetic engineering has much convincing advantages that make its broad practical application in fisheries and aquaculture for increasing productivity. By switching off specific genes of a particular animal, it becomes easier to pinpoint which phenotypic characteristics are influenced and controlled by the gene of interest. This makes our knowledge about relationships and dependencies between phenotype and genotype, which ultimately enhances the chances of success in breeding performance and breeding lines.

3. Biotechnology in fish breeding

The best known biotechnological tool that revolutionized induced breeding technology of fish is gonadotropin-releasing hormone (GnRH). GnRH plays a major role in regulation and initiation of reproductive cascade in all vertebrates [7]. Synthesis and release of GnRH (tropic peptide hormone) occurs at GnRH neurons within the hypothalamus. GnRH was first isolated from sheep and pig having the ability to induce follicle-stimulating hormone (FSH) and pituitary release of luteinizing hormone (LH) [8]. Release of FSH and LH was governed by the size and frequency of GnRH pulses and feedback from androgens and estrogens. High-frequency GnRH pulses help in LH release, whereas low-frequency GnRH pulses stimulate FSH. In most placental mammals, only 1 form of GnRH has been discovered, whereas, in nonplacental animals (except guinea pig), a total of 12 variants of GnRH have been identified. In fish, a total of eight variants of GnRH have been identified [9–12]. Till date, several chemical analogues of GnRH have been experimented for fish breeding, among which the salmon GnRH analogue is the most successful.

4. Transgenesis

The concept of transgenesis refers to insertion of transgene/exogenous gene obtained from one animal to another animal. The receiver animal is known as transgenic animal. Through transgenesis, foreign trait could be introduced to an animal through genome manipulation. Palmiter et al. [13] produced the first transgenic animal in which the researchers introduced metallothionein human growth hormone fusion gene (mT-hGH) into the mouse egg, resulting in a dramatic increase in the growth of the modified mouse. This prompted a series of experimentation on gene transfer to different economically important animals including fishes.

The first transgenic fish developed was goldfish, in which a novel gene was inserted through microinjection into the fertilized egg [14]. The researchers claimed that at the age of 50 days, the ratio of positive transgenic fish is about 50%. Since then, there has been revolutionary development in the technique and it has been successfully experimented in different fish species. Among different fishes, in salmonids, growth enhancement by transgenesis is very encouraging. Devlin et al. [15] revealed transgenic adult salmon growing 3–5 times higher than the nontransgenic salmon. Furthermore, during the first few months of culture, the growth enhancement compared to control goes up to 10–30 times higher. Gene transfer into fish embryos was experimented in several fish species, such as tilapia (*Oreochromis niloticus*), rainbow trout (*Salmo irideus*, Gibbons), medaka (*Oryzias latipes*), catfish (*Parasilurus asotus*, Linnaeus), common carp, zebrafish (*Danio rerio*), and loach (*Misgurnus anguillicaudatus*) [16,17]. In the process of development of transgenic fish, more emphasis has been given for efficient production of sterile progeny simultaneously, so that the environmental risk of transgenic fish can be minimized. Extreme cold temperature during winter has been an age-old problem in aquaculture, making cold tolerance of fish always a top priority for the researchers [18]. Cold-water temperatures are the major stressor to different fishes, and very few fish species are able to survive water temperatures below 0–1°C. Interestingly, in nature, it has been observed that few marine teleosts have high levels (10–25 mg/mL) of serum antifreeze proteins (AFPs) or glycoproteins, which play a major role in effectively reducing the body freezing temperature by preventing formation of ice crystals. The gene encoding for liver AFP in winter flounder has been well characterized and subsequently introduced into the genome of Atlantic salmon. The AFP gene was found to be well integrated in Atlantic salmon and passed into the offspring where considerable expression was found in the liver [17]. Furthermore, introduction of AFP gene construct into goldfish successfully increased their cold tolerance compared to control fishes [19]. Similarly, in milkfish and tilapia, injection and oral administration of AFP successfully increase the cold resistance [20]. Hence, development of fish species tolerant to cold temperature will definitely boost the potential of temperate region by improving productivity during the colder seasons. In addition, green fluorescent protein (GFP) was transferred into fertilized eggs of zebrafish as a reporter to produce transgenic zebrafish [21].

Embryonic stem cell (ESC) technology is the recent update for the development of transgenic fish and is being explored extensively. ESCs are totipotent and undifferentiated, which makes them easier for in vitro manipulation. The process enables introduced trait to be stabilized or deleted [22]. For development of genetic homogenous strain of transgenic fish, nuclear transplantation with gene-targeted embryonic cells is used effectively. Additionally, in model fishes, i.e., zebrafish and medaka, stem cell–like lines are successfully demonstrated [23–25]. Although experimentally significant development has been made in fish transgenic development, still few issues need to be resolved before successful commercialization of transgenic fishes in aquaculture, particularly for food fishes where ethical issues play a major role.

5. Chromosome engineering

Chromosome engineering helps in manipulating a new genetic attribute of a given individual by modifying its original genome for better performance in natural environment. In aquaculture, chromosome sex manipulation is extensively used for production of triploid or tetraploid individuals and uniparental chromosome inheritance (gynogenesis and androgenesis) [26,27]. These techniques help in improvement of fish breeding quality as they provide a rapid method for sex control, gonadal sterilization, improvement of hybrid viability, and clonation. Somatic cells of most vertebrates are diploid in nature, containing two complete chromosome sets. However, polyploidy refers to individuals containing one or more additional chromosome sets, which turns the chromosome number to three in triploids, four in tetraploids, and so on. In fisheries and aquaculture, induced triploidy is the most effective method for producing sterile fishes. The techniques used to induce triploids and other chromosome manipulations in fishes and their applications to aquaculture and fisheries management are well described [26,28]. Not only in fish but also in triploid Pacific oysters, *Crassostrea gigas* showed better growth and sterility compared to diploid [29]. Other than triploidy, tetraploid breeding lines are also beneficial for aquaculture. Simple interploidy crosses between tetraploids and diploids have the potential to produce large numbers of

sterile triploid [30,31]. Although in many fishes experimentally tetraploidy has been induced, the viability of tetraploids was low in most of the instances [32].

In teleosts, for inducing sterility, exogenous hormone treatment is also a very lucrative biotechnological venture [33]. However, limitation associated with exogenous hormone is governmental regulation and a lack of consumer acceptance of hormone-treated fish and fishery products. Triploidy can be achieved by exposing freshly fertilized eggs to physical or chemical treatment to inhibit extrusion of the second polar body [34,35]. Theoretically, triploid fishes are sterile because of the failure of homologous chromosomes to synapse correctly during the first meiotic division. Triploid induction can be achieved by exposing fertilized eggs to hydrostatic pressure shock, temperature shock (hot or cold), or chemicals such as cytochalasin B, colchicine, or nitrous oxide. Gynogenesis is used to yield all-female populations in a species with female homogamety. It is suitable to use gynogenetic all female progenies (instead of normal bisexual progenies) for sex inversion experiments. Combined use of induced gynogenesis along with hormonal sex inversion has been developed for several aquaculture species [36]. Androgenesis can be used for generating homozygous breeding lines of fish and in the recovery of lost genotypes from the cryopreserved sperms. Although the success of androgenesis is very low in spite of few species, it has been experimented in cyprinids, cichlids, and salmonids [37].

6. Cryopreservation of gametes or gene banking

The method of cryopreservation aimed for long-term storage of biological material at a very low temperature (usually at -196°C). The principle behind the technique is that at very low temperature, the physio-biochemical activities of cells get immobilized, thereby making it likely to keep them viable for a longer period. In aquaculture, cryopreservation of fish spermatozoa has been adopted for longtime preservation of milt. Blaxter [38] for the first time successfully preserved *Clupea harengus* milt at ultralow temperature and subsequently fertilized *C. harengus* eggs with frozen–thawed milt. The spermatozoa of most of the cultured fish species have now been cryopreserved successfully [27]. Cryopreservation solved the all-time fish breeding problem of male fish maturing before the female, which makes selective breeding and stock improvement easier and efficient, and aids in the species conservation [39]. Till date, cryopreservation of fish milt is only standardized, whereas there is no viable technique available for preservation of finfish eggs and embryos. Interestingly, freezing of shrimp embryos has been reported [40,41]. Gene banking of all the commercial fish milt and egg is the need of the hour, which will further boost the sector toward sustainability.

7. Optimized feed and effective health protection

Feed plays a significant role in the development and economic sustainability of the aquaculture industry, and it accounts for more than 60% toward cost of production of fish. In the advent of high cost of fish meal or reducing its use in fish feed, an alternate source of it is the demand of the day. In this context, biofortification of fish feeds, insect larvae as alternate ingredient, and fermentation technologies are coming up to cater the sectoral demand. The use of pre- and probiotics, genetically modified yeasts to produce more carotenoids, and exogenous source of digestive enzymes particularly in the larval diet seems to be very promising. Plant-based protein sources are increasingly in use as a substitute to fish meal, although the antinutritional factors like phytates, protease inhibitors, saponins, tannin, lectins, oligosaccharides, phytoestrogens, alkaloids, gossypol, cyanogens, mimosine, and antivitamin limit their use without proper processing. GMOs to eliminate antinutritional factors and increase certain amino acids and fatty acids are now possible by the use of biotechnological tools. Genetically modified microbes to produce certain high-quality enzymes with temperature and pH stability that can be used through granulation technology in pellet feed preparation would certainly revolutionize the feed industry sector. Trace minerals are now being attached to oligopeptides for increasing their bioavailability. Nutraceuticals with growth and health benefits derived from waste products are now having tremendous demand in the feed industry. The new trend is to develop diets on digestible amino acid levels, thereby reducing the requirement for protein. Lysine is produced by microbial fermentation and methionine is chemically synthesized, both of which are in routine use in fish feeds. Genetically enhanced microorganisms are being used to produce threonine and tryptophan on a commercial basis, and in near future, other essential amino acids also would become available. By using these dietary amino acids, a substantial load on protein replacement could be achieved to reduce the feed cost. The concept of an ideal protein blend from genetically modified (GM) feedstuffs and feed additives (such as amino acids and enzymes) will greatly help in decreasing the amount of nitrogen excreted in animal waste [42]. Toxin binders, fish feed attractants, and pro- and probiotics are few other areas that have huge scope in future for healthy and quality fish production. Modern biotechnological tools like polymerase chain reaction (PCR), biomonitoring and DNA/gene-based diagnostics, HPLC, and ELISA-based assays could

be used for the detection of pathogens, toxins, heavy metals, pesticides, biotoxins, and other contaminants in fish feeds and feeding environment.

Toward health protection of fishes and increasing productivity, biotechnology plays a major role. Rapid diagnosis is one of the keys for controlling diseases in aquaculture in the advent of molecular methods, i.e., PCR, real-time PCR, and nucleic acid sequence–based amplification, that have enabled rapid detection, identification, and quantification of very low levels of infecting pathogens [43]. Furthermore, technologies like microarray offer a new dimension to multiplex screening for pathogens and host response [44]. Recently, loop-mediated isothermal amplification technique has drawn the attention of the diagnostic laboratories as proved to be more advantageous tool than PCR because it needs only a single thermostatically controlled water bath as a source of constant tube temperature and is more specific [45]. Immunological-based diagnostic methods, i.e., immunohistochemistry, fluorescent antibody technique, indirect fluorescent antibody technique, ELISA, and dot blot or western blot, help in the rapid and specific detection of infecting pathogens without the need of the firsthand isolation of pathogen [46]. In these tests, specific monoclonal antibody supplements with ideal standardized reagents are used and many are now available commercially [44]. Due to the development of specific pathogen-free and specific pathogen-resistant whiteleg shrimp (*Litopenaeus vannamei*), there is a huge increase in shrimp production worldwide. In addition, different vaccines that safeguard fish from deadly diseases are increasingly used in the field of fish health management. Worldwide researchers are working for development of highly effective new-generation vaccines which no longer consist of “inactivated/attenuated pathogens,” but of genetically modified microorganisms, recombinant genetic material, protein subunits, and DNA fragments. Although several experimental successes have been observed for new-generation vaccines, very few are commercialized and in use for aquaculture farms. The future of biotechnology lies with commercially viable multivalent vaccines for economically important pathogens of diverse cultivated aquacultured species. For the development of “natural product research,” biotechnology has a great potential for synthesis of pharmaceutical products such as oils, pigments, alginates, sterols, and agarose from micro- and macroalgae. Recently, an antitumor peptide, CIGB-552, has been developed as a candidate for cancer therapy, which was isolated from the blood plasma and tissue extracts of horseshoe crabs (*Limulus polyphemus*) [47]. The peptide developed is under phase I clinical trials as anticancer drug and also has possible application for other metabolic and inflammatory diseases.

In comparison to the vertebrate immune system, shrimp immune system is weakly developed, and in this case also, biotechnologically engineered molecules are of great use for augment of shrimp immune status. Microbial products such as lipopolysaccharides, peptidoglycans, or glucans have shown great potential in stimulating nonspecific defense system of different shrimp species [48]. In the absence of vaccination strategy for shrimp and danger of several viral etiologies in shrimp that have crippled the shrimp aquaculture sector, there is a tremendous scope for developing alternative strategies to combat deadly diseases. A potential area in understanding the dynamics of microbial communities that are being modulated during disease development process through metagenomics approach may lead to not only understanding favorable microenvironment but also has tremendous scope for better health management.

8. Traceability, residue analysis, and value chain in aquaculture

Traceability is an emerging challenge in aquaculture. Traceability means “the ability to follow the movement of a food through specified stage(s) of production, processing and distribution,” and it is used with issues or problems associated with food safety and quality assurance, preventing risk in the business and improving consumer trust. Biotechnology industry has a tremendous role in tracking the whole process to deliver a safe product to the consumer after complete antibiotic and other microbes or their toxin residues in the value chain process. The recent tools for estimating toxins or screening microbes through PCR- or ELISA-based methods are quite promising. Industrial aquaculture poses significant human health and food safety concerns. The increased use of antibiotics, pesticides, and others to protect from fish diseases in fish farming can accumulate in fish tissues. Furthermore, the feed used in commercial aquaculture is made from wild-caught or trash fish that may be heavily contaminated with dioxins or other toxins. Hence, the development of biotechnological tools for their easy detection or screening would be beneficial in delivering a safe product to the end users.

9. General challenges

9.1 Genetically modified food adoption

Genetically modified animal adoption has been an empirical problem. The public acceptance has been the big concern as there are many myths circulated and fixed in the public mind. For the safety of foods and food products produced from GM animals, harmonized guidelines have been published by the Food and Agriculture Organization/WHO Codex

Alimentarius. Recommendations provided by Codex Alimentarius and the European Food Safety Authority GMO Panel are comparing safety assessment of GM products with non-GM counterparts with a scope of safe use, in addition to the molecular characterization of the introduced genetic material. Other traits needed for testing are potential toxicity (of the introduced or altered components), occurrence of unintended effects alongside original targeted modification, horizontal gene transfer, and nutritional value [49]. More than 35 species of fish including salmon, catfish, tilapia, trout, striped bass, and flounder are being genetically modified in the world for arrays of traits (growth, disease resistance, larger muscle, and temperature tolerance) suiting industrial aquaculture on experimental scale. However, in 2015, FDA approved the AquaAdvantage salmon produced by AquaBounty Technologies for commercial use in spite of possible danger of environmental concern and health safety. It remains as a major challenge to the biotechnologists to answer these negative comments while solving the global protein demand of the ever-growing population.

9.2 Societal concerns

For the acceptance of GM and transgenic food, still several societal stigmas need to be clear. Novoselova et al. [50] reported higher consumer preference of conventional pork over GM pork. The negative perception of GM pork may be compensated by improvements in increased animal health, quality and welfare [51], less residues, a lower impact of the environment, and discounted price. Increased animal welfare along with additional health benefit has the most positive effect on consumer preferences, whereas improvement in environments receives the lowest utility. In the case of GM salmon, it has been commonly observed that discount in the product price is the most quoted personal benefit for acceptance [52,53]. Other benefits offered by GM salmon are potential health benefits resulted from higher level of omega-3 [54,55] and environmental benefits, by reducing the use of chemicals [53] or the use of lesser fodder [52].

9.3 Environmental harm

Greatest threat related to environmental harm of the GMO is escape of genetically engineered organisms to wild and mixing of them with the natural flora and fauna. There is also inherent uncertainty among researchers in identifying the potential environmental problems at initial stage and the difficulty in successful remediation once a threat has been identified. The negative impacts of introducing genetically engineered animals can be either direct, i.e., resulting from direct competition for limited food, habitat, and other resources, or indirect, caused by changes in other biotic and abiotic factors utilized or needed by different ecologic community. To assess the risk factors of GE fish, the following data are utmost important: (1) the ability of the GE fish to escape and disperse into diverse communities, (2) the effect of the transgene on the “fitness” of the fish within the ecosystem into which it is released, and (3) the stability and resiliency of the receiving community.

9.4 Production/laboratory safety

Laboratory safety of both experimental animal and working researchers is also very important. The laboratory should not have any loop hole for escape of any genetic material from the laboratory, which may create havoc. Developing such facilities and model aquatic animals remains a big challenge for biotechnologists although whole-genome information of few species is available. The future era will bring out whole-genome and metabolome information of wide array of economically important both food and ornamental fish species.

9.5 Ethical issues

Concerns for animal welfare, including ethical issues, can arise at every stage in the generation and life span of a genetically engineered aquatic animal. The main concerns are as follows: (1) invasive procedures for the generation of a new genetically engineered animal line, (2) large numbers of animals required during embryonic engineering of animals, and (3) unanticipated welfare concerns. By inserting gene that encodes the GFP from sea anemone and jellyfish, zebrafish have been genetically engineered to express fluorescent proteins with attractive colors, commercially termed “GloFish.” As ornamental pet fish, GloFish began to be marketed in the United States during 2003. However, later their sale sparked controversial ethical debates in California and then become the only US state to prohibit the sale of GloFish as pets. There is a need for free flow of information, public awareness and expression, transparency, and accountability to address the ethical issues for responsible fisheries.

10. Other challenges

Lastly, the biotechnology industry is also facing few other challenges, which include the following:

- a. **Length of patent time of each product and marketing issues:** The time taken to get a patent for any developed product is more than a year and sometimes it goes beyond a decade. It creates lots of agony to the developer as well as the investor. Furthermore, the private entrepreneurs or aquaculture industry is not so established for easy commercialization of developed product. For example, in the absence of any aquaculture policy on drug use or routine screening of samples for disease diagnosis or strong biosafety guidelines, particularly in the case of freshwater aquaculture, many of the developed products do not see its commercialization. Again, most of the products that are commercialized are not gaining momentum at the industry level. There is again only a handful of aquaculture biotechnology industry in India, even though it stands second in aquaculture production, dealing with fishery technologies.
- b. **Investment and return on investments:** Fish being a commodity has less or region-specific demand, although it meets to a substantial protein demand of human beings. However, there is less emphasis in all biotechnology sectors starting from pharma to health products or breed improvement tools. Furthermore, wide phylogenetic variation among food or ornamental fish species also plays a big role on wide activity of any of the products for large diverse fish species. The players involved in health or pharma industry are very meagre. Hence, there is a big question on return of investment of any technologies as there is no clear-cut guidelines existing for routine health monitoring of any fish farm, strong quarantine system that requires screening of border movement of pathogens, residual levels of antibiotics in fish used for consumption within the country, drug residues in food fish, etc. Hence, none of the industries are venturing to invest in those sectors because of lack of demand.

11. Conclusion

Biotechnology has made a mark in the development of the aquaculture sector. Although it has made a slower progress in the application unlike terrestrial animal or human sector, its revolutionary role in selective breeding and marker-assisted selection for achieving higher growth, meat quality and disease resistance in fish, biodiversity conservation, molecular diagnosis and vaccine development, transgenics, and gene editing is noteworthy. Furthermore, there is enough scope for application of this technology in aquaculture by bringing in new global policy reforms for easier trade and market, further engineering of developed tools to suit to local or regional needs, and reducing the dependency on heterologous species information in product development. At the same time, the ethical and biosafety concerns that would come up need to be addressed on the global front.

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Genetically improved aquaculture species in Bangladesh: advances and challenges

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1. Introduction

In Asia, the third largest aquatic biodiversity belongs to Bangladesh, behind China and India, and it is globally considered to be one of the suitable regions for fisheries. The species diversity has been attributed to the world's largest flooded wetland (Bengal Delta) and three main river systems viz. Ganges, Brahmaputra, and Meghna that flow from the Himalayan mountain into the Bay of Bengal. Because of land-based opportunities are limited, vast and rich water resources offer the best possibilities for food security and income generation for the millions of people living in the country. In particular, fresh and brackish water fisheries resources have potentials for producing increased yields of fish through managed aquaculture practices. In 2018, Bangladesh was globally ranked third in inland water capture fisheries and fifth in aquaculture production [1]. Presently, the government is making serious efforts to rehabilitate the inland fisheries and marine fisheries under the recent concept of blue economy development [2,3]; meanwhile, it has focused its attention on the technology-based aquaculture in the closed freshwater and marine water bodies including marine aquaculture [4], which has tremendous opportunity in the country. There is no doubt that modern genetics and biotechnology represents a range of advanced and frontier technologies that extend opportunities to develop improved fish breeds having increased growth rate; to improve nutrition of aquafeeds; to improve and protect fish health; to help in the sustainable use, management, and conservation of fish stocks and other aquatic resources; and to restore and protect aquatic environments, which should have wider implications for increasing overall productivity of the fisheries sector.

The commonly used seven fast-growing carp species for composite carp culture/polyculture systems in the country are catla (*Catla catla*), rohu (*Labeo rohita*), mrigal (*Cirrhinus mrigala*), silver carp (*Hypophthalmichthys molitrix*), grass carp (*Ctenopharyngodon idella*), common carp (*Cyprinus carpio*), and silver barb (*Barbonymus gonionotus*). Carp species dominate particularly in total annual aquaculture production (2019–20) and presently share 65.39%, where indigenous major carps contribute about 37.23%, exotic major Chinese carps contribute about 19.48%, and other carps share about 8.68% of the total aquaculture production [5]. The recent advancement of freshwater aquaculture particularly in the carp polyculture and other related freshwater fish farming is mainly due to the advent of hypophysation technique(s) during the mid-1960s. In Bangladesh, the native major carp seed production through artificial breeding has become a common practice since that time [6]. Meanwhile, Chinese major carps, exotic barbs, and catfishes have also been introduced, and a large number of hatcheries both in public and private sectors have been established. It is estimated that there are about 1038 carp hatcheries (both public and private hatcheries) in the country. These hatcheries are currently producing 664,020 kg of carp spawn. Today, the hatchery-produced fries/fingerlings are greatly contributing to the overall aquaculture production of the country. It has been observed that while some of the hatcheries are careful in selecting and maintenance of their brood stock, practices followed by most of other hatcheries result in inbreeding and genetic deterioration of stocks [7]. Once a hatchery is constructed, the general temptation is to stock with whatever materials that are easiest to acquire. This is a short-sighted and unscientific approach as pedigree of the farmed stocks can be an important determinant of its performance of

future profitability of the operation. Moreover, small number of brood fish for species with high fecundity like carps and their replacement by succeeding generation can give rise to uncontrolled inbreeding often leading to inbreeding depression and decrease in growth rate, fecundity, and survival of fish in most of the small hatcheries. The problems that are caused by uncontrolled inbreeding are usually accompanied by loss of alleles via genetic drift. When a breeding program is used, genetic changes are planned and desired because they will lead to improved growth rate, survival, etc. When no breeding program is conducted, genetic changes are unplanned and random, and those caused by inbreeding and genetic drift can ruin the population [8]. So, many fish breeding experts suggest that the use of selective breeding technique(s) is the simplest, promising, and useful method(s) to improve desirable traits in a founder stock with high genetic variability and that it may ultimately be of benefit to fish breeding and aquaculture by increasing the growth rate, fecundity, survival, and disease resistance of commercially important fishes like carps and other cultivable fish species [7].

Genetic improvement research in tilapia and carp species especially in silver barb and rohu has already been initiated and made a good progress in Bangladesh. Both mass selection and family selection techniques were in practice to improve their stocks, and significant genetic gain in growth across several generations, meanwhile, has been achieved in genetically improved farmed tilapia (GIFT) strain, genetically improved silver barb strain, and genetically improved rohu at Bangladesh Fisheries Research Institute (BFRI) and WorldFish, Bangladesh and South Asia. In fact, BFRI initiated the genetic stock improvement of silver barb, *B. gonionotus*, in 1996 and continued until 2008 using both mass selection and family selection breeding techniques [9,10]. In family selection trials, cumulative genetic gain (%) at harvest weight was estimated over the three generations of selection and F₃ generation attained 36% weight gain over the control group. For genetic stock improvement program of GIFT strain, although BFRI conducted mass selection or individual selection during 1999–2004, since 2005, the institute redesigned the program for further stock improvement through family selection until 2019 [11–13]. Thirteen generations have already been developed from 2007 to 2019, resulting in G₁₃ generation attaining 64.44% harvest weight gain than that of the average base population (Dr. Kohinoor pers. communication). The improved GIFT and silver barb strains have already been released since their stock improvement for farming and shown significant impact on quality seed production and aquaculture production in the country. BFRI also initiated rohu, *L. rohita*, genetic improvement program using selection technique(s) since 1999 [14] and continued until 2018. Meanwhile, genetically improved G₁, G₂, G₃, and G₄ generations of rohu have been developed by BFRI, and G₄ generation rohu strain achieved 20.12% harvest weight over the existing strain. On June 10, 2021, G₄ improved generation of rohu was named as “Subarna rohu” by BFRI, which has, meanwhile, been released for quality seed production to support carp polyculture among government and private hatcheries (Dr. Yahia Mahmud, personal communication). Meanwhile, WorldFish, Bangladesh and South Asia, came forward through some of its projects, viz. United States Agency for International Development (USAID) Aquaculture for Income and Nutrition (AIN) and USAID Bangladesh Aquaculture and Nutrition Activity (BANA) funded projects and redesigned Rohu Genetic Improvement Program (RGIP) and initiated to improve rohu strain using family selection technique since 2013 [15], and G₁ rohu generation of the fish has already been produced in 2016 [16], and G₂ and G₃ rohu generations, respectively, in 2018 and 2020. WorldFish-improved G₃ strain of rohu has also been released and distributed among private hatchery owners in Jessore and other regions since October 2020 (Dr. Mathew Hamilton, pers. communication).

So, there are plenty of opportunities to include more indigenous major and minor carps and exotic major and minor carps including other fin fish species mostly used in aquaculture to improve their stocks through genetic improvement program using family selection or combined selection technique(s). Among major carp species along with rohu, other potential species like catla, mrigal, silver carp, and bighead carp could be prioritized by BFRI, WorldFish, and public universities/private research agencies.

This chapter has been designed and attempted to review genetically improved fish in Bangladesh, highlight the advances and challenges, and touch their impact on aquaculture production in the country.

2. Breeding plans and genetic stock improvement of carp species

2.1 Background information

Genetic improvement of carp species has already been initiated in Asia. Nguyen reported that selective breeding programs have been carried out in important species such as silver barb (*B. gonionotus*) in Bangladesh and Thailand [9,10,17,18]; rohu (*L. rohita*) in Bangladesh [14–16,18] and India [19,20]; and common carps (*C. carpio*) in China, Indonesia, and Vietnam [21]. For estimating genetic merit and select replacements, the majority of these countries have established systematic genetic improvement programs, and fully pedigreed populations are maintained by fitting an individual animal model. This enables rigorous conduct of the genetic improvement program as well as the necessary control of inbreeding to

secure long-term response to selection. Across carp species examined so far, genetic gain ranged from 8% to 20% per generation [17,20,22]. There is also considerable genetic variation in these populations, with the heritability for body weight ranging from 0.20 to 0.40, providing valuable stock for future selection [23].

In this section, an attempt has been made to present comprehensive description of genetic studies and stock improvement program on carp species so far conducted in Bangladesh.

2.2 Genetic stock improvement of rohu using mass selection and family selection techniques

Rohu is extremely popular and the preferred species among the indigenous major carps naturally available in the major river systems, viz. Padma, Brahmaputra, Jamuna, Tista, Meghna, and their tributaries, and the fish mostly used for carp polyculture farming in Bangladesh. This Indo-Gangetic riverine species is also the natural inhabitant of the vast river systems of northern and central India and the rivers of Pakistan and Myanmar [24]. Rohu is one of the fast-growing species after catla farming in perennial ponds and confined water bodies along with other carps in the country, which shares 16.4%, 28.2%, and 44.8%, respectively, in total aquaculture production, total carp production, and total indigenous carp production [25]. After the advent of artificial breeding technique during the mid-1960s, seed production for aquaculture was mainly supported by the carp hatcheries. But recently it has been observed that while some of the hatcheries are careful in selecting and maintenance of their carp brood stock by collecting from either rivers or known sources, practices followed by most of other hatcheries result in inbreeding and genetic deterioration of stocks. Since the middle of 1990s, stock deterioration in carp hatchery population has also been reported because of poor brood stock management and inbreeding depression [7].

To take advantage of modern genetic techniques and to become independent of deteriorated hatchery stocks and reduced natural stocks, genetic stock improvement of carp species could be sustainable solutions for aquaculture in Bangladesh, following the breeding of improved brood stocks and rearing of the quality seeds in grow-out ponds and other closed water bodies. In view of this, rohu has been identified as first priority species of major carps for genetic stock improvement using selective breeding techniques.

BFRI conducted rohu breeding program for genetic stock improvement through individual selection or mass selection technique(s) in 1999 [14,18] and subsequently continued through family selection program until 2018. WorldFish, Bangladesh and South Asia, came forward with a well-designed RGIP under its USAID-funded AIN project and started using within-family selection technique(s) since 2013 [15,16].

2.2.1 Rohu genetic stock improvement at Bangladesh Fisheries Research Institute

BFRI initiated RGIP since early 2000, and it took one and half decades to produce four generations using selection breeding techniques. As the maintenance of each generation was done carefully, year gaps in producing subsequent generations were not a major constraint. The entire research program has been narrated in the following sections.

2.2.1.1 Origin of stocks

In view of genetic stock improvement, land races of rohu, *L. rohita*, were collected from different river systems i.e., from Brahmaputra and Jamuna rivers of the country. However, the third stock of rohu was taken from Freshwater Station (FS), BFRI, Mymensingh. The collected stocks were reared separately in three ponds until maturity and screened by investigating differences in extrinsic genetic traits by means of morphological assessment.

2.2.1.2 Development and rearing of base population

The two wild stocks, Jamuna and Brahmaputra, along with an existing domesticated hatchery stock, were mated to produce three crossbred lines through a 3×3 incomplete diallel crossing design in 1999. The crossbred lines so produced using the three different stocks were the Hatchery ♀ x Jamuna ♂, Jamuna ♀ x Brahmaputra ♂, and Brahmaputra ♀ x Jamuna ♂ [14,18]. These lines were reared in separate nursery, rearing ponds, and grow-out ponds until they were being used for the production of next generation.

2.2.1.3 Breeding protocols and production of new generations through selection

During the breeding season of 2004, two crossbred lines, i.e., Hatchery♀ x Jamuna ♂ and Jamuna ♀ x Brahmaputra ♂, were taken to use for the production of G₁ generation. From sexually matured females and males, at least 10% of best 50 pairs were mass selected from each of the crossbred lines. G₁ generation progenies were produced by reciprocal single pair mating in the hatchery, i.e., crosses between females and males from two lines. Immediately after ovulation, at least 100 g

of fertilized eggs from each of the 50 pair mating (in each line) was collected and mixed together and incubated in two circulated spawning arenas. Subsequently, the two pool batches of G_1 generation fry were reared separately in primary, secondary, and tertiary nursing systems and finally grown until maturity by proper feeding and water quality management in two earthen ponds (1000 m² each).

During the breeding season of 2008, an attempt was made using G_1 generation breeding stocks. A nested pair mating design was followed to produce 70 families of G_2 generation using best selected 70 females and 35 males from either line of G_1 rohu stocks in the FS, BFRI carp hatchery, in Mymensingh. Individual progeny families were nursed separately in primary, secondary, and tertiary hapas in earthen ponds for 2, 4, and 6 months, respectively. Fingerlings (>25 g size) of randomly chosen 40 families were taken and 600 individuals were Passive Integrated Transponder (PIT) tagged (15 fingerlings from each family) and communally stocked in an earthen pond for further rearing until sexual maturity. Before stocking, the body weight, standard length, and PIT tag numbers of the fingerlings were recorded in both the trials. After stocking, the fish were taken intensive care by regular feeding and water quality management in all the communal grow-out ponds. Throughout the entire grow-out period, the fish were fed two times daily with formulated feeds at recommended estimated body weight. After every 30-day interval, sampling of 50 growing fish was made to check the growth and adjust the feeding rate. After 18 months of communal rearing, at harvest, the body weight, body depth, body width, and standard length of individual G_2 generation rohu have been measured.

During the breeding season of 2013, from sexually matured females and males, at least 10% of best 50 pairs were mass selected from each of the 70 families of G_2 generation. G_3 generation progenies were produced by single pair mating in the hatchery, i.e., crosses between females and males at least from two separate families. Immediately after ovulation, at least 100 g of fertilized eggs from each of the 70 pair mating was collected and mixed together and incubated in two circulated spawning arenas. Subsequently, the 70 pool batches of G_3 generation fry were reared separately in primary, secondary, and tertiary nursing systems and finally grown until maturity by proper feeding and water quality management in two earthen ponds (1000 m² each).

In view of producing G_4 generation of rohu, further mass selection protocol was followed using G_3 generation stock during the breeding season of 2018. The 10% best females and males were selected from 50 randomly taken brood stock of G_3 generation and used for producing the next generation. At least 50 pairs of mass-selected breeders were used for single pair mating to produce the G_4 generation progeny. Similar nursing and rearing protocols were followed for the 50 pool batches of G_4 generation and finally grown until maturity by proper feeding and water quality management in two earthen ponds (1000 m² each).

2.2.1.4 On-station evaluation of growth performance of improved generations of rohu strains produced from selective breeding programs

In this family selection trial of rohu, growth evaluation of improved generations was carried on station at communal grow-out ponds (1000 m² each) and cumulative genetic gain (%) at harvest weight was estimated. It was observed that the new G_1 , G_2 , G_3 , and G_4 selected groups attained, respectively, 7.5%, 12.38%, 16.83%, and 20.12% weight gain over existing nonselected groups (Fig. 3.1). This trend of improved growth has some high significant impact for quality brood stock

FIGURE 3.1 Cumulative genetic gain (%) by weight of selected groups over the existing or nonselected groups in different generations of rohu.

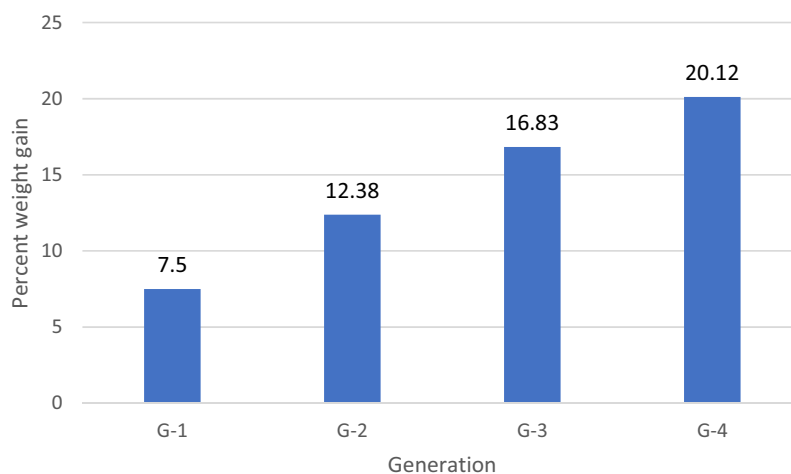




FIGURE 3.2 Image of generically improved “Subarna rohu” strain recently declared by Bangladesh Fisheries Research Institute in Bangladesh. Photo: Dr. Enamul Hoq.

development in the carp hatcheries as well as is promising for further genetic improvement for growth and other specific trait(s) in rohu.

On June 10, 2021, BFRI named the G_4 generation rohu as “Subarna rohu” (Fig. 3.2) and officially handed it over to one public carp hatchery and one private carp hatchery of Mymensingh region in Bangladesh. Subsequently, BFRI has a plan to distribute gradually this genetically improved strain to all other carp hatcheries throughout the country.

2.2.2 Rohu genetic stock improvement at WorldFish, Bangladesh and South Asia

To take advantage of modern genetic selection technology, reduce the risk of inbreeding depression in hatchery-produced carp seeds, and become independent of reduced natural stocks for supporting polyculture systems in Bangladesh, WorldFish, Bangladesh and South Asia, first initiated RGIP, under the USAID-funded AIN project of WorldFish, Bangladesh and South Asia, in 2013 [15,16,26]. WorldFish, Bangladesh and South Asia, continued RGIP under USAID AIN project until 2017. During 2018–2020, this program was financially supported by another USAID BANA project. Since mid-2021, RGIP and other carp genetic improvement programs of WorldFish, Bangladesh and South Asia, are being funded and supported under USAID Feed the Future Innovation Lab for Fish, Mississippi State University, USA.

It is worthy to mention that WorldFish recently initiated as well catla and silver carp genetic stock improvement side by side with RGIP.

2.2.2.1 Origin of stocks from riverine sources and their maintenance

Fertilized eggs of three wild rohu stocks had been collected directly from Halda, Jamuna, and Padma rivers during the breeding season in 2012 and reared in a number of farmer’s ponds in Faridpur and Jessore. Subsequently, during the last week of October to second week of November 2013, the required number of juveniles of three riverine rohu founder stocks were transported live using two fiber glass tanks having the facility of aeration to Jessore. From this collection, a total of 300 Halda + 300 Jamuna + 300 Padma stocks, total = 900 juvenile rohu (>200 g each), were PIT tagged and communally stocked in two earthen grow-out ponds. All the 900 PIT-tagged rohu that have been stocked for growing in a common farm environment have been used to collect the tissue samples, i.e., the fin clips. These samples were required to assess the relationship of the fish in order to be able to exclude mating close relatives when mating was undertaken in the following generations. For that reason, the fin clip samples need to be taken in time to allow marker development and genotyping prior to any further breeding season. In fact, all those 900 fin clip samples were sent to the laboratory of Diversity Arrays Technology Pvt. Ltd., University of Canberra, Australia, for DNA analysis using single nucleotide polymorphism (SNP) markers.

These stocked fish were divided in two equal groups, i.e., 150 Halda + 150 Jamuna + 150 Padma stocks, total = 450 fish in each group, and the first group of 450 fish were stocked in an earthen pond having an area of 84 decimal at

Mukteshory Fish Farm, Kazipur, Jessore, and the second group of 450 fish were stocked in a pond having an area of 55 decimal at Niribili Fish Farm, Chachra, Jessore. In these ponds, at the time of stocking, the body weight, standard length, and tag numbers of the individual fish were recorded. Throughout the entire grow-out period (December 2013–May 2014), the fish were fed with formulated feeds twice daily at recommended estimated body weight.

2.2.2.2 Development and rearing of base population

During the mid of July 2014, a total of 210 full-sib families were produced through single pair matings from these stocks using complete diallel crossing design. During the last 6 months (August 2014–January 2015), progenies of 210 full-sib families were separately stocked and reared until the fingerlings reach to the tagging size under two different nursery systems (i.e., hapas [nylon nets] and chambers [small partitions of the pond made by nylon nets set with bamboo frames] set up in a number of earthen ponds). Meanwhile, 50 fingerlings (25 from hapa + 25 from chamber) from each of the families were PIT tagged and communally stocked for monoculture until harvest in four replicated grow-out ponds in Jessore. At the same time, in another trial of 70 full-sib families of the all-possible combinations between the 3 stocks of rohu (complete diallel crosses), 20 fingerlings (10 from hapa + 10 from chamber) from each of the families were PIT tagged and communally stocked for polyculture until harvest along with catla and mrigal fingerlings in 3 replicated ponds. Throughout the entire grow-out period, the fish were fed two times daily with formulated feeds at recommended estimated body weight. After every 30-day interval, sampling of 50 growing fish was made to check the growth and adjust the feeding rate. All these trials have been attempted to verify the genotype by environment interactions (GXE) for growth and survival of rohu under mono- and polyculture systems and finally to establish a base population (i.e., a population having broad genetic variability) for the genetic improvement of rohu in Bangladesh.

2.2.2.3 Breeding protocols and production of new generations through family selection

At first harvest, during October–November 2015, the body weight, body depth, body width, and standard length of individual rohu have been measured and recorded, including their PIT tag numbers in all the grow-out ponds of monoculture and polyculture trials in order to estimate their individual EVB. Moreover, survival of pond-wise and family-wise fish also has been calculated and recorded.

In 2016, the data were analyzed using best linear unbiased prediction (BLUP) to get the estimated breeding values (EBVs) of the fish for growth [16]. From the 210 families, a total of 160 families were identified for high EBV for growth, which was decided to keep for the breeding program (between family selection). The best candidates for growth from each family will be used in single pair mating to produce the first generation of the improved strain (within-family selection). A control line from fish with average EBV for growth will be created for future comparison with the selection line and estimation of the response to selection [16]. During the month of June 2016, as per the mating list, a total of 152 selection lines, 43 control lines, and 10 negative lines of G_1 generation were successfully produced. Both G_1 generation selection line and control line families were nursed in primary, secondary, and tertiary hapas in selected ponds until becoming tagging size. Subsequently, after tagging, comparative growth performance of selection and control line was evaluated for the estimation of genetic parameters and realized response to selection of G_1 generation.

For the production of the F_2 and F_3 generations in the breeding seasons of 2018 and 2020, the very best individuals of rohu were chosen in terms of EBV and within-family selection protocol was followed. But a compromise was maintained between selection intensity and effective population size (i.e., NE). At least 200 independent families were further formed and maintained to continue subsequent selective breeding programs. In each subsequent generation, a control line was maintained by mating individuals with average EBV and progeny was compared with that of selected line individuals with greatest EBV. The harvest weight of genetically improved rohu achieved cumulative genetic gain at least 30% across the three generations of selection with approximately averaging 10% selection response per generation.

2.3 Genetic stock improvement of silver barb at Bangladesh Fisheries Research Institute

Silver barb (*B. gonionotus*, Bleeker) is an exotic popular minor carp species among the fish farmers in Bangladesh because it grows well on low-protein diets, whether feeding on certain aquatic plants or given supplementary feeds, and can tolerate a wide range of environmental conditions. The fish was first introduced into the country from Thailand in 1987 [18]. It is mostly cultured in perennial ponds, seasonal ponds, roadside pits or ditches, rice fields, etc., in both polyculture and monoculture systems. In Bangladesh, breeding of *B. gonionotus* is mainly carried out by hypophysation in the hatcheries during April–July for seed production. Brood stock are normally used for two to three breeding seasons and spawning be repeated two to three times with an interval of 40–60 days in a single season.

As *B. gonionotus* gains popularity for culture and farmers mostly depend on private and public hatcheries for collecting stocking materials, i.e., seeds, the likelihood of inbreeding is great because female and male spawners are chosen from finite population for mating. The close inbreeding causes homozygosity to increase rapidly with a typical loss of genetic vigor. Such genetic deterioration of hatchery population of the species all over Bangladesh has been reported (Hussain, 1988). Silver barb has been susceptible to diseases, in particular, epizootic ulcerative syndrome, that particularly occur at the onset of the winter season, perhaps because of temperature stress to this tropical species (Lilley et al., 1998). It is presumed that good generic management and genetic stock improvement has the potential to help overcome both of these problems. Genetic stock improvement through genetic selection is one of the most useful ways of enhancing desirable traits in a founder stock with high genetic variability to reduce inbreeding in a hatchery population [40]. In view of that, BFRI came forward to develop genetically improved strain(s) of *B. gonionotus* using both individual selection or mass selection and family selection.

2.3.1 Genetic improvement of silver barb using mass selection techniques

For the first time in Bangladesh, selective breeding was carried out to genetically improve silver barb as one of the most popular productive minor carp species for aquaculture. The breeding program was initiated in 1996 at FS, BFRI, under the first phase of Asian Development Bank (ADB)—funded project “Genetic Improvement of Carp Species in Asia” of WorldFish Center (formerly International Center for Living Aquatic Resources Management [ICLARM]). The program was conducted involving two wild silver barb stocks obtained from Indonesia and Thailand and existing local stock in Bangladesh [17]. The breeding program was the follow-up using mass selection protocol continued up to the seventh generation. The improved seventh generation of silver barb showed 32% increased body weight in comparison to the local strain [9,10].

2.3.1.1 Development of base population derived from diallel crosses

In 1996, a parental base population was made through a complete 3×3 diallel cross to produce nine genetic groups. For each of the reciprocal crosses, 10 pairs of breeders (F:M, 1:1) were mated separately and an equal number of fertilized eggs were pooled together and incubated in a series of funnel jars; the hatchlings were kept separately in nine hapas until the first feeding [17]. All the matings were performed within 3 days. Next, the nine progenies were nursed in earthen ponds separately. Individuals of the three purebred (control) and six crossbred groups derived from the diallel crosses were tagged using PIT tag at advanced fingerling stage (average weight ranging from 20 to 40 g) and stocked communally (10 individuals from each of the genetic groups) in the same pond. A total of six ponds were selected on the basis of their overall ecological conditions (productivity, depth, and other physical features) and categorized into “Good,” “Medium,” and “Poor” ponds. Each of the test environments had two replicated ponds (300–400 m² each pond) having the same stocking density (1 fish/4 m²).

2.3.1.2 Breeding protocol and production of further generations through individual selection or mass selection

In 1997, the F₁ generation of progeny was produced from the mature parental base populations of each stock [10]. For each of the six reciprocal crosses, five to eight pairs of brood stock were mated and their progenies were kept separately in hapas placed in hatchery conditions. The best 3 progeny groups were selected, to make 18 full-sib progeny lines, which were then communally stocked by mixing 125 larvae from each line and grown in nursery, rearing ponds, and grow-out ponds. The control group was maintained through stocking larvae from all the mated pairs.

During the breeding season of 1998, when all the F₁ generation progenies were matured at the age of 10 months, the 20% largest and heaviest females and males were mass selected and held separately in earthen ponds until they were used to produce the next generation in the breeding program. In the process of individual selection or mass selection with respect to sexes, empirical assessment of the breeders was always followed on the basis of their large size, good health and shape, and shiny colors. The production of F₂ generation of silver barb was carried out by pool mating of at least 150 pairs of mass-selected breeders and 50 pairs of nonselected control breeders in separate spawning arenas. The mating operations of both selected and nonselected breeders were completed within 2–3 days, involving 40–60 pairs per batch.

During the breeding season of 1999, further mass selection was carried out among all of the mature F₂ crossbred fish. The 15% best females and males were selected and used for producing the next generation. About 182 pairs of mass-selected breeders and 50 pairs of nonselected control (BxB) breeders were separately pool mated to produce the F₃ generation of progeny.

During the breeding season of 2000, mass selection was carried out among the mature F₃ crossbred fish, and the 10% best females and males were selected for producing the F₄ generation.

During the breeding season of 2001, 2002, and 2003, respectively, the F₅, F₆, and F₇ generations were produced following the same mass selection protocol of breeding.

2.3.1.3 Evaluation of growth performance of improved generations produced from mass selection

A 7.5% genetic gain in growth performance was attained by the F₁ selected group over the nonselected control group. The F₂, F₃, F₄, F₅, F₆, and F₇ selected groups attained 2.3%, 12.1%, 6.3%, 2.5%, 1.2%, and 0.1% cumulative weight gain, respectively, over six generations (Fig. 3.3 [10]). The average gain per generation across seven generations of selection for growth performance in weight was estimated as 4.57%. Under this mass selection program, the improved seventh generation of silver barb showed 32% superiority in growth rate over the nonselected strain of silver barb in Bangladesh.

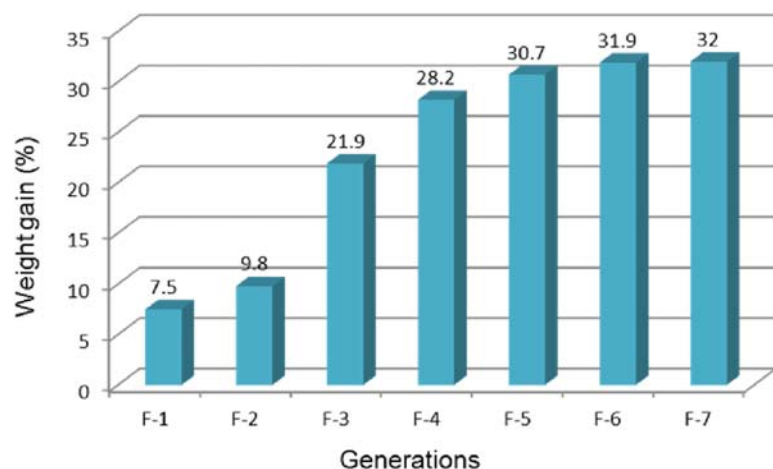
2.3.2 Genetic improvement of silver barb using family selection techniques

The rate of genetic gain in silver barb was greater up to fourth generation and the trend was slower for the following generations while using the mass selection technique. The reason behind such a less growth might be the accumulation of inbreeding. Therefore, the genetic improvement strategy for silver barb was redesigned under the second phase of ADB-funded project “Achieving Greater Food Security and Eliminating Poverty by Dissemination of Improved Carp Strains to Fish Farmers” since 2004 using family selection protocol. In that program, fish were individually identified, full pedigrees were maintained, and BLUP selection procedures were implemented [9,10]. This breeding program was run until further three generations through family selection protocol. The improved third generation of silver barb showed 36% increased body weight in comparison to the local strain of Bangladesh.

2.3.2.1 Breeding protocol and production of new generations through family selection

The brood fish used as base population from the seventh generation of silver barb descended from mass selection experiment of the first phase of the carp project “Genetic Improvement of Carp Species in Asia” [9]. In this program, during June–July 2004, a total of 114 full-sib families were generated followed by single pair mating design of breeding. The newly produced F₁ generation larvae of each family were kept separately in the primary, secondary, and tertiary hapas by reducing their stocking densities as well as full-sib family numbers and reared up to tagging size (10–15 g). The fingerlings were finally harvested for PIT tagging. During March–April 2005, 50 fingerlings from each of 50 families were PIT tagged and stocked in an 1800-m² pond and 10 fingerlings from each of 50 families were PIT tagged and stocked in another 800-m² pond for communal rearing. After 5 months rearing (until September 2005), all the fish were harvested and the final body weight, sex, and tag number of individual fish were recorded. Sex-wise growth data of individual fish belonging to the 50 families were reckoned and tabulated. These data were analyzed by incorporating statistical package SAS and ASR email software to EBVs and ranking the fish to prepare mating list for further breeding programs. EBV of

FIGURE 3.3 Cumulative genetic gain (%) by weight of mass-selected groups over the nonselected groups in different generations of *Barbonymus gonionotus*.



individual fish was ranked and two lines were established, one line was selected based on highest EBV (called the selection line) and the other was selected based on average EBV of the population (called the control line). The mating lists were prepared for the selection line (104 pairs including both male and female breeders) and control line consisting of 55 pairs. The EVB for female and male in the selection line ranged from 0.05 to 13.5 and 0.49 to 5.46, respectively, whereas the values ranged to zero in the control line. For selection line, a total of 92 females and 90 males were kept in conditioning cages, and for control line, a recovery of 42 females and 47 males was made [9,10].

The next breeding program was initiated during the second week of July to the first week of August 2006. The same protocol of 2004 was followed to generate F₂ full-sib selection and control lines [9]. A total of 75 full-sib families of selection line and 37 full-sib control lines were produced using breeding of single pair mating. For nursing and rearing of families, the newly produced F₂ generation larvae of each family were kept separately in the primary, secondary, and tertiary hapas by reducing their stocking densities and reared up to tagging size (10–15 g). A total of 60 full-sib families of selection line and 24 full-sib families of control line were maintained and nursed in the secondary and tertiary hapas. The F₂ fingerlings were harvested and identified family wise for PIT tagging in similar ways during March 2007 and stocked for communal rearing until June 2007. In this breeding program, in each generation, a control group was maintained by mating individuals with average breeding value and progeny was compared with that of selected individuals with the highest breeding value. At harvesting, the final body weight, sex, and tag number of individual fish were recorded both for select line and control line. These data were analyzed statistically accordingly to estimate the breeding value (EBV) of all the progeny groups. The mating lists were prepared for the selection line consisting of 50 pairs and control line of 20 pairs to produce F₃ generation fish.

During the entire month of July 2007, next-generation breeding program was performed following similar protocols of producing previous generations to generate F₃ full-sib selection and control lines. In the newly produced F₃ generation, 50 full-sib families of selected line and 20 families of control line larvae/fries were kept separately in the primary, secondary, and tertiary hapas by following the same rearing protocols of previous generations. The fingerlings (10–15 g) were finally harvested for PIT tagging during March 2008 and stocked for communal rearing until June 2008. At harvest, all the fish were harvested and the final body weight, sex, and tag number of individual fish were recorded. In these breeding programs, as mentioned earlier, in each generation, a control group was maintained by mating individuals with average breeding value and progeny was compared for growth evaluation with that of selected individuals with the highest breeding value.

2.3.2.2 On-station evaluation of growth performance of improved generations produced from family selection

In this family selection trial of silver barb, growth evaluation of improved generations was carried on station at communal grow-out ponds and cumulative genetic gain (%) at harvest weight was estimated. It was observed that the new F₁, F₂, and F₃ selected groups attained, respectively, 33%, 35%, and 36% weight gain over control group (Fig. 3.4). This trend of improved growth has some high significant impact for quality brood stock development in the carp hatcheries as well as is promising for further genetic improvement for growth and other specific trait(s) in silver barb (Fig. 3.5).

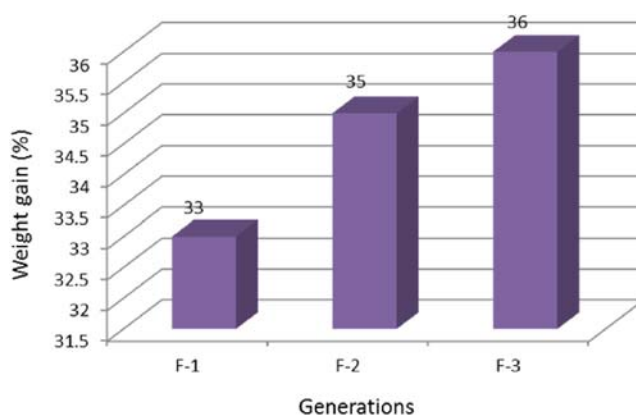


FIGURE 3.4 Cumulative genetic gain (%) by weight of selected groups over the nonselected groups of *Barbonymus gonionotus* across the three generations of family selection.

FIGURE 3.5 Bamboo basketful of generically improved silver barb, *Barbonymus gonionotus*, in Bangladesh. Photo: Dr. M. Gulam Hussain.



2.3.3 On-farm evaluation of genetically improved silver barb with other carps at different agroecological zones in Bangladesh

Mean harvesting weight of silver barb in different agroecological zones such as Trishal, Muktagacha, Paikgacha, and Parbatipur are presented in Fig. 3.6. In case of genetically improved strain of silver barb, the harvested weight was $149.37 \pm 16.01\text{g}$, $168.6 \pm 18.06\text{g}$, $198.00 \pm 14\text{g}$, and $230.00 \pm 9.25\text{g}$, whereas the harvested weight of local strain of silver barb was $113.47 \pm 15.52\text{g}$, $136.28 \pm 20.66\text{g}$, $170.00 \pm 17\text{g}$, and $205.00 \pm 12.29\text{g}$ for Trishal, Muktagacha, Paikgacha, and Parbatipur, respectively. Local strain showed less weight gain because the brood stock being used by the hatchery operators might be genetically deteriorated or inbred. However, the BFRI-improved stock showed superior growth rate due to its additive genetic gain attributed through several generations of selection.

2.3.4 Stock improvement of silver barb using chromosome manipulation and sex inversion techniques

In silver barb, *B. gonionotus*, female fish is faster than males; therefore, mass production of all-female population is desirable for the benefit of aquaculture [14]. In view of developing and producing all-female silver barb using the techniques of gynogenesis and sex reversal, the FS of BFRI, Mymensingh, participated in a collaborative project with the University of Stirling, Scotland, and the National Aquaculture Genetics Research Institute (NAGRI), Thailand.

It had previously been demonstrated that the meiotic gynogens of this species were all female, suggesting female homogamety (bearing XX genotype) [27]. The protocol for the next step in such an approach was to produce neomales

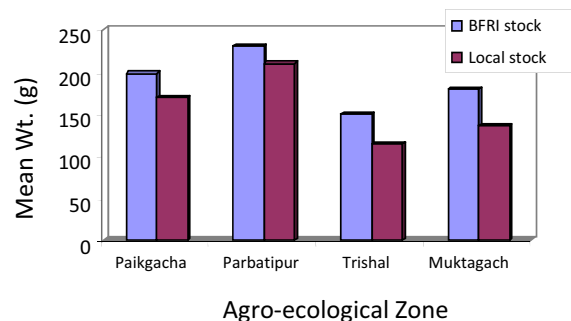


FIGURE 3.6 Mean weight of genetically improved silver barb strain and existing local strain in four on-farm locations in Bangladesh.

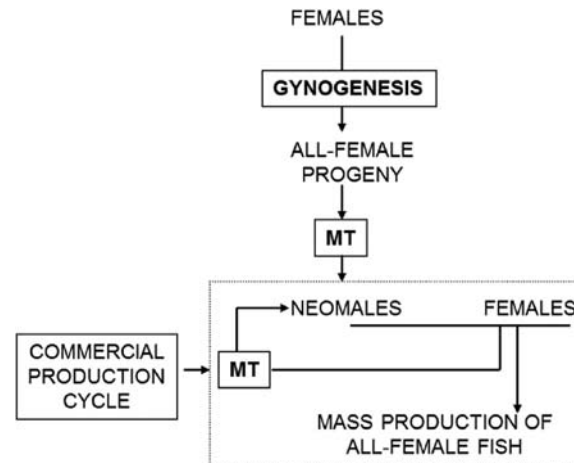


FIGURE 3.7 The protocol for commercial production of producing all-female silver barb using the technique of gynogenesis and sex reversal.

(phenotypic male having XX genotype) by feeding androgen hormone—treated feed to the gynogenetic fish. These neomales could then be crossed with normal females for mass production of all-female seeds of silver barb (Fig. 3.7).

Thus, for the production of all-female silver barb, *B. gonionotus*, along with large-scale neomale, research was conducted using imported neomales from NAGRI, Thailand, in view of mating them with females belonging to wild germplasm of Thai and Indonesian stocks. Moreover, production of additional batches of neomale through gynogenesis and sex reversal was initiated [28].

At FS, BFRI, a study was also conducted on the sexual dimorphism for weight in *B. gonionotus*. This was initially done through sampling of different populations from different culture systems. The sexual dimorphism index for weight (SDIW) was determined by the ratio of mean weight of females to the mean weight of males within one such sample. The SDIW values for weight in *B. gonionotus* at the age of approximately 1 year were found to range between 1.1 and 1.7 in different populations and in different culture systems. Proximate composition of the carcass of female and male fish was also compared, where insignificant ($P > .05$) differences were found in the case of moisture, protein, and ash content; however, fat showed a significant difference ($P < .05$) between the sexes [29].

2.4 Molecular studies for population genetic structure in indigenous major carps and detection of hybridization in exotic major carps

2.4.1 Population structure study in indigenous major carps

Studies on population genetic structure of three major river populations of catla, *C. catla*, and rohu, *L. rohita*, using microsatellite DNA markers have recently been conducted in Bangladesh, respectively, by Alam et al. [30,31]. In case of catla, Alam and Islam [30] developed eight microsatellite loci (Cc6, Cc7, Cc8, Cc9, Ccat C3, Ccat A12, Ccat G1, and Ccat G2) and used to analyze the genetic variation in three river populations, viz. Halda, Jamuna, and Padma rivers, and one hatchery used population. They observed that seven of the eight loci analyzed were polymorphic in all the populations. In particular, they found that locus Ccat G2 had the highest numbers of alleles (8), while the locus Cc9 had the lowest (2). Among the four populations, average numbers of alleles were observed and differences were found in heterozygosity; no difference was observed in proportion of polymorphic loci (P_{95}) among the populations. Due to the deficiency of heterozygosities, all the studied populations deviated from Hardy–Weinberg equilibrium proportions at a number of loci. A low level of population differentiation (F_{ST}) was observed among populations; however, significant differentiation was evident only between the Halda and hatchery populations. Alam et al. [35] finally concluded and revealed in their study that there was a relatively low level of genetic variation at microsatellite loci within and between catla river populations, and hatchery population even showed genetic variation lower than the river populations. In case of rohu, Alam et al. [31] used and analyzed four dinucleotide microsatellite loci to study and detect population genetic structure of rohu, *L. rohita*, collected from three major rivers, viz. Halda, Jamuna, and Padma rivers in Bangladesh. In all the studies populations, the four loci were polymorphic (P_{95}). Among all the loci analyzed, the populations varied in the number and frequencies of alleles as well as heterozygosities. They observed population differentiation (F_{ST}) value between the Halda and the Jamuna populations, which was significant ($P < .05$). Between the Padma and the Jamuna populations, relatively high level of

gene flow and low level of F_{ST} values were observed. Two different clusters were found between Halda and Jamuna population while genetic distance was plotted and measured using the unweighted pair group method with averages dendrogram. Finally, the results also revealed a relatively low level of genetic variability in the river populations of rohu in Bangladesh.

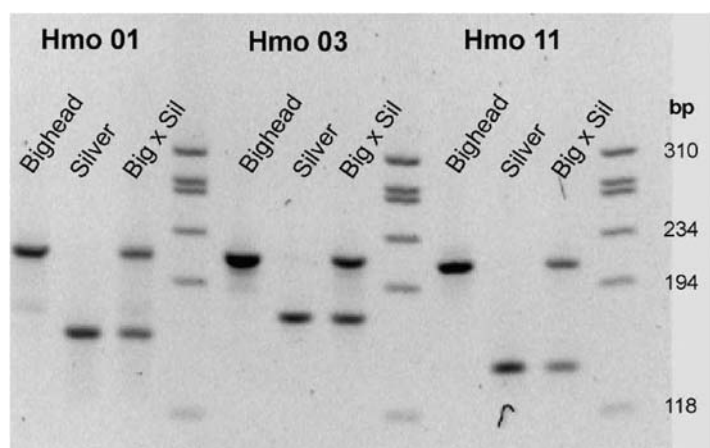
Information obtained from the above studies regarding population structure of the two important river populations of indigenous major carp species will certainly be very useful tools for the scientists and hatchery management workers who are planning and working on their genetic stock improvement program for supporting the promotion of aquaculture in Bangladesh.

2.4.2 Detection of hybridization using DNA microsatellite loci in Chinese major carps

Hybrid introgression is thought to have occurred in some Chinese major carp populations in Bangladesh. Hybridization between silver carp, *H. molitrix*, and bighead carp, *Aristichthys nobilis*, has been reported to occur fairly frequently in commercial hatcheries [18]. To detect hybridization between these two species, a survey was designed and conducted, in which fin samples and information about hatchery management techniques were collected from the reference populations at the Department for International Development–funded Northwest Fisheries Resource Development and Management Project (NFRDMP), Parbatipur, Dinajpur, of Department of Fisheries (DoF) and several hatcheries in each of the five major hatchery areas, viz. Mymensingh, Bogra, Chandpur, Jessore, and Comilla [32].

Microsatellite markers or loci have been developed for silver carp from a stock held by the NFRDMP. Both silver carp and bighead were held there and originated from direct importation from Yangtze River wild population in China. These stocks might have been hybridized due to inferior and inconsistent management practices. Preliminary report showed that some these loci could be used as species-specific markers to distinguish between silver carps and bighead carp. According to Mia et al. [32], the names assigned to the microsatellite loci were derived from the Latin name of the species, *H. molitrix*, and a serial number. Four loci were initially identified as potentially being diagnostic for the silver carp and bighead carp, but one of these, i.e., *Hmo5*, was discarded as it was giving an inconsistent, multiple banding pattern in particular for bighead carp. Three remaining loci, viz. *Hmo1*, *Hmo3*, and *Hmo11*, were used for that study. Tissue samples were collected and removed from the edge of a fin using scissors of either silver carp or bighead carp (those were physically recognized by the hatchery technicians and workers) and placed on tissue paper to remove excess water or mucus and kept into a microcentrifuge tube containing approximately 1 mL of 95% ethanol. The ethanol solution was discarded and replaced later the same day. During transportation, the samples were stored at ambient temperature and at 4–6°C in the laboratory until DNA extraction and analysis. In the NFRDMP hatchery at Parbatipur, the deliberate hybrid crosses of silver carp and bighead carp were produced and samples were collected and fixed the same way as the fin samples and analyzed. The DNA was extracted using a protocol based on either phenol or chloroform [33] of Chelex resin [34] and three loci were amplified by the polymerase chain reaction (PCR) in separate reactions to estimate allele sizes in selected samples and to confirm results from agarose gels [32]. The analysis revealed that 8.3% of 422 hatchery brood stock that were morphologically identified as silver carp had bighead carp allele(s) at one or more of the three microsatellite loci, while 23.3% of the 236 fish morphologically identified as bighead carp had silver carp allele(s) at one or more loci (Fig. 3.8). The results suggested that while some of these fish might be F1 hybrids, others had more complex genotypes,

FIGURE 3.8 Electrophoresis of polymerase chain reaction—amplified microsatellite loci *Hmo1*, *Hmo3*, and *Hmo11* shows an example of pure *Hypophthalmichthys molitrix*, pure *Aristichthys nobilis*, and hybrid (Big x Sil) genotypes for each locus. The righthand lane of each set contains molecular weight standards, with the sizes in base pairs (bp) given at the extreme right of the figure.



suggesting further generations of hybridization or introgression between the species in hatcheries, with potentially damaging consequences for the integrity of these stocks and their performance in aquaculture.

3. Breeding plans and generic stock improvement of tilapia species

3.1 Background information

During 1999 to 2019, there has been a tremendous progress in tilapia farming in Bangladesh. It has been reported that Bangladesh was ranked third after China and Indonesia among the top eight tilapia-producing countries in the Asia–Pacific region [35,36]. Production increased from 2140 tonnes in 1999 to 370,000 tonnes in 2019 [37]. Meanwhile, >500 tilapia hatcheries and >20,000 small, medium, and large commercial tilapia farms have been established all over the country. These hatcheries are producing >5.0 billion monosex fry every year. The majority of these fry are used for commercial farming in the country, and there is a report that >150 million tilapia fries and fingerlings are sold to farmers in the neighboring countries such as India and Nepal [36]. In Bangladesh, tilapia farming was not popular before 1993, but it got momentum immediately after the introduction of GIFT strain, *Oreochromis niloticus*, under the “Dissemination and Evaluation of Genetically Improved Tilapia in Asia (DEGITA)” project of WorldFish Center (formerly ICLARM), a systemic strain of *O. niloticus*, in 1994, from the Philippines [38]. The GIFT was developed by WorldFish Center through several generations of selection from a base population involving eight different strains of Nile tilapia [39,40]. BFRI received the first batch of GIFT strain in 1994; second batch in 1996; and third batch in 2005 through WorldFish Center (formerly ICLARM). In on-station and on-farm trials of BFRI, the first batch of GIFT strain was reported to show 35%–57% superior growth than that of the existing strain of the country [41]. Meanwhile, GIFT strains were extensively disseminated among the private tilapia hatchery owners and farmers for commercial seed production and farming in the country.

3.2 Genetic stock improvement of GIFT strain at Bangladesh Fisheries Research Institute

Nevertheless, in private hatcheries, repeated use of the same parent population led to poor brood stock management. Additionally, no new or poor brood stock replenishment resulted in the production of inferior quality seeds and genetic drift in hatchery population inhibiting sustainable tilapia production. To mitigate the existing situation, during 1999–2004, BFRI conducted an individual or mass selection research using 116 families of GIFT introduced as the second batch. Through mass selection, six generations (G_1 to G_6) of improved GIFT strain were produced. Through mass selection technology, the G_6 generation of GIFT strain achieved 3.7% harvest weight gain over the existing nonselected GIFT strain. The rate of genetic gain in weight of fish was greater up to third generation, but it decreased gradually until sixth generation. The reason behind such a decrease in genetic gain in particular for body weight might have been the accumulation of inbreeding.

Under the DEGITA project, BFRI received further 30 families of third batch of GIFT strain in 2005 from WorldFish Center, Malaysia, which led to the development and continuous improvement of strains by genetic selection at the institute. Therefore, the genetic improvement strategy for GIFT was redesigned, and during 2007–2019, BFRI conducted a well-designed family selection research at BFRI’s closed tilapia breeding nucleus (TBN). In this study, relative to the base population, the gain achieved after 13th generation of family selection was 64.44%, averaging 4.95% weight gain per generation.

In view of supporting huge demand of genetically improved GIFT brood stock for quality seed production, during 2012–16, WorldFish, Bangladesh and South Asia, joined to establish and run seven outdoor TBNs at private sector tilapia hatcheries in Bangladesh [36].

3.2.1 Genetic improvement of GIFT strain using mass selection techniques

3.2.1.1 Development of base population

During the breeding season (March–July of 1998), a base population of the GIFT strain was formed through pool breeding of equal number of males and females taken from 116 families in a breeding pond (1000 m²). After breeding, required numbers of fingerlings were collected for nursing and rearing by using supplemental feeding until the size of sexual maturity (60–70 g) was attained.

3.2.1.2 Further stock improvement through mass selection

Among the base population, 400 individuals, 250 females and 150 males, were randomly taken for mating in breeding pond to produce G₁ generation (1999). After first spawning, 1260 individuals of G₁ progeny were collected from the breeding pond, reared, and sexed as 920 females and 340 males. The largest and heaviest individuals of F₁ generation to the descending order at 10% of each sex were selected on the basis of their fast and higher growth, good look and shape, and having shiny color and disease resistance and stocked in a breeding pond for the production of G₂ generation (2000). The same procedure of selection and breeding protocols was followed for the production of G₃, G₄, G₅, and G₆ generations, respectively, in the years 2001, 2002, 2003, and 2004.

3.2.1.3 On-station evaluation of growth performance of improved generations of GIFT strains produced from mass selection

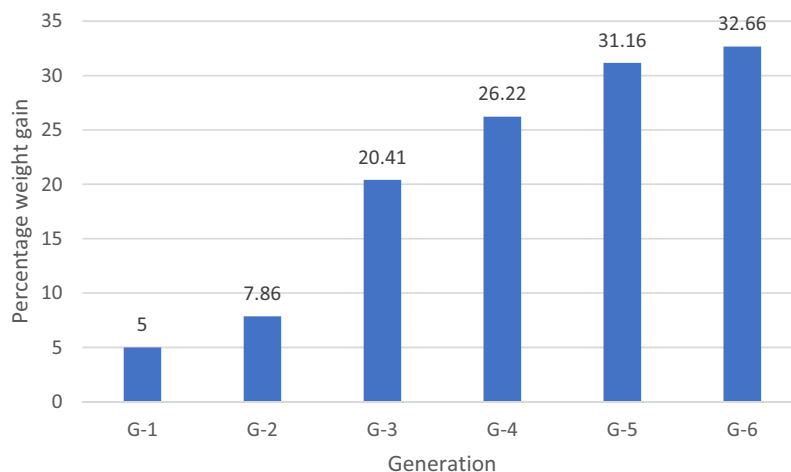
On-station evaluation of growth performance was carried out through comparative trials between selected and nonselected average existing stock of GIFT strains [38]. The progeny of each generation was evaluated in comparison to the average population of the GIFT in both cistern (2.0 m³) and pond hapa (2.0 m³) for the period of 5 months. In both the cases, there were two treatments with three replicates at a density of 5 fish/m³. The fish were fed twice a day with rice bran (80%) and mustard oil cake (20%) at recommended body weight. Fortnightly sampling was done to adjust the feed allowances and also to observe the health condition of the fish. After 5 months, in each generation, both the experiments were terminated. In these evaluation trials, a 5% genetic gain in growth performance was attained by the G₁ selected group over the nonselected average group of GIFT strain. The G₂, G₃, G₄, G₅, and G₆ selected groups attained 7.86%, 20.41%, 26.22%, 31.16%, and 32.66% weight gain, respectively, over six generations (Fig. 3.9). The average gain per generation across six generations of selection for growth performance in weight was estimated as 5.44%.

3.2.2 Genetic improvement of GIFT strain using family selection techniques

3.2.2.1 Origin of founder stock, tagging, and communal rearing

A new founder stock comprising 30 families having 300 individuals of new generation of GIFT strain has been further introduced from Malaysia through WorldFish Center in March 2005. The founder stocks were tagged by using PIT and reared in a 100-m² hapa for 3 months. After these periods, the tagged fish were stocked for communal rearing in a pond (1000 m²) and fed with supplementary feed 6 days in a week (28% crude protein) at the rate of 4%–5% of estimated biomass. Fish sampling was made at fortnightly interval to assess the growth and feed adjustment. Water was supplied once in a week to maintain water depth at 1.0 m. The pond was fertilized fortnightly with urea and triple superphosphate (TSP) at the rate of 12.5 and 25.0 kg/ha, respectively. After 4 months of rearing, the fish were recaptured through seine netting and pond drying. The final body weight, sex, and tag number of all harvested fish were recorded. In view of female and male GIFT strain family selection, EBVs were estimated for individual fish in a full pedigree.

FIGURE 3.9 Weight gain (%) of mass-selected group of genetically improved farmed tilapia (GIFT) strain over nonselected average GIFT strain across the six generations.



3.2.2.2 Breeding protocols and production of new generations through family selection

For the production of G_1 generation, the best 40 males were crossed with 40 best females (from 30 families). The breeders were selected on the basis of EBV of the founder stock. In the breeding pond, 40 breeding hapas (1.0 m^3) were set up with bamboo poles. A pair of female and male breeders (1:1) was stocked in each breeding hapa for mating. After 12 days of stocking, fertilized eggs were collected from brooding females. Subsequently, collected eggs were transferred to the hatchery for incubation. Immediately after hatching, the larvae were shifted to a series of trays and were kept until their yolk sac resorption stage. From each family, 300 fries were transferred to 40 individual fine mesh nursery hapas (2.0 m^3) already set in the pond. The progeny was fed with nursery feed containing 30% protein @30% of estimated body weight. The mean weight of the fry was $2.80 \pm 0.42\text{g}$ after 45 days of nursing. Subsequently, in each progeny group, 150 fries were transferred to 40 individual rearing hapas (2.0 m^3 in size). At first sexual maturity of the reared fish, from each progeny group, 25 male and 25 female fish were selected and tagged using PIT. Tagged fish from 40 families (2000 fishes) were stocked in a pond having 1000 m^2 area for communal rearing in July 2007. Supplementary feed (25% crude protein) was administered regularly @6% of estimated biomass. The fish were harvested and tag number, weight, sex, and body depth were recorded after 6 months of grow out in the pond. Then, EBVs of G_1 generation were estimated from the complete data set. Side by side, the nonselected GIFT population (200 breeders) was stocked in a 300-m^2 pond for pool breeding. Fry, fingerling, and grow-out fish were reared separately to continue generation-wise breeding stock maintenance as well.

For the production of G_2 generation, the best 60 males were crossed with 60 females of G_1 generation (selected on the basis of EBV). For breeding, 60 breeding hapas (1.0 m^3) were set up in a pond. A pair of female and male breeders (1:1) was stocked in each breeding hapa. After 21 days of stocking, 300 larvae from each progeny group were shifted to a series of hapas in a pond. The progeny was fed with nursery feeds with the quantity used at G_1 fry nursing. After 30 days of nursing, the mean weight of the fry was $2.46 \pm 0.81\text{g}$. From each progeny group, 150 fries were transferred to 70 individual 2.0-m^3 size rearing hapa. Supplementary feed was applied in all the hapas @10%–15% of estimated biomass. From each of the progeny groups, 20 males and 20 females were PIT tagged. Tagged fishes from 60 families (2400 fishes) were stocked in a pond (1000 m^2) for communal rearing in July 2008. Supplementary feed was supplied regularly at the same rate used at G_1 grow-out fish rearing. After 4 months of rearing, fish were harvested and tag number, weight, sex, and body depth were recorded. Then these data were analyzed through statistical analysis for EBV estimation.

More or less similar breeding protocols and family selection procedures were used for the production of G_3 , G_4 , G_5 , G_6 , G_7 , G_8 , G_9 , G_{10} , G_{11} , G_{12} , and G_{13} generations (2009–2019).

3.2.2.3 On-station evaluation of growth performance of improved generations of GIFT strains produced from family selection

Comparative trials were conducted for evaluation of growth performances in each generation of genetically improved GIFT strains through family selection group and nonselected group of GIFT strains for 5 months during June–October each year from 2007 to 2019. Three replicated ponds were selected and used, having an area of 1000 m^2 . Every time, prior to the initiation of evaluation trials, the ponds were cleaned and limed at the rate of 250 kg/ha . After 3 days of liming, ponds were fertilized with urea and TSP at the rate of 12.50 and 25.0 kg/ha , respectively. In each generation trial, both genetically improved GIFT strain fries (average weight $>4.5\text{ g}$) and nonselected group fries (average weight $>4.5\text{ g}$) were stocked together for communal rearing. In each group, equal stocking density (i.e., 600 selected + 600 nonselected, total = 1200 fry) was maintained. Fries of genetically improved population of GIFT strain were tagged only using PIT tags. After stocking, fries were fed initially with nursery feeds containing 28% crude protein. Fries were sampled at fortnightly interval to assess the growth as well as for adjustment of feeding rate. During the first month, fries were fed @10% of estimated body weight, and in the consecutive months, feed ration was reduced to 8%, 6%, 4%, and 3% in second, third, fourth, and fifth month, respectively. After 5 months of rearing, fish were harvested through repeated netting followed by pond drying. During the harvesting time of each experimental pond, group-wise tag numbers, individual weight, sex, body depth, etc., were recorded.

The average harvest body weight of the genetically improved population was 191.8 g . The survival rate was rather high over the grow-out period of 5 months (92.6%). In these evaluation trials, genetic gain was measured as EBVs for each generation of selection. A 7.17% genetic gain in growth performance was attained by the G_1 selected group over the nonselected group of GIFT strains [12,42]. Relative to the base population (nonselected), the gain achieved after 13 generations of selection was 64.44%, averaging 4.96% per year or generation (Fig. 3.10). After many generations of selection, GIFT strain has become a “super GIFT strain” for its sustainable great performances all over Bangladesh (Fig. 3.11).

FIGURE 3.10 Genetic gain (%) achieved from the family selection program for harvest weight of selected groups over the nonselected groups (i.e., base population) of genetically improved farmed tilapia strains across the 13 generations.

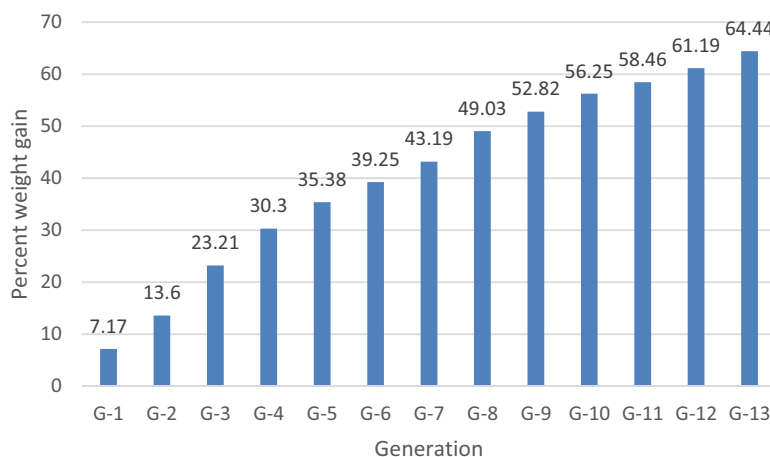


FIGURE 3.11 Farm yard display of genetically improved superstrain of genetically improved farmed tilapia, *Oreochromis niloticus*, in Bangladesh. Photo: Dr. M. Gulam Hussain.



3.2.3 Maintenance of rotational breeding of genetically improved strain of GIFT strain at the outdoor TBNs

From 2012 to 2016, WorldFish, Bangladesh and South Asia, established a number of TBNs under RFLDC/DANIDA, USAID Aquaculture Income for Nutrition (AIN), and CSISA-BD projects. The base population having 56 families of 11th generation of the improved GIFT strain was collected from Jitra Station, Malaysia. A total of seven TBNs were successfully established and run in seven small-, medium-, and large-scale commercial private hatcheries in Jashore, Narail, Bagerhat, Barisal, Rangpur, and Mymensingh regions of the country [13,36]. Both selective breeding and rotational breeding techniques have been applied in these TBNs to produce generation-wise improved GIFT fry. In this case, individual selection or mass selection protocol was followed and 10%–20% of best fish by weight, color, shape, etc., were considered for matting in each generation. Only selected males were rotated during odd and even generations among eight cohort (combination of unrelated seven families in each cohort) breeders (Fig. 3.12 and Fig. 3.13). In 2016, a total of 2.36 million improved GIFT fries were produced and distributed/sold from these TBNs among 47 tilapia hatcheries in Rangpur, Jessore, Narail, Faridpur, Khulna, and Barisal regions. In 2017, all these TBNs produced >3.0 millions of improved GIFT

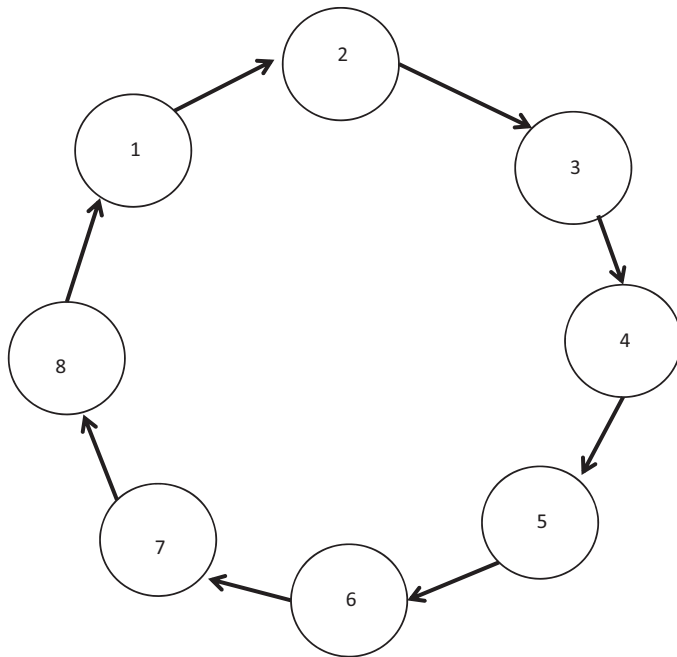


FIGURE 3.12 Rotation of genetically improved farmed tilapia males for generations 1, 2, 3, and so on.

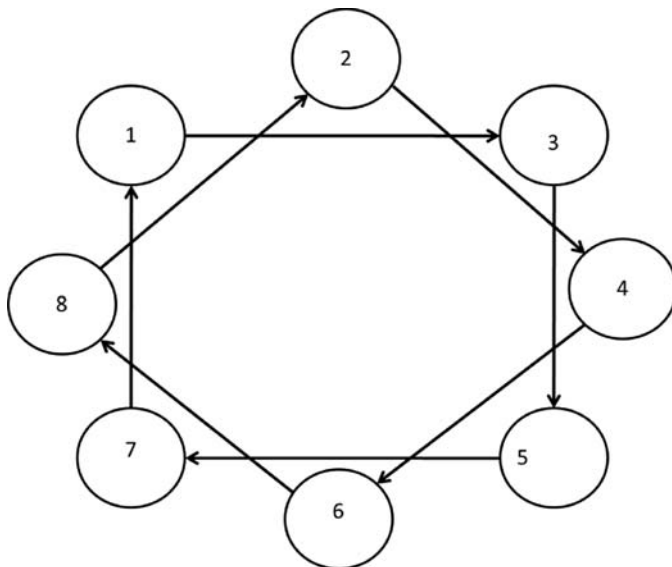


FIGURE 3.13 Rotation of genetically improved farmed tilapia males for generations 2, 4, 6, and so on.

fries and those were distributed among 70 multiplier tilapia hatcheries in southern, northern, and greater Mymensingh regions as well as beyond. Afterward, these private TBNs owners have been well trained and build up their technical capabilities and used to run the systems independently. One of such successful TBNs has been operated by Eon Aquaculture Ltd. at its two well-established tilapia hatcheries in Muktagacha, Mymensingh, and Subarnachar, Noakhali, after signing an MoU with WorldFish, Bangladesh and South Asia, in late 2016. Eon Aquaculture Ltd. has isolated TBN complexes at its vast hatchery compounds just to maintain properly selective breeding (i.e., mass selection) plus rotational breeding protocol each year. It is reported that these two TBNs are producing about 1.5–2.0 million improved GIFT germplasm and selling/distributing >100 tilapia hatcheries all over Bangladesh.

4. Impacts of genetically improved aquaculture strains in aquaculture

In Bangladesh, genetically improved strains have, meanwhile, impacted greatly in some species, viz. rohu, silver barb, and tilapia, in aquaculture production. The introduction of the GIFT strain of Nile tilapia (*O. niloticus*) provided in 1994 by WorldFish (formerly ICLARM) under the then DEGITA project and a second introduction in 2005 from Malaysia led to the development and continuous improvement of strains by genetic selection at BFRI. Since 2007–2019, BFRI already developed 13 generations of genetically improved strains of GIFT. In comparison to the base population, the gain achieved after the 13th generation of family selection was 64.44%, averaging 4.95% weight gain per generation. This was followed by dissemination of these improved strains in each generation and adoption of low cost and appropriate breeding and aquaculture technologies in a large number of tilapia hatcheries and farms throughout Bangladesh, which has shown huge impacts on tilapia production. The Food and Agriculture Organization [35] reported that Bangladesh was ranked third after China and Indonesia among the top tilapia-producing countries in the Asia–Pacific region. Within a span of 15 years (2005–19), tilapia production increased more than 20.5 times in Bangladesh, from 0.019 million tonnes in 2005 to 0.37 million tonnes in 2019 ([37], Fig. 3.14).

Inland aquaculture in Bangladesh mostly revolves around cultivation of endemic carps (viz. rohu, *L. rohita*; catla, *C. catla*; mrigal, *Cirrhinus cirrhosis*; calbasu, *Labeo calbasu*; and some minor carps) and exotic major carps (viz. silver carp, *H. molitrix*; grass carp, *C. idella*; common carp, *C. carpio*; and silver barb, *B. gonionotus*). Currently, annual total aquaculture production (2019–20) is 2,583,866 metric tonnes (mt) in the country, among which carp species are playing a dominating role (65.39%). The contribution of rohu is the highest, 17.28% (446,483 mt), followed by silver carp, 10.42% (269,295 mt); catla, 10.00% (258,497 mt); mrigal, 9.94% (257,069 mt); common carp, 6.88% (177,740 mt); and silver barb, 3.82% (98,565 mt). Other species like grass carp, bata, gonia, calbasu, and other exotic carps are contributing >3% in aquaculture production (Fig. 3.15 [5]). It is roughly estimated that if available generically improved strains of rohu are widely used for farming, then about 80,000–115,000 mt of fish can be added in the production figure in the near future. However, the contribution of silver barb in aquaculture was almost nil before year 2009–10. Afterward, while genetically improved silver barb strain were developed and released by BFRI, the species gradually was gaining popularity due to its short-cycled and fast-growing characteristics and farming practices maintained by the rural farmers either at monoculture or polyculture systems mostly in seasonal water bodies. Within a span of 8 years (2013–20), silver barb production increased more than 38,000 mt in Bangladesh, from 60,540 mt in 2013 to 98,565 tonnes in 2020 [5].

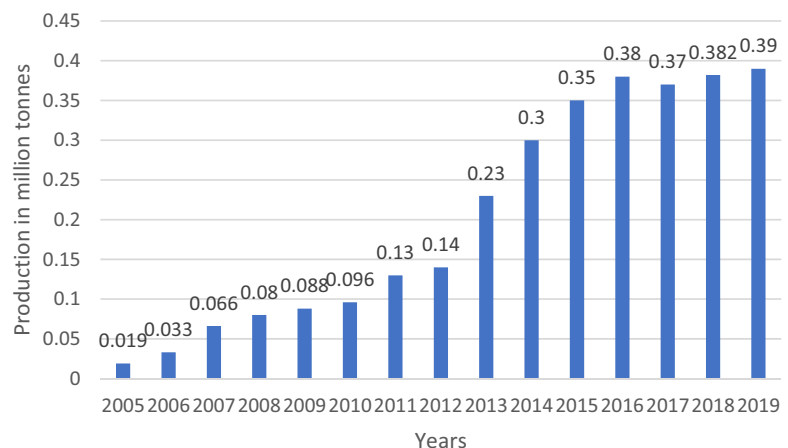
5. Major strategies, challenges, and way forward

5.1 Strategic guidelines

5.1.1 Institutional framework

The development of a strong aquaculture breeding plan and genetic research in Bangladesh will initially require a strong coordination between the various stakeholders, from research institutions/universities to industry. An effective strategy in this regard needs to promote development and utilization of genetically improved breeds and products at three levels:

FIGURE 3.14 Tilapia production over the 15 years (2005–2019) in Bangladesh.



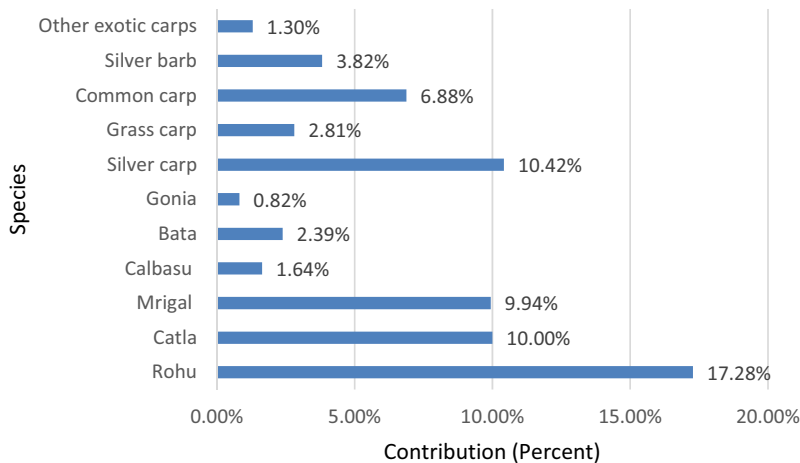


FIGURE 3.15 Species-wise contributions in annual aquaculture production (2019–20) in Bangladesh [5].

- Development of infrastructure for genetics and biotechnology research.
- Basic fish breeding and genetic stock improvement research and technology development.
- The commercialization and marketing of the improved breeds/products nationally and internationally.

A strong aquaculture breed(s) development center/industry, therefore, requires not only coordination between people and institutions but also a multidisciplinary approach involving scientists, economists, and business managers. Such an effective coordination can only be achieved through an institutional framework.

The main authority for implementing the fish breeding plan strategy will be the “Steering Committee on Aquaculture Breed Development and Dissemination Board (SCABDDDB) that needs to be established. The SCABDDDB will function under the directive of the Ministry of Fisheries and Livestock, Government of Bangladesh.” The SCABDDDB will approve for release and dissemination of any genetically improved aquaculture breed(s) developed by the research institutes/universities/private research institutions.

5.1.2 R&D priorities for fish genetics and biotechnology research

- Development of improved strains through selective breeding or combined breeding techniques
- Development of monosex population (all male or all female) using both sex inversion and chromosome-set manipulation techniques
- Production of genetically manipulated sterile fish
- Development of quantitative trait loci—specific molecular marker and marker-assisted selection for all major cultivated species
- Genome sequencing of all major cultivated species using SNP markers
- Cloning of genes of economic traits from fish and production of transgenic fish for enhanced growth and disease resistance
- Development of cryogenic gene banking for conservation of genetically improved strains and threatened species
- Development of PCR-based molecular technique(s) for rapid and effective diagnosis of infectious diseases
- Development of genetically engineered vaccines for protecting devastating pathogens

5.1.3 Capacity building for fish genetics and biotechnology research

- One important feature of fish genetics and biotechnology is that it is highly research-intensive and technically demanding. In most cases, such type of research requires well-equipped laboratories. There is no alternative of capacity buildup in laboratories conducting genetics and biotechnology research. The Government will fund for capacity building for fish genetics and biotechnology research at different research institutes and universities. Whenever necessary, the Government may explore the possibilities of obtaining foreign assistance to build up and upgrade the national capacity.

5.1.4 Dissemination strategy for improved carp breed(s)

The regional station(s) of BFRI, lead hatcheries of DoF, and Aquaculture Research Platform, WorldFish, Bangladesh, will act as carp breeding nucleus (CBN). The overall breeding programs like selective breeding and family testing will be performed at the nucleus unit.

Government Fish Seed Multiplication Farms and some selected private hatcheries having good hatchery, nursery, and grow-out facilities can function as satellite centers of the CBNs to produce large-scale improved breeds having increased rate of heritability and genetic variability of specific trait (i.e., growth) for distribution to multiplier stations. Satellite stations can also sell excess larvae, fries, and fingerlings to grow-out farmers.

Mostly, the selected private sector hatcheries will act as prime seed producers using improved breeds and become multiplier centers (MCs). The DoF will take responsibility to keenly guide and monitor the activities of multipliers. The MC will distribute the larvae and fries/fingerlings to a large group of grow-out producers/operators (the farmers including the commercial nursery owners).

Ultimately, the grow-out producers (i.e., the fish farms) of the country will be the last place of proving the genetic gain of the improved carp strain(s) attained by the systematic breeding program and equitable and effective dissemination of improved breeds for quality seed production for fostering sustainable production of aquaculture species. Improved strains/breeds production and dissemination model of carp species is shown in Fig. 3.16.

5.2 Challenges

- Lack of skilled and devoted aquaculture breeding and genetic specialists who sustain their long-time services
- Difficulties for careful isolation and maintenance of all breeding families and lines
- Difficulties for continuation of longtime breeding plan and genetic stock improvement programs for selected aquaculture species by the respective research institute/universities
- Lack of adequate laboratories and field facilities
- Poor on-station and on-farm trails and dissemination
- Lack of interinstitutional coordination

5.3 Way forward

Aquaculture breeding plans and genetic stock improvement programs are long-term processes. The sustainable breeding and genetic improvement require sequential steps to follow as below:

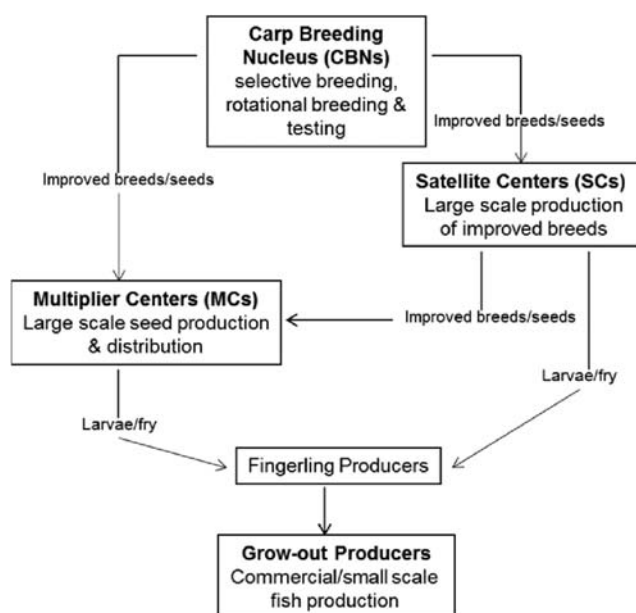


FIGURE 3.16 Improved strain/breed production and dissemination model of carp species.

- Species identification and prioritization
- Designing the breeding plan to address the technique(s), develop breeding and selection strategies, and identify the traits
- Selection of the testing and evaluation environment
- Recording and initiating the calculation of EBVs
- Developing the best breeding stocks on the basis of EBVs
- Production of subsequent generation(s)
- Testing and dissemination of genetically improved population among the target groups/institutions/industries

6. Conclusions

Genetically improved fish breeds have shown, so far, some positive impacts on aquaculture production in particular for salmon and tilapia production in Norway and Southeast Asian countries including Bangladesh, respectively. Some of such progresses have also been attributed in carp production in Bangladesh, China, India, and Vietnam with generically improved carp species like rohu, common carp, and silver barb. Bangladesh is one of the leading countries in Asia where freshwater aquaculture industry is booming due to the development of huge numbers of carp and tilapia seed production facilities recently (i.e., hatcheries and nurseries). In view of addressing inbreeding depression and genetic deterioration in hatchery population, meanwhile, breeding plan and genetic stock improvement research has been conducted and achieved most positive success to develop genetically improved breeds of most popular freshwater species of carps (i.e., rohu and silver barb strains) and tilapia (GIFT strain). Such genetically improved strains have been utilized and positively impacted for high-quality seed production to support sustainable aquaculture production in the country, which has been highlighted comprehensively in this chapter. As breeding plan and genetic stock improvement programs are continuous process, the respective national research institute like BFRI and respective public universities and international organization like WorldFish, Bangladesh and Southeast Asia, should continue their efforts for sustaining aquaculture genetics/biotechnology research with presently developed aquaculture breeds and include more other commercially important carps and other aquaculture species in Bangladesh.

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Application of molecular markers in aquaculture

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1. Introduction

The origin of genetic markers could be traced to the pioneering work of Alfred H. Sturtevant in 1913 in *Drosophila melanogaster* while he was still an undergraduate student in the laboratory of the famous biologist Thomas Hunt Morgan. Sturtevant produced the very first chromosome map in the fruit fly that included six sex genes (he called them sex-linked factors) along with their relative spacing, thereby laying the foundation and logic for genetic mapping [1]. Since then, considerable research has gone into constructing genetic maps in plants, animals, and fishes. It was only after electrophoretic studies were introduced in the 1960s that the search for markers started picking momentum. Initially, considerable work was carried out in plants, insects and animals especially with protein markers like allozymes, which were identified using starch gel electrophoresis. In the early 1990s and beginning of 2000, the polymerase chain reaction (PCR) took center stage, the work becoming relatively easier in unraveling polymorphism at the DNA level. The restricted fragment length polymorphism (RFLP) was first described while constructing a genetic linkage map in humans [2]. The random amplified polymorphic DNA (RAPD) as a molecular marker was introduced in the year 1990 [3]. The problem with RAPD was the lack of reproducibility, which was later overcome by another marker, the amplified fragment length polymorphism (AFLP) [4]. We also know about short tandem repeats or microsatellites which are quite abundant in genomes. In addition to these, we have the mitochondrial markers especially from select genes of the mitochondrial DNA, viz. 12S rRNA, 16S rRNA, cytochrome *b*, D-loop, and COI. Unraveling genetic polymorphisms using PCR entailed laborious electrophoretic work, and although markers could be identified, they were too less in number for covering the whole genome, which was one of the drawbacks of this study [5]. From late 2000, techniques like next-generation sequencing became center stage, which has helped in unraveling markers like single nucleotide polymorphisms (SNPs) that are found abundantly in the genomes.

Genetic improvement programs have been in vogue since long in plants, crops, and animals. It uses the conventional tools of breeding for selecting individuals for a particular trait or traits. The main idea of genetic improvement is selecting and breeding those individuals that exhibit higher performance in terms of growth or production so that their progenies also perform better. Selection is usually carried out for quantitative traits that show a continuous distribution and also have heritability estimates ranging from low to high. Once artificial selection is practiced, the gene frequency of the trait selected for, increases in the selected population. The increase that is obtained in one generation is carried over to the next generation, which is why selection has to be continuously carried out. In aquaculture, it is only in the early 1970s that selective breeding principles were first applied in Norway to rainbow trout in 1973 and Atlantic salmon in 1975 [6]. These programs have indeed come a long way since then and as on date have added disease resistance as well as several other traits like carcass quality too. The selective breeding programs in Norway stand out as a fine example of how fish can be bred for increased growth and improved disease resistance. A few other selective breeding programs in vogue are those in Pacific white shrimp, tilapia, and Asian sea bass. The change and reach that these programs have shown to the world is commendable. The Pacific white shrimp is now a major contributor to the world shrimp production, and the genetically improved farmed tilapia (GIFT) is produced and cultured in 14 countries across 5 continents [7]. The next-generation sequencing technologies that came up since the late 1990s have opened up a new vista for genetics research. The

information available post sequencing is immense, and it is up to the breeder to make maximum use of it in the breeding program. One such proposition was the utility of sequence information to be incorporated into the breeding program [8]. This concept was picked up by plant and animal breeders and the idea of using genomic information in selection was put into practice, the term being referred to as genomic selection. Usually in conventional selection, the information on the individual, pedigree, and relatives are used to estimate heritability, which would then be used to compute the breeding values. The breeders to be used as parents for the next generation are selected on the basis of the breeding values. Once genomic information in terms of DNA markers is incorporated into the breeding value, it is termed genomic breeding value [9]. Compared to the breeding value, the genomic breeding value is more robust in terms of sequence information incorporated, consequent to which we can expect higher genetic gains. The use of DNA markers makes the task of selecting parental lines more efficient [10] and enhances the genetic gains. Hence in essence, the genetic gains are accrued at a faster pace than when conventional selection is used. This could also be termed marker-assisted selection. Marker-assisted selection can be used for improving genetic gains in traits that exhibit low heritability, for example, resistance to white spot syndrome virus (WSSV) in shrimp. It has to be borne in mind that marker-assisted selection cannot be a substitute to conventional selection, but can only supplement it. In the following sections, an attempt has been made to discuss the molecular markers that have been unraveled in aquatic species with special emphasis on the Indian species. In addition, an effort has been made to showcase how genetic markers have enhanced the production of fish and shellfish globally.

2. Molecular markers in aquaculture

Before we delve deep into the utility of molecular markers, it would be pertinent to first comprehend what exactly is a molecular marker. Molecular markers can be defined as a heritable and observable variant of a gene or DNA sequence that is associated with a particular gene or trait [6]. The DNA polymorphisms when used as markers have a remarkable property in that they are largely unaffected by the environment, the only difficulty being them unraveling from the genome. However, the next-generation sequencing technology has made this task easier and cheaper, resulting in a very large availability of much-needed data specifically at the molecular level. There is also a requirement from the population genetics point of view that the marker alleles need to have a frequency of more than 1% in a population [11]. Looking from the linkage and recombination perspective, the marker should invariably be situated at a distance of less than 5 cM (centimorgan) from a gene or quantitative trait loci (QTL) of interest. It is not sufficient to just identify molecular markers. In India, there has been a major effort in identifying molecular markers especially in fishes. However, unless the identified markers find use in construction of dense linkage maps, decipher population genetic structure, parentage assignment and genome-wide association studies (GWAS) leading to QTL identification of economically important traits in fishes, this effort will largely remain futile.

Molecular markers associated with genes of known function, viz. allozymes and RFLP, are called type I markers, whereas those associated with anonymous gene segments like RAPD, AFLP, SNPs (unless they are developed from coding SNPs), and microsatellites (unless they are associated with genes of known function) are known as type II markers [12]. All the above mentioned markers are from the nuclear DNA. In aquaculture research, several DNA markers are used for different purposes. It would be of interest to comprehend as to what are the important molecular markers that are being used in aquaculture.

2.1 Single Nucleotide Polymorphism markers

As on date, SNPs are indeed making a huge impact in marker development and marker-assisted selection in plants, animals, and fishes, especially when sequencing is becoming cheaper and sequence information is becoming readily available. The polymorphic information content (PIC) (which is the value of a marker for detecting polymorphism) of SNPs when compared with multiallelic microsatellites is low, but this drawback gets balanced by their sheer abundance across the genome. Furthermore, in a simulation study, it was proven that the low PIC is offset by their high abundance in the genome and a map of 700–900 moderately polymorphic SNP markers is equivalent to 300–400 microsatellite marker sets [13]. The SNPs can reveal hidden polymorphisms that are not detected by other markers and methods [14]. This is a very important characteristic of SNPs. They are also useful for GWAS by which the genome is scanned for SNPs/QTLs that have association with economic traits. Once the association of the marker with a trait is established, it would strengthen selective breeding through marker-assisted selection, resulting in higher genetic gains. The SNP markers can either be type I or type II, are codominant and follow a Mendelian pattern of inheritance. They are biallelic [13], and it is only in the late 1990s that their importance was revealed when gene chip technology was introduced [14]. It is now possible to

manufacture a genotyping array containing thousands of polymorphic SNPs and use this chip to genotype individuals and obtain molecular information.

2.2 Microsatellites

Microsatellites or short sequence repeats are abundant in fish. These markers have multiple alleles at a given locus and are highly polymorphic among individuals. They follow the Mendelian inheritance and are codominant in nature. They can be used for studying species diversity and parentage assignment as well as in genetic mapping [14]. Microsatellite markers are useful in aquatic animals as genetic tags also. Selective breeding entails maintenance of a large number of families that need to be reared communally in a common environment to elicit genetic variation. For communal rearing, physical tagging of a large number of fishes will not be feasible; hence, microsatellites could be utilized for parentage assignment, provided the microsatellite information on parents is also available.

2.3 Mitochondrial DNA markers

The mitochondrial DNA is maternally inherited and has been used extensively by aquaculture geneticists to study the stock structure of fishes [14]. For population genetic studies, the mitochondrial genes, viz. COI, 12s rRNA, 16s rRNA, cytochrome *b*, and ATPase 6/8, are used. It has been observed that the overlapping ATPase 6/8 genes are quite effective in picking up the genetic differences in teleosts from different locations. RFLP carried out using the 16S rRNA mitochondrial PCR product of two mud crab species, *Scylla serrata* and *Scylla tranquebarica*, using the Hind III restriction enzyme revealed a clear distinction in the restriction pattern between the two species [15]. Comparative study of the mtDNA of Asian sea bass from the Indian subcontinent, Myanmar, Singapore, Malaysia, Thailand, Indonesia, and Australia revealed the existence of two distinct species: one representing the Indian subcontinent along with Myanmar and the second representing Southeast Asia (Singapore, Malaysia, Thailand, and Indonesia) and Australia [16]. The markers used in the study were cytochrome *c* oxidase subunit 1 (COI), 16S rRNA, and D-loop (control region). Samples of tiger shrimp from the east and west coasts of India were studied for genetic variability by amplifying a partial fragment of 16s rRNA mitochondrial gene. Sequence analyses of the amplified 520 bp of 16s rRNA mitochondrial gene segment revealed similar sequence identity and no apparent sequence variation could be observed in this amplified segment [17].

3. Molecular markers in aquatic species

3.1 Penaeid shrimp

The tiger shrimp is an important species in the Indian shrimp industry. Till the introduction of the specific pathogen-free (SPF) Pacific white shrimp into India in 2009, tiger shrimp was dominating the shrimp culture scenario. Ever since 2009, the culture of SPF *Penaeus vannamei* has increased tremendously overtaking the tiger shrimp, and to date, this exotic species contributes substantially to the export basket of India. Presently, the production of tiger shrimp in India is quite low, but there is a possibility of the production being on the rise due to the availability of SPF seed. A major challenge in tiger shrimp is the reproduction aspect, the obstacle being the closed thelycum in females. Consequent to this bottleneck, it has become very difficult to produce a large number of sibling families that are necessary during the execution of a selective breeding program. However, genomic resources are now available in tiger shrimp in the form of a linkage map constructed using cSNPs. An Illumina iSelect genotyping array containing 6000 SNPs was developed in India for the first time and used to genotype 1024 offspring belonging to 7 full-sibling families. A total of 3959 SNPs were mapped to 44 linkage groups corresponding to the haploid number of chromosomes in tiger shrimp. The female map was 28% longer than the male map [18]. In the same study, nine linkage groups were found to harbor QTL significantly associated with *hours of survival* after a challenge with WSSV. Another interesting observation was that these QTLs showed homology to immune-related genes. The QTLs once validated could be used for selecting broodstock, which can resist WSSV infection. Furthermore, several SNPs significantly associated with sex were found on linkage group 30 and specifically about three SNPs were found located in a 0.8-cM stretch between positions 43.5 and 44.3 cM corresponding to the feminization genes of *Caenorhabditis elegans*. These SNPs have future implications for the shrimp industry in identifying monogametic female tiger shrimp for production of an all-female monosex population. The information from this study can go a long way in identification of QTLs or markers and association studies for commercially important traits in penaeid shrimp [19].

Pacific white shrimp is a very important species of shrimp that dominates the world shrimp production today. Research on this species especially for improved growth as well as SPF status has resulted in the dissemination of this shrimp to

several countries. Studies revealed that growth and this resistance to WSSV were negatively genetically correlated [20]. In another study from India, it was observed that heritability for resistance to WSSV in tiger shrimp was very low and nearing zero, indicating that selection for improving this trait would not be effective [21]. In a study from a breeding program in China, 14 QTLs were identified for growth-related traits like body weight, total length, partial carapace length, first and third abdominal segment depth, and first abdominal segment width in Pacific white shrimp. These QTLs explained 2.62%–61.42% of the phenotypic variation in these traits [22]. In a breeding program at Colombia, information from challenge tests with WSSV in families of Pacific white shrimp and SNP marker information were incorporated into the computation of breeding values and genomic breeding values thus obtained were used for selecting individuals as parents for propagating the next generation. It was observed that the progeny obtained from mating of parent shrimp having high genomic breeding values for resistance to WSSV exhibited a higher survival compared to that from mating of parent shrimp having low genomic breeding values. This could possibly be a very useful procedure for developing shrimp with increased resistance to WSSV using SNP markers [9]. The advantage here is that the breeding candidates can be selected on the basis of genomic breeding values and the selected shrimp need not undergo the challenge test, thereby preventing any possibility of infection to the broodstock.

In a study at Australia, the AFLP marker was utilized to unravel QTLs affecting body weight, total length, and carapace length in a full-sib family of kuruma shrimp *Penaeus japonicus* [23]. An interesting finding was that two QTLs were detected in the male shrimp: one QTL responsible for part of the variation in total length and carapace length mapped to linkage group 1 and another QTL affecting the same traits being found in linkage group 25. This incidentally was the first study that has reported a QTL related to growth in penaeid species [23].

3.2 *Labeo rohita*

The Indian major carps, viz. rohu, catla, and mrigal, together produced 5.95 million tonnes in 2019–20 [24]. *Labeo rohita* or rohu is an important species in India. In 1992, a genetic improvement program had been initiated at ICAR-Central Institute of Freshwater Aquaculture in Bhubaneswar and selection for growth was the main objective. In 2009, as part of this program, resistance to the pathogen *Aeromonas hydrophila* was studied by challenging the fish with these endemic motile pathogenic bacteria that cause hemorrhaging and ulceration when fish are under stress. Fish challenged with these bacteria were followed closely for mortality, which was recorded in terms of *hours of survival*. A dense linkage map was constructed for the first time in *L. rohita* using 3193 SNP markers apportioned to 25 linkage groups, which corresponds to the haploid number of chromosomes in this species. A GWAS revealed that 21 SNPs were mapped to 10 linkage groups and covered 12 distinct regions in total, showing significant associations with the trait *hours of survival*. Most of these SNPs showed homology to genes of known immune function [25]. An important observation was that the enzyme ceruloplasmin was 4.58 times more highly expressed in resistant than in susceptible lines of rohu. The level of ceruloplasmin could therefore be used as an indirect marker for selection of *A. hydrophila*–resistant rohu [26]. Using these QTLs for selecting parents for propagation of the next generation would result in progeny harboring genes that are resistant to aeromoniasis. Furthermore, it would be interesting and possible to evaluate these QTLs on other carp species also for developing aeromoniasis-resistant lines [25].

3.3 Asian sea bass

Lates calcarifer is an important food fish that is cultured in India on a small scale, but of late is steadily picking up. Although this species has been successfully bred in captivity and quality seed is being produced by government agencies in India, selective breeding has not been initiated so far. However, selective breeding programs are already in place in countries like Vietnam [27] and Singapore [28]. In the selective breeding program at Temasek Life Sciences Laboratory in Singapore, 11 genes potentially associated with growth traits were identified from a QTL region in the Asian sea bass, and association studies revealed that SNPs in 3 specific genes could be used as markers for selecting fast-growing fish at the fingerling stage itself [29].

3.4 Atlantic Salmon

Earlier, infectious pancreatic necrosis (IPN) had led to more than 90% mortality especially when the fingerlings were in hatcheries or had just been transferred to sea cages for farming [30]. Atlantic salmon has two phases in its life cycle, the larval phase is spent in freshwater after which they migrate to the seas. A very large QTL on the Atlantic salmon chromosome 26 was discovered, which was found to have a highly significant effect on IPN by explaining 80–100% of the

genetic variation for this trait in experimental trials both in freshwater and seawater [31–34]. The requirement of carrying out challenge tests was circumvented by high-throughput sequencing and unraveling SNPs affecting IPN [35,36]. Consequently, a marker-assisted selection procedure was put in place, wherein fish were selected for a favorable allele, i.e., resistance to IPN [32,36], resulting in a very great reduction in the occurrence of IPN and in some cases, this disease was totally eliminated [37,38].

3.5 Tilapia

Tilapia, a cichlid from the family Cichlidae, is found abundantly in Africa and Middle East countries. There are several species that inhabit lakes in Egypt, Malawi, Ghana, Ethiopia, Zimbabwe, South Africa, Israel, Tanzania, Zambia and Southern Sri Lanka. There is constant crossing taking place between the species and it is necessary to identify the species involved in hybridization [39]. The sequence analysis of mitochondrial gene COI and a panel of 24 SNPs were utilized to distinguish between four species of tilapia, viz. *Oreochromis niloticus*, *Oreochromis aureus*, *Oreochromis mossambicus* and *Oreochromis urolepis hornorum*. In addition, a larger number of SNPs were found, which could distinguish between the pairs of this species within this subset [39]. Tilapia lake virus (TiLV) is a pathogen affecting the Nile tilapia (*O. niloticus*) and is considered an important one as tilapia is a species cultured globally [40]. The authors used data on GIFT tilapia that had got infected with this virus in ponds. The fish were categorized as either survived or dead and genotyped using a 65K SNP array. A significant QTL could be unraveled for this trait on chromosome *Oni22*. Individuals homozygous for the susceptible and resistant alleles could be identified. Candidate genes within this QTL for host response to the viral infection were also discovered [40]. These markers could thus be utilized to select for tilapia fish resistant to TiLV and use them to propagate the next generation, obviating the need for carrying out any challenge tests on the broodstock fish.

4. Future prospects of using DNA markers

The future for enhancing fish production lies in having selective breeding programs in select fish species that are important commercially, provided their life cycle is closed and production of a large number of families becomes easy and feasible. Selective breeding programs the world over target growth, which is the most important trait from a commercial point of view. Once a conventional selective breeding program is in place, the search for DNA markers could be explored. The markers that are unraveled need to be polymorphic and also have substantial effects on the trait concerned. In addition, there could be several other traits that become important as the program gets executed, for example, resistance to a particular disease. Markers when identified and tested would be very useful in aiding the breeder and strengthen their hands to choose the best animals as parents for the next generation. The genetic gains accrued would be much faster when important markers are incorporated into the breeding program. Such a procedure would speed up the genetic gains and the traits selected for will improve substantially.

5. Conclusions

Molecular markers have been used quite extensively in plants, especially crops and terrestrial farm animals, because of the fact that selective breeding programs are already in vogue in these species for quite some time. Aquaculture has started reaping the benefits of selective breeding programs only recently, which is corroborated by the fact that to date, less than 10% of global aquaculture production comes from genetically improved stocks. In one way, it is a good learning experience for people involved in aquaculture because the obstacles faced by breeders of crops and animals can be addressed while planning such programs for improving aquatic species. Well-planned conventional genetic improvement programs in commercially important fishes would greatly enhance the availability of improved strains of fish that can ultimately boost culture, leading to increased fish production. Simultaneously, it would be useful if molecular markers for growth and resistance to specific diseases are unraveled, verified for their contribution to the respective trait, and thereafter incorporated into the breeding program for eliciting higher genetic gains.

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An update on induced breeding methods in fish aquaculture and scope for new potential techniques

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1. Introduction

Aquaculture is the world's fastest-growing food-producing sector. The sector's expansion began in the 1970s, stimulated by advances in hatchery technology and pond husbandry. The future of aquaculture lies in production of species that are able to grow fast, adapt to changing climatic conditions, and also remain capable of withstanding the rigors of an enhanced production system. India is enriched with vast inland water resources and vast stretch of sea in its three borders. As a consequence, natural fisheries (capture) resources are scattered all over the country. The contribution of aquaculture to global supplies of fish, crustaceans, mollusks, and other aquatic animals increased from 3.9% of total production by weight in 1970 to 27.1% in 2000 and 32.4% in 2004, and thereafter it is increasing on a day-to-day basis. The projected fish production of India in 2020 was 11.9 million tons, consisting of 7.4 million tons from inland production and 3.5 million tons from the marine sector. Development strategies need attention toward diversification of aquaculture through production of locally available fish species. Carps, catfish, prawns, molluscs, and ornamental fish provide options for diversification of species to be cultured. Success in captive reproduction and hatchery-based seed production is fundamental for the development of sustainable aquaculture. Fish seed is the critical and basic input for successful fish culture operations, and until the 1960s, a major part of the seed required for culture was collected from the riverine sources. Generally, fishes do not breed in captive conditions, and artificial breeding technique is an important fisheries operation. In this technique, brood fish are stimulated by pituitary gonadotropins (luteinizing hormone [LH]) or any other agent that stimulates LH. The stimulation promotes timely release of sperm and eggs. In this chapter, an overview of old and current induced breeding agents used in fish aquaculture is presented and the scope for newer ones is discussed.

2. Need for induced breeding in fish aquaculture

For successful fish culture, quality fish seed is one of the most important prerequisites. Fish culturists and farmers often face problems in its procurement as spawn collected from natural water is not pure and of the desired quality. The presence of undesirable wild species and sorting of pure seed are quite impossible among mixed seed sources of various species.

Availability of required quantity of fish seed of desired species is one of the most important prerequisites for successful fish farming. The widely cultured Indian major carps in inland waters like catla (*Catla catla*), rohu (*Labeo rohita*), mrigal (*Cirrhinus mrigala*), and calbasu (*Labeo calbasu*), and Chinese carps like silver carp (*Hypophthalmichthys molitrix*), big head (*Aristichthys nobilis*), grass carp (*Ctenopharyngodon idella*), black carp (*Mylopharyngodon piceus*), and mud carp (*Cirrhinus molitorella*), normally do not breed in confined waters. They do mature there, but breed only in the flooded shallow areas along the course of the rivers during monsoon months, which are their natural habitat.

In most teleost species, reproductive processes are by no means fully impaired in captivity. The progressive development of the gonads remains, in general, uninhibited up to the final stages of gamete maturation, and it is only at the point of gamete release that the process is arrested. Both gonadal maturation and spawning behavior have long been known to be

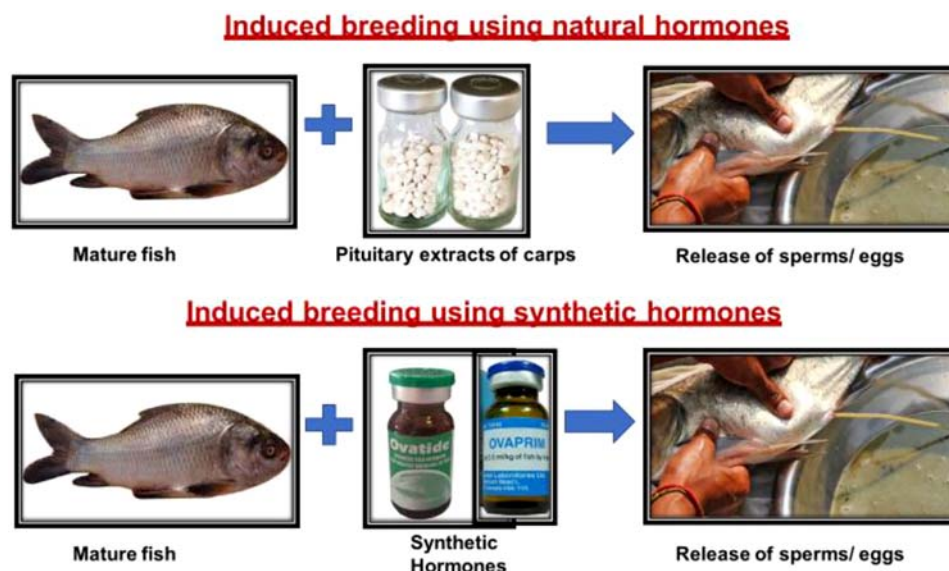


FIGURE 5.1 Approaches of induced breeding in fish. Images were collected from [slideshare.net](https://www.slideshare.net) assembled and re-presented as figure.

responsive to environmental stimuli—temperature, hours of daylight (photoperiod), and the amount of rainfall, among them. The fish culturists are lucky that the intervention is required at the terminal stage when the needed environmental cues are lacking and an artificial means is needed to push the process to completion. This has been accomplished through the technique of induced breeding. In general, **induced breeding is a process where the fish, which do not breed in stagnant water bodies, will do so under the influence of some stimulants or hormones injected in to their bodies.** The technique of breeding the fish by administering pituitary gland extract injection is known as **hypophysation**. In this process, the stimulating agents or hormones are injected into the ripe brood fish and the stimulation promotes timely release of eggs and milt from the ripe breeders under captive conditions. Agents used in induced breeding techniques are natural or synthetic products (Fig. 5.1). The pituitary gland secretes several hormones, of which gonadotropin (GtH) is the most important one for breeding. Whatever the agents used, the production of endogenous GtH, or supply of exogenous GtH, is central and basic to the success of the technique.

3. Historical developments in induced breeding methods

The technique of induced breeding was first developed in Argentina using pituitary extract by B.A. Houssay in 1930 ([1]; Fig. 5.2). He was initially successful to breed frogs with the help of pituitary extract. He injected some viviparous fish with pituitary gland extracts removed freshly from other species of fish and induced premature birth of the young. Brazil was the first country to develop successfully the technique of hypophysation in fishes. The injection of crude piscine pituitary extract was first successfully demonstrated in channel catfish by von Ihering and coworkers in Brazil in 1934 (vide., [2]). In the period between 1937 and 1970, many scientists in different countries have worked to adapt the technique for different fish species. Some notable contributions are made by N.L. Gerbilsy (Soviet Union), E. Woynarovich (Hungary), L.S. Ramaswami and B.I. Sundararaj (India), H. Chaudhuri and K.H. Alikunhi (India), and K.E. Sneed and H.P. Clemens (USA). Gerbilsy and Woynarovich have worked out the detailed procedure for commercial applications.

This technique still forms the backbone of many induced breeding operations developed over the years to understand the complex problems of reproductive refractoriness and to find solutions. Parallel to it, research in the neuroendocrine control of GtHs has contributed substantially to the development of new-generation techniques. Methods were developed for preserving fish pituitary glands and extracts, and for purifying GtHs. Subsequent research explored the feasibility of using steroid hormones that act directly at the gonadal level, and the use of antiestrogens as estrogen feedback inhibitors. In the early 1970s, the characterization and synthesis of mammalian gonadotropin-releasing hormone (GnRH) and its superactive analogues led to its successful use in some species. This was followed by the synthesis of piscine GnRH, particularly salmon GnRH and its superactive agonists. Later research has shown that, in several groups of teleosts, a dopamine (DA) antagonist administered along with GnRH potentiated the action of GnRH and achieved successful spawning [3–6].



FIGURE 5.2 Bernardo alberto houssay (April 10, 1887–September 21, 1971) was an [Argentine physiologist](#) who, in 1947, was a corecipient of the [Nobel Prize for Physiology or Medicine](#) for his discovery of the role played by [pituitary hormones](#) in regulating the amount of blood sugar ([glucose](#)) in animals (Wikipedia).

4. Fish gonadotropins and gonadotropin-based techniques

Two forms of GtH are present in most teleosts, follicle-stimulating hormone (FSH) and LH (see reviews [6–8]). In catfishes, as an exception, only a single form of GtH, i.e., LH, is present. FSH is concerned with gametogenesis and gonadal growth and is secreted under natural environmental conditions of light and temperature. In females, FSH is concerned with [oocyte development, steroidogenesis, and vitellogenesis](#), whereas in males, it is concerned with steroidogenesis and spermatogenesis. LH is concerned with the final process of gonadal maturation (final oocyte maturation [FOM] and ovulation in females) and sperm maturation and spermiation in males. Spawning is initiated by the surging production of LH (LH surge) and induced by the right spawning conditions. Under captive conditions, many fishes do not breed due to the lack of the right set of spawning stimulus or stimuli, resulting in the failure of spawning, i.e., release of the gametes. They either suffer from confinement stress or critical environmental cues are missing in captivity, resulting in the inhibition of reproduction. Hence, spawning is to be induced artificially. The pituitary gland is the storehouse of several hormones including LH and FSH. The hormones are released when appropriate stimuli are received from the environment via the brain sensory systems. Trial-and-error experiments using pituitary preparations in the past have shown that the extract stimulated spawning in a variety of fishes [3]. Supplementation with mammalian pituitary hormones showed that all except LH lack the property to stimulate spawning activity in fishes. In 1959, K.E. Sneed and H.P. Clemens (USA) used human chorionic gonadotropin (hCG) to induce spawning of channel catfish. Since then, mammalian LH and hCG have been used as inducing agents side by side with pituitary extract (see Ref. [9]). Pregnant mare serum gonadotropin failed or had low response in this regard, although it has LH- and FSH-like activities. Purification of fish GtHs has been attempted in the intervening period. Salmon GtH was partially purified by Sephadex G-100 and was found effective to stimulate spermatogenesis and spermiation and vitellogenesis and ovulation in goldfish and catfish [10,11]. This GtH preparation has been tested in different fish species and established the role of GtHs in fish reproduction. However, due to its nonavailability in markets, pituitary extract and hCG (in different trade names) remained the mainstream hypophysation agents until the discovery of LHRH (GnRH) in mammals in the early 1970s and superactive analogues.

5. Fish GnRHs and GnRH-based techniques

Mammalian LHRH was discovered in 1969 by Andrew Schally and Roger Guillemin. Since then, GnRH research has picked up momentum in nonmammalian vertebrates at the molecular and anatomical level. In teleost fishes, multiple forms of GnRH (up to 3) and GnRH receptors (up to 5) occur [12]. GnRH1 is the hypophysiotropic form in the two-model groups (e.g., catfishes), and GnRH3 is the hypophysiotropic form in the three-model species (e.g., seabream). The midbrain GnRH2

(chicken II GnRH) is conserved in all vertebrates. All of them have a decapeptide structure proteolytically cleaved from a large precursor molecule coded by specific genes. The different forms vary in amino acid composition giving varied functions and potency. Native GnRH stimulates gonadotropes in the anterior pituitary and releases GtHs to stimulate gonadal functions (Fig. 5.3). A GnRH surge stimulates corresponding preovulatory LH surge to control ovulation and spermiation in vertebrates. The identification of the amino acid sequence of GnRH led to the development of agonistic and antagonistic analogues through suitable substitutions that have far-reaching research and clinical implications. The GnRH agonists are more potent and have a longer half-life than native GnRH. They produce an initial stimulation of pituitary gonadotropes that results in the secretion of FSH and LH and the expected gonadal response. This response is followed by downregulation and inhibition of the pituitary—gonadal axis. As compared to GnRH agonists, GnRH antagonists promptly suppress pituitary GtHs by GnRH receptor antagonism, inhibiting the initial stimulatory phase. Discontinuation of GnRH antagonist treatment leads to a rapid and predictable recovery of the pituitary—gonadal axis. Agonists would be used as strong sustained stimulators of GtH secretion, and the antagonists promised to be a potential tool for chemical hypophysectomy of the reproductive axis.

Investigations primarily in goldfish (*Carassius auratus*), rainbow trout (*Salmo gairdneri*), and African catfish (*Clarias gariepinus*) have led to existence of dual control of GtH secretion: by stimulatory GnRH and the inhibitory DA systems [4,6,13–16]. The two systems interact at the hypothalamic and pituitary levels to regulate the GtH secretion. DA uses two receptor subtypes: D1 subtype to inhibit GnRH synthesis at the perikaryal level and D2 subtype at the axonal endings on the gonadotropes in the pituitary to inhibit GnRH release. Consequently, DA receptor blockers can augment GnRH synthesis and release, leading to blockade of GtH secretion. In other words, the use of DA antagonists like domperidone and pimozide stimulates GnRH release several folds and induces the LH surge in mature ready-to-spawn fish [4,13–15,17–19]. The use of the combination of a GnRH analogue and DA2 receptor blocker has led to the development of a new hypophysation method known as the “LINPE” method, named after the inventors Dr. Lin and Dr. Peter. The LINPE method was first successfully used in cooperative farming of carps in China [4,15]. The Syndel laboratory in Canada developed the first spawning kit known as Ovaprim (OVP) and marketed it for commercial use. OVP contains a salmon GnRH analogue and a DA2 receptor antagonist, domperidone. Many years of research went into the development of OVP [20–22]. Although sGnRH alone is effective for inducing ovulation and spermiation in numerous species, some fish groups also require a DA blocker to augment spawning activity. Based on the LINPE method, other spawning aids such as Ovopel, Ovapel, Ovatide, and Wova-FH were formulated and are commercially available for fish breeding practices (see below). These formulations have been tried by different workers in different species and operational protocols have been developed (Tables 5.1, 5.2).

In India, the first use of a GnRH analogue (des-Gly¹⁰[D-Ala⁶]-LHRH ethylamide) and a DA receptor antagonist (pimozide) to induce ovulation was reported in the catfish *Clarias batrachus* [36]. The combination resulted in a spawn weight of 21.15 ± 2.6 g with a high rate of ovulation (86%), hatching rate (92%), and high yield of normal fry (86%). In a

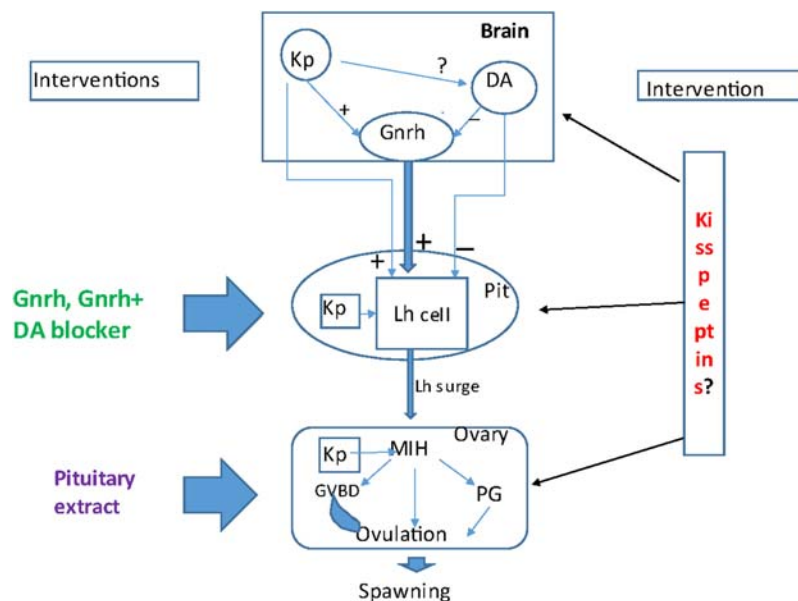


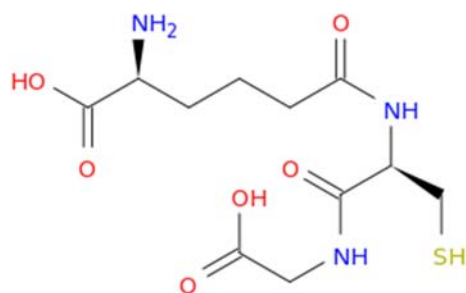
FIGURE 5.3 Brain—pituitary—ovarian axis in a catfish showing kisspeptin (Kp), gonadotropin-releasing hormone (GnRH), and dopamine (DA) relationships in the control of luteinizing hormone (LH) surge. *MIH*, maturation-inducing hormone; *PG*, prostaglandins.

subsequent study in the catfish *Heteropneustes fossilis* [37], the combination was demonstrated to elicit a highly significant increase in plasma LH level (preovulatory surge). This study also revealed that the mammalian (m) GnRH analogue alone was effective at high doses and pimozone can reduce effectively the dose of mGnRH analogue to induce ovulation. A detailed study on the effects of OVP (0.5 μ L/g body weight), two intraperitoneal injections 24 h apart, was conducted in *H. fossilis* (50–60 g) in our laboratory [38]. OVP induced 100% ovulation from 6 h onward up to 48 h. The fertilization rate was high between 6 and 18 h and declined significantly from 24 h onward. At the pituitary level, the treatment upregulated the expression of GtH subunit genes differentially. The expression of *gpa* and *lh β* peaked at 6 and 12 h and declined at 18 and 24 h after the first injection. The second OVP injection at 24 h elicited only a transient increase in the *gpa* expression at 6 h and a sustained increase in the *lh β* expression from 6 to 18 h after the second injection, but both transcripts decreased subsequently compared to the first dose. The *fsh β* expression responded to the OVP treatment from 12 h onward and maintained a constant level from 18 to 36 h after the first injection; the second dose had little effect. Plasma steroids were differentially altered: the levels of estradiol-17 β decreased while that of 17,20 β -dihydroxy-4-pregnen-3-one (maturation-inducing hormone, MIH) increased, causing the steroidogenic shift preceding FOM and ovulation. Thus, this study showed that *lh β* expression coincided with the LH surge and ovulation response.

Pituitary gland extract or hCG supplementation simulates directly the preovulatory LH surge necessary for downstream gonadal events that culminate in spawning activity. OVP and similar spawning agents, however, induce an endogenous LH surge within few hours of the injection, leading to FOM and ovulation in females and spermiation in males. In either case, the subsequent gonadal events follow a common pathway that in females induces a steroidogenic shift from E₂ to a progesterone derivative, 17,20 β -dihydroxy-4-pregnen-3-one (MIH in most fishes) or 17,20 β ,21-trihydroxy-4-pregnen-3-one (the MIH in some species like Atlantic croaker) [39,40]. The MIH initiates a series of changes in the signaling pathways to promote the synthesis of a maturation-promoting factor (MPF), which is a heteromer of cdc2 kinase and cyclin B. The MPF initiates a series of cellular changes such as germinal vesicle (GV) migration, GV breakdown, and resumption of meiosis arrested at the prophase 1 stage. This is followed by ovulatory changes involving breakdown of the follicular envelope and release of ova into the ovarian cavity. The MIH also promotes prostaglandins that participate in the ovulatory response. Similar mechanisms occur in the testis to initiate spermiation [8]. The changes in the hormonal milieu ignites spawning activity, and gametes are released to the exterior.

6. Some common inducing agents used in fish aquaculture

- i) **Pituitary gland extract:** This is simple and crude but still used effectively to obtain pure seed of cultivable fishes practiced on extensive scale in India and other countries in the world. It involves injecting mature female and male fishes with extracts of pituitary glands taken from own or other mature fishes. Homologous and heterologous preparations are useable. It contains all pituitary hormones including GtHs. Disadvantages are difficulty in collection, storage, lack of potency optimization, and immune responses on prolonged use.
- ii) **hCG:** It is a glycoprotein hormone produced by the placenta in the pregnant woman. During early pregnancy, the hormone appears in the urine in large quantities. It is LH-like and more potent and long acting. When it is injected to brood fish, the hormone is known to cause maturation and release of gametes. The effects of the hormone in inducing sperm release and ovulation is a synergistic action with the circulating pituitary hormones. When hCG is injected singly, it is not so effective as when it is injected together with pituitary gland extract. It is available in different trade names and easily available in pharmacies.



ChemEssen.com

- iii) **Sumaach:** Infar Ltd. (India) has brought out a product that is cheaper compared to the pituitary gland extract and has a long shelf life. The product is grinded in distilled water (2 mg in 0.2 mL) and the mixture is centrifuged. The supernatant is then used for injection.
- iv) **Synahorin:** It is another preparation of chorionic gonadotropin and mammalian hypophysial extract.
- v) **OVP (Syndel Lab, Canada):** OVP is a mixture of salmon GnRH and the DA2 receptor antagonist, domperidone, in a stable solution. It is prepared in glycerin and alcohol at particular proportions. This kit is available commercially since 1988 and is extensively used. These are introduced into the fish with a dose of 0.3 to 0.5 mg/kg body weight of female and 0.01–0.3 mg/kg body weight of male. OVP can be stored at ambient temperature even in the tropics for more than a year.
- vi) **Ovatide (Hemmo Pharmaceuticals Pvt Ltd., India):** It is an injectable inducing hormone consisting of salmon GnRH analogue and the DA antagonist, pimozone. It is efficient in induced spawning of carps and catfishes.
- vii) **Wova-FH (Biostadt India Ltd.):** It is a combination of sGnRH and domperidone with glycerol as carrier. It is injectable and the dose is 0.20–0.80 mL/kg of fish in females and 0.10 to 0.60 mL/kg in males. It is used for breeding of different fishes like carps, trout, and salmon.
- viii) **Ovopel (Unic-Trade Kft., Hungary):** It is a combination of mammalian GnRH analogue (D-Ala⁶, Pro⁹NEt-mGnRH) and a DA antagonist, metoclopramide [41]. It is administered in one or two doses according to the fish [42–44].
- ix) **Ovapel:** It was developed by the Hungarian University of Agriculture and Life Science, Gödöllő, Hungary. It is a combination of mammalian GnRH analogue and DA receptor antagonist, lactosum. The recommended dose is 1–2 pellet/kg of fish for carps [45].

The success and efficacy of the various formulations are assessed by certain fixed criteria [15], which are as follows:

- High spawning (ovulation/spermiation) rate
- Complete ovulation, i.e., no retention of eggs
- High rate of spawning repeatable from one group to another within a species
- Short and predictable latency time after injection
- High fertilization and viability of eggs
- No adverse effects on the brood fish

7. Kisspeptins and potential role as an induced breeding agent

Kisspeptin (KISS1) gene was first discovered in human in the context of cancer, specifically melanoma, as a suppressor gene of metastasis. The smallest biologically active peptide is a decapeptide (KISS 1-10) cleaved from the large precursor parent protein, the product of *KISS1* [46]. KISS1 is identified as an endogenous ligand of the G protein–coupled orphan receptor GPR54 [46–48]. In mammals, a majority of GnRH neurons express GPR54 and KISS1 is considered the upstream regulator of GnRH secretion [49,50]. It belongs to the RFamide peptide family and shares an Arg-Phe (RF) amide sequence motif at the C terminus. KISS1 plays a critical functional role in reproduction in timing puberty onset, dynamic control of GnRH secretion via stimulation of GnRH neurons, transmission of the negative and positive feedback effects of sex steroids, metabolic regulation of fertility, and the control of reproductive function by environmental (photoperiodic) cues. Detailed neuroanatomical studies have documented the existence of discrete populations of KISS1 neurons in key hypothalamic areas, the arcuate/infundibular nucleus, and the steroid-sensitive anteroventral periventricular nucleus [51–54]. While research on KISS1 has focused its role as the upstream regulator of GnRH to control GnRH secretion, parallel studies in gonads and placenta have shown that the KISS1/receptor system is active in peripheral tissues [55,56]. In the ovary, KISS1/KISS1R is expressed in the theca–granulosa/luteal granulosa cells [55,57]. Saadeldin et al. [58] reported that KISS1 enhanced oocyte maturation and subsequent developmental competence in an autocrine–paracrine manner in porcine cumulus–oocyte complexes. KISS1 mRNA expression increased 82.2-fold at the acquisition of oocyte meiotic capacity, and when the oocyte progressed through the first and second divisions of meiosis (MII), the expression decreased to 5.4- and 12-fold, respectively [59]. In rat, COX2 (prostaglandin synthase) inhibitor downregulated KISS1 mRNA expression and suppressed the efficiency of ovulation (Cao et al. 2019). KISS1 stimulated progesterone secretion by rat luteal cells and by chicken and porcine granulosa cells. Recombinant KISS1 significantly enhanced basal and hCG-induced progesterone levels in cultured rat luteal cells and upregulated the transcription of key steroidogenic enzymes (StAR, CYP11A, and 3 β -HSD) by increasing the phosphorylation of the mitogen-activated protein kinase Erk1/2, but not of P38 MAPK and Akt [60]. Taken together, KISS1 is an important local modulator of follicular development, steroidogenesis, and follicular maturation, and its role in ovulation may be indirect by influencing the prostaglandin pathway.

Following the kisspeptin research in mammals, intensive studies have been undertaken in lower vertebrates especially fishes. Molecular cloning and characterization of the KISS system has been taken up in a large number of fishes and has

revealed the complexities of the system. In lower vertebrates with the exception of birds, Kiss genes occur in multiple forms, coding for different peptide precursors and for different GPR54. Teleosts exhibit two patterns, species with *kiss1* and *kiss2* and species with only *kiss2* [6,51,61–64]. *Kiss2* is considered the hypophysiotropic form concerned with reproductive activity. The Kiss/Kiss receptor system is expressed in the brain, pituitary, and gonads [64–70], indicating its involvement at multiple levels. In the catfish *H. fossilis*, a *kiss2* gene is characterized and is expressed in the brain, pituitary, and gonads [65]. The *kiss2* transcripts were localized in major hypothalamic nuclei (nucleus preopticus [NPO], nucleus recessus posterioris, and nucleus recessus lateralis), pituitary, and follicular layer of ovarian oocytes. In the pituitary, the *kiss2* transcripts were localized in one type of proximal pars distalis cells, suspected to be the GtH cells. The expression of the Kiss/Kiss receptor genes has been shown in relation to developmental stage of the fish/gonads and in different seasons [71–73]. The Kiss expression is influenced by steroid feedbacks and photothermal conditions. In goldfish, only *kiss2* neurons in the preoptic area were upregulated by ovarian estrogen [74]. In the prepubertal zebrafish, estradiol treatment increased the expression of both *kiss1* and *kiss2* [69]. In *H. fossilis*, ovariectomy inhibited the expression of brain *kiss2* and E2 treatment reversed the ovariectomy effect and elevated the expression [65]. In this species, long photoperiod and high temperature elevated the *kiss2* expression [75].

Physiological studies have shown that the Kiss peptides exhibit varied effects on the expression/levels of GnRH and GtH. In goldfish *C. auratus*, intraperitoneal administration of *kiss1* resulted in an increase in serum LH, while *kiss2* had little effect [76]. Alternatively, in European sea bass, *Dicentrarchus labrax*, intramuscular injections of *kiss2* exerted higher effects on LH secretion over *kiss1* [66]. In orange-spotted grouper, *Epinephelus coioides*, intraperitoneal injection of *kiss2* significantly increased *gnrh1* transcript levels in the hypothalamus and *fsh β* transcript levels in the pituitary [25]. *Kiss1* mRNA levels in the brain gradually increased in female zebrafish during the first 2–8 weeks of life to peak in fish with large mature vitellogenic follicles at 12 weeks [69,77]. The highest brain *kiss2* mRNA expression was reported either before or during the spawning season in a number of species.

Investigations on the direct effect of Kiss peptides on gonadal physiology are meager. In the perch *Channa striata*, the administration of kisspeptin-10 (mammalian kisspeptin-10) stimulated gonadal maturity and E2 levels [78]. In a detailed study in the catfish *H. fossilis* using synthetic human *kiss1* and catfish *kiss2*, it has been reported that the Kiss peptide treatments in vivo and in vitro stimulated *gnrh1* and *gnrh2* expression in the hypothalamus, pituitary, and ovary; stimulated GtH subunit expression; stimulated E2 and MIH (17,20 β -dihydroxy-4-pregnen-3-one) levels; and stimulated FOM and ovulation (Fig. 5.3). In all these, catfish *kiss2* was more effective than human *kiss1*. The GPR54 receptor blocker peptide 234 inhibited the stimulatory effects of the Kiss peptides, and the Kiss peptide supplementation with the inhibitor reversed the inhibitory effects of peptide 234 (11, under review). The results obtained with the Kiss treatments are encouraging and the research needs to be extended to field studies to explore the feasibility of the peptides as inducers of breeding in cultured fish.

8. Neurohypophysial peptides and potential role as inducing agents

In teleosts, vasotocin (VT) and isotocin (IT) are the neurohypophysial nonapeptides, which are the homologues of mammalian vasopressin (VP) and oxytocin (OT), respectively. These peptides are encoded by specific genes on large precursor proteins. Both VT and IT genes have been cloned and characterized in a number of teleosts. The gene transcripts/peptides are localized in the NPO, and the axonal fibers run toward the pituitary where the peptides are stored or released into the circulation for peripheral actions. Obviously, the pituitary extracts contain these peptides and they influence the induction of spawning overtly. In a pioneering study, Singh and Joy [79] reported that, apart from the brain–pituitary system, the nonapeptides are localized in the follicular layer of the ovary of the catfish. The peptides showed seasonal variations in both ovary and testis, and the levels increased with gonadal development and maturation. The nonapeptide levels are lower in the testis and the IT levels are lower than VT. In later studies in the same species, the genes for VT, IT, and a novel neutral peptide, sevotocin (ST), were cloned and characterized in the ovary [80–82]. The VT receptor genes *v1a1* and *v1a2* were localized in the follicular layer of the ovarian follicles and *v2a* at the junction between the granulosa and zona pellucida [83,84]. The VT receptor genes were also localized in the testis [84]. Taken together, like the brain, a gonadal nonapeptide system exists in teleosts, as in higher vertebrates.

The reported physiological roles of the nonapeptides are in controlling osmoregulation and cardiovascular, behavioral, chronobiological, metabolic, and reproductive functions. The involvement of VT in ovulation, oviposition/parturition, and spawning behavior has been documented in fishes [85–87]. But its dynamic involvement in gonadal development and maturation has been investigated and documented only recently [88]. Ovarian VT is regulated by hCG, E2, progesterone, and 17,20 β -dihydroxy-4-pregnen-3-one (17,20 β -DP) [89]. VT modulated ovarian steroid hormone secretion in a season-specific manner and stimulated FOM, oocyte hydration, and ovulation [89–91]. In preparatory phase, VT stimulated E2 levels dose-dependently, low doses stimulated and high doses inhibited the secretion. In spawning phase, VT inhibited E2 and testosterone secretion simultaneously stimulating MIH secretion (steroidogenic shift), causing FOM and ovulation

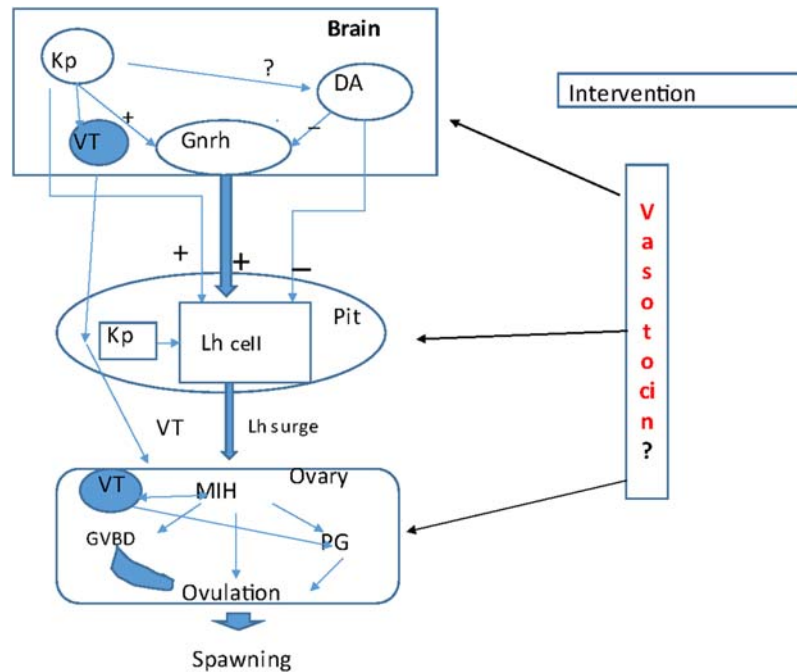


FIGURE 5.4 Brain–pituitary–ovarian axis in a catfish showing kisspeptin (Kp), gonadotropin-releasing hormone (GnRH), and vasotocin (VT) relationships in the control of final oocyte maturation and ovulation. *MIH*, maturation-inducing hormone; *PG*, prostaglandins.

[90,91]. These studies indicated a gonadotropin-like action of VT, which is physiologically relevant in catfishes as only a single gonadotropin, i.e., LH, has been characterized. Catfish FSH protein has not been demonstrated [92] though the genes for *fshβ* and the cognate FSH receptor have been functionally characterized [93,94]. In all these studies, the neutral peptide IT has elicited only a low response, implying VT is the potent reproductive hormone. At the pituitary level both VT and IT modulated GtH subunit gene expression differentially [95]. The results of this study showed that VT modulated both *fshβ* and *lhβ* expressions, influenced by the sex and reproductive stage. The neutral peptide IT and ST exerted insignificant effects on the *fshβ* expression regardless of sex or season. Both VT and IT/ST elicited a significant effect on the *lhβ* expression in late recrudescence phase especially in females.

The OVP treatment stimulated brain and ovarian VT levels, indicating functional interaction between GnRH–DA–VT systems. In vivo, OVP induced ovulation and increased significantly brain and plasma VT levels in a time-dependent manner. Plasma steroids were differentially altered; the levels of E2 and testosterone decreased, and the MIH (17,20β-DP) level increased time dependently. In order to demonstrate whether OVP acts at the level of the ovary directly, the incubation of ovarian slices/follicles with OVP stimulated GVBD and VT level in a concentration- and time-dependent manner with a maximal increment at 16 h. Ovarian testosterone and E2 levels decreased concurrently with the dose- and duration-dependent rise in the MIH level. The direct OVP–VT cascade has the potential to stimulate FOM and ovulation, side lining the pituitary glycoprotein hormone (LH) surge. The results of studies strongly indicate that the basic nonapeptide VT is a critical neuropeptide in the activation of the hypothalamo–hypophysial–gonadal axis. Structurally, VT has the ring structure of OT and the tail structure of VP. The neutral nonapeptide IT has lesser effects in the catfish. VT is a potential candidate for induced breeding of fishes alone or in combination with other agents. VT can provide the much-needed signals to initiate spawning behavior (Fig. 5.4).

9. Conclusions and future perspectives

Hypophysation methods have evolved over the years from the simple pituitary extract supplementation to commercially marketed hormones and drug combinations, thanks to advancements in our understanding of reproductive endocrinology and development of biochemical and molecular biology tools. As a result, the success and efficacy of breeding methods have predictably increased. There are still concerns as to the question on what prevents natural spawning of fishes under captivity conditions! Is it the confinement stress that impede the spawning signals? Kisspeptins and VT are two promising molecules whose involvement in reproductive activity is established in laboratory studies. The potential role of these neuropeptides in the induction of spawning is to be established in field studies, and further research on these neuropeptides may solve some of these questions.

10. Appendix

TABLE 5.1 Summary of data using Ovotide as the induced breeding agent in various fish species (the list is not exhaustive).

Female body weight (g)	Dose (mL/kg BW)	Male body weight (g)	Dose (mL/kg BW)	Latency period (h)	Ovulation (no. of eggs)	Fertilization rate (%)	Hatching rate (%)	Fish species	References
4500–6000	0.5	4250–10,000	0.5	5–8	1,00,000–720,000	90–93	50–88	<i>Catla catla</i>	[23]
3000–4000	0.5	4800–6000	0.5	6–10	90,000–7,20,000	85–95	65–94	<i>Labeo rohita</i>	[23]
1800–2400	0.5	2400–4800	0.5	6–8	35,000–4,00,000	85–90	50–83	<i>Cirrhinus mrigala</i>	[23]
1000–1700	0.5	2000–3200	0.5	6–8	20,000–200000	90–93	60–94	<i>Labeo calbasu</i>	[23]
—	0.4 mL	—	—	—	—	90.6	91	<i>Channa punctatus</i>	[24]
201–207	0.6–1.0			18–19	4788–4919	67.7–82.3	48–55	<i>Clarias batrachus</i>	[25]
—	0.3 mL	—	—	—	—	90.2	92	<i>Anabas testudineus</i>	[26]
8,000–11,000	0.3–0.5			8.00–8.30	4.1–7.4	77–85	49–80	<i>L. rohita</i>	[17]
4500–5000	0.3–0.5	—	—	8.30–9.00	1.7–2.7	71–90	57–82	<i>L. calbasu</i>	[17]
5000–6000	0.3–0.5	—	—	8.00–9.00	1.6–3.9	54–85	41–77	<i>C. mrigala</i>	[17]
10,000–12,000	0.3–0.5			8.30–8.45	3.0–8.7	60–94	40–84	<i>C. catla</i>	[17]
330–1078	0.10–0.25	317–542.40	0.10–0.25	8.0	6276–6826	87.54–95.43	31–65	<i>Clarias gariepinus</i>	[27]
255–256	0.3–0.5	174–176	0.1–0.3	7–10	3,0,850–63,400	55.2–93.4	59–85	<i>Osteobrama belangeri</i>	[28]
340–500	0.06–0.1	—	—	8	632–645	67.11	57	<i>Clarias anguillaris</i>	[29]
326–408	0.06–0.1	—	—	8.10	661–695	63.3	55	<i>C. gariepinus</i>	[29]

TABLE 5.2 Summary of data using Ovaprim as the induced breeding agent in various fish species (the list is not exhaustive).

Female body weight (g)	Dose (mL/kg BW)	Male body weight (g)	Dose (mL/kg BW)	Latency period (h)	Ovulation (no. of eggs; lakhs)	Fertilization rate (%)	Hatching rate (%)	Fish species	References
—	0.3–0.7	—	—	—	—	95–98	Complete	<i>Channa striata</i>	[30]
—	0.3–0.5	—	—	—	—	73–75	50–65	<i>Channa punctatus</i>	[31]
—	0.3–0.7	—	—	—	—	70–75	50–60	<i>Heteropneustes fossilis</i>	[31]
—	1.5	—	—	—	—	100	—	<i>Anabas testudineus</i>	[32]
—	1.0	—	—	—	—	92	63.4	<i>Horabagrus brachysoma</i>	[33]
—	1.0–2.0	—	—	—	—	71–73	60–55	<i>Clarias batrachus</i>	[34]
8000–11,000	0.3–0.5	—	—	7.30–8.30	2–7.0	55–86	30–75	<i>Labeo rohita</i>	[35]
4500–5000	0.3–0.5	—	—	7–8	1.5–3.9	58–90	43–79	<i>Labeo calbasu</i>	[35]
5000–9000	0.3–0.5	—	—	7.30–8.30	2.0–3.9	60–85	43–71	<i>Cirrhinus mrigala</i>	[35]
10,000–12,000	0.3–0.5	—	—	7.30–8.00	3.5–6.5	54–84	47–79	<i>Catla catla</i>	[35]
256–258	0.3–0.7	174–177+3	0.1–0.3	7–10	24,533–6733	55–96	52–87	<i>Osteobrama belangeri</i>	[28]

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Biofloc technology in aquaculture

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1. Introduction

Intensification has become the key to increasing aquaculture production with enhanced access to knowledge and better technology while reducing the use of land and water resources. The role of intensive aquaculture in addressing socio-economic issues, particularly hunger, poverty, and malnourishment, is well recognized [1]. However, intensification comes with many challenges. First, higher input, and hence the higher output, of effluents from aquaculture is considered detrimental to the environment, leading to severe water pollution issues, particularly eutrophication that encourages algal blooms due to the high nutrient loading (e.g., nitrogen and phosphorus). Eutrophication often results in oxygen depletion, killing living organisms in the water. In addition, more hazardous pollutants like chemicals and antibiotics also disturb the environmental balance.

Water pollution associated with aquaculture is not a new topic; there has been evidence of aquaculture activities damaging aquatic ecosystems [1,2]. Management of the stock in an intensive aquaculture system presents several challenges particularly associated with maintaining optimum husbandry conditions, including water quality and feed efficiency. Many environmental factors may deteriorate dramatically, and an imbalance of these parameters is detrimental to animal health. Because of the intimate relationship of aquatic animals and the water environment in an aquaculture system, the environmental parameters are crucial to monitor and adjust within the optimum ranges for each species, for example, dissolved oxygen (DO), pH, alkalinity, temperature, ammonia ($\text{NH}_3\text{--N}$), nitrite ($\text{NO}_2\text{--N}$), and nitrate ($\text{NO}_3\text{--N}$). Despite helping maintain rearing conditions, regular water exchange has become unsustainable with its perceived environmental costs and biosecurity concerns [3,4].

Factors affecting the health of reared animals in intensive aquaculture are many [5,6]. Disease outbreaks in aquaculture systems are associated with the imbalance of three factors: the environment, the overall health of the animals, and the abundance of pathogens. As the environment in an intensive aquaculture system tends to fluctuate significantly, it causes stress to the animals and ultimately undermines animal health; such imbalances contribute to the surge of disease outbreaks in aquaculture. The consequent use of therapeutic chemicals and antibiotics has also resulted in antimicrobial resistance (AMR) [8], rendering antibiotics ineffective and severely impacting human health globally.

Apart from the threat of water pollution (Fig. 6.1), aquaculture predominantly uses the scarce water resources for food production, with on-farm water use of approximately 45 tons/kg of fish produced from pond farming compared to other livestock [4]. However, intensive aquaculture could considerably reduce this water consumption by efficiently recycling water using filters as in a recirculating aquaculture system (RAS) or generating some portion of the supplementary feed from within the system by efficient bioconversion of waste materials into protein as in a biofloc production system. Both these systems also facilitate maintaining the health and well-being of the culture stock, significantly contributing to aquatic animal welfare.

The solutions for technology advancement to counter the inherent issues of intensive aquaculture systems should address the issues mentioned above—poor water quality, high nutrient discharge, biosecurity, and animal welfare, for which the application of biofloc technology has emerged as a potential solution for sustainably intensifying the aquaculture practices [4,7]. This chapter elucidates the theory and practice of biofloc technology in aquaculture, emphasizing the key benefits, and the emerging biotechnological innovations underpinning the transformation of biofloc application into a more robust technological solution to overcome the challenges facing aquaculture intensification.

FIGURE 6.1 Sediments from a harvested shrimp pond bottom, washed and discharged to the environment. *Credit: K.R. Salin.*



2. Basic concepts of biofloc technology

Biofloc is an aggregate of living and nonliving matter formed by the flocculation of uneaten feed, fish feces, and several tiny aquatic animals stabilized by a microbial matrix suspended in water in a biocolloidal form. Biofloc (bio = life, floc = a clump) is thus primarily an aggregate of living organisms, such as algae, bacteria, protozoans, zooplankton, and organic matter, into a symbiotic structure. Biofloc technology refers to forming the flocs from particulate waste, making water cleaner and highly nutritious for animal consumption. This can be regarded as a self-cleaning system with microbial activity controlling water quality by removing toxic metabolites. In conventional pond culture, water quality is regulated predominantly by microalgal removal of nitrogenous wastes, often resulting in unstable water quality, particularly in a fluctuating pH. However, in a biofloc system, bacteria, predominantly of the heterotrophic group, control the water quality, aided by regular addition of organic carbon compounds providing the base for their proliferation utilizing the available nitrogen from the culture system. Thus, the stability of a biofloc system depends on the carbon:nitrogen (C:N) ratio that ensures maintenance of an adequate bacterial population to stabilize the rearing environment.

As an intensive aquaculture system, biofloc is suitable for several finfish and shellfish, including tilapia, catfish, and shrimp. High stocking densities and intensive aeration are required for developing and maintaining a stable biofloc throughout the crop period. Unlike an RAS that depends on the activity of chemoautotrophic bacteria in a biofilter removing the nitrogenous metabolites, it is the heterotrophic bacteria with much more bioconversion efficiencies than autotrophic bacteria that predominate in biofloc production systems. Therefore, the water quality in a biofloc system is often stabilized much more efficiently than in an RAS without mechanical/biological filters or pumps and recirculation. However, biofloc units need continuous monitoring by technically sound personnel for long-term stability and successful operation.

Like the biofloc system, the RAS exploits the knowledge of microbiology to maintain inorganic nitrogen in the water, but the operation is different. The RAS's features resemble a 'clear water' system, in which the majority of suspended solids are filtered out of the system. Vigorous aeration removes dissolved nitrogen by a series of processes in which ammonia is first transformed to $\text{NO}_3\text{-N}$ by nitrifying bacteria, then turned to the gaseous form of nitrogen (N_2) by the denitrifying process before being diffused out of the system. However, in the biofloc system, solid waste is partially recycled and becomes nutritious food for cultured animals. It is also noteworthy that in the biofloc system, animals are immersed with microbial community, which is influenced by the composition of this diversity, while in the RAS, a separate mechanism is used for the growth and application of the nitrifying bacteria, the biofilter.

2.1 Feed the pond, not the fish

In aquaculture, managing the concentration of inorganic nitrogen ($\text{NH}_3\text{-N}$, $\text{NO}_2\text{-N}$, $\text{NO}_3\text{-N}$) is crucial as these ions are detrimental to aquatic animals [8]. This waste can be efficiently recycled in a biofloc system with the involvement of the microbial community. For a sustainable future for aquaculture, the use of water should be optimized, and there is a need for a paradigm shift in the aquaculture practice, 'feeding the pond, not the fish' as envisioned by Verdegem et al. [3],

emphasizing the need for a closed aquaculture system to save water and reduce effluents. Feed for aquatic animals ends up 50%–70% wasted; hence, the key for this outlook complies with recycling the waste for better use. ‘Feed the pond,’ to be precise, is to feed microorganisms that may rely on the 70% wasted resources to thrive. The biofloc system fits this description and, with our knowledge of the microbial relationships, has demonstrated its ability to recycle nitrogenous waste to provide natural food for fish and shrimp [4,7].

Supplementary feeding is one of the most critical components in intensive aquaculture, affecting animal productivity and economics of its operation. Wasting a substantial portion of the feed during the crop is detrimental to sustainable food production apart from its impact on the environment, resulting in environmental pollution and emission of greenhouse gases contributing to climate change. While aquaculture’s share of its impact on climate change is much less than terrestrial crops, its potential future contribution to climate change could be higher, given its rapid expansion as the fastest-growing food production system [9]. Therefore, production technologies that can reduce the impact of feed wastage by converting organic waste back to feed by nutrient recycling are feasible to ensure aquaculture sustainability. Biofloc typically suits this requirement as a futuristic technology for intensive aquaculture.

2.2 Microbial community in the water, biofloc formation

The three crucial microorganism groups thriving in aquaculture systems include phytoplankton, chemoautotrophic (nitrifying) bacteria, and heterotrophic bacteria. Each of them has its function and specific mutual relationships within the system. Bioflocs either are generated from the existing microbes within the system or can be introduced as an inoculum prepared separately. In the initial phase of biofloc formation, the phytoplankton dominate the system, followed by a transformation to the heterotrophic bacterial dominance indicating biofloc formation. Nutrient inputs substantially affect the growth of these microorganisms and need to adhere to the C:N ratio. The addition of a suitable carbon source is necessary to maintain the balance of heterotrophic bacteria as they need organic compounds for growth. The nutritional quality of the flocs is also influenced by several parameters such as the carbon source, C:N ratio, and DO content, among others, which ultimately contribute to the beneficial characteristics of the biofloc system, particularly improving water quality, animal growth, and pathogen control and maintaining high meat quality of the farmed animal (Fig. 6.2).

Chlorophytes, diatoms, and cyanobacteria are the common phytoplankton groups that initially dominate a biofloc system [10]. This group uptakes ammonia and nitrate, and carbon dioxide, to produce organic biomass and releases oxygen into the water. Microalgae usually persist in the culture system from the beginning to the end. However, high nitrogen and phosphorus

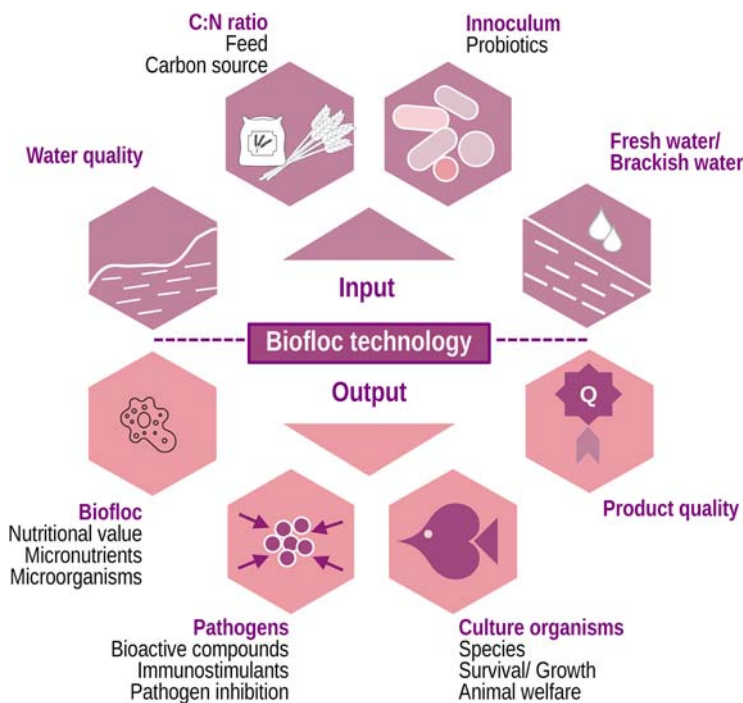


FIGURE 6.2 Various factors and their interrelationships in a biofloc system. Credit: N.T. Vinh.

FIGURE 6.3 Dominance of greenish microalgae (right) and brownish heterotrophic bacteria (left) in adjacent shrimp ponds. Credit: K.R. Salin.



concentrations can trigger an uncontrollable algal bloom, which causes oxygen depletion coupled with low pH, mainly during the early morning hours. The low light intensity can limit algae growth, and hence the potential benefits of microalgae in biofloc vary based on season and weather. Microalgae composition is highly divergent across culture systems and influenced by factors that can change over time; for example, in tilapia culture, *Chlorophyta* emerges first, followed by *Cyanobacteria*, both groups remaining till the end of an 8-week trial with the predominance of chlorophytes [11]. Similarly, in white shrimp culture, *Chlorophyta* dominated from the beginning to the late stage of the biofloc system [12]. Low salinity (2–4 ppt) might result in a high density of chlorophytes, while diatoms dominate in salinity of 25 ppt [13].

Heterotrophic bacteria are usually dominated in the later stage of biofloc (Fig. 6.3), indicating the change of water color from green to brown [14] and also the development of foam in water (Fig. 6.4), indicating the transition from an algal-dominated to bacterial biofloc [4]. This group can directly assimilate organic compounds and toxic ammonia nitrogen to biomass for other higher trophic species [4]. Unlike microalgae, heterotrophic bacteria must rely on organic compounds as the carbon source to grow. In intensive systems, despite high input, the growth of heterotrophic bacteria is limited by the amount of organic carbon. Additional carbon sources (e.g., molasses and wheat) must be added to stimulate heterotrophic bacterial growth and reach the suitable C:N ratio required for bacterial cell structure. Heterotrophic bacteria have a short generation time of 30 min compared to 12 h for chemoautotrophic bacteria [15]. Therefore, a high C:N ratio can often stimulate the growth of heterotrophic bacteria, and as they need nitrogen for cell construction, nitrogen can be removed much faster [14].

FIGURE 6.4 Foam formation in a biofloc pond during the transition from algal to bacterial phase. Credit: K.R. Salin.



TABLE 6.1 Stoichiometry for the metabolism of different microorganism groups on 1.0 g NH_4^+ –nitrogen.

		Photoautotrophic algae	Autotrophic bacteria	Heterotrophic bacteria
Consumption (g/gN)	Alkalinity (HCO_3^-)	3.13	7.05	3.57
	Carbon dioxide (CO_2)	18.07	—	—
	Oxygen (O_2)	—	4.18	4.71
	Carbohydrate	—	—	15.17
Yield (g/gN)	VSS ^a	15.85	0.2	8.07
	Carbon dioxide (CO_2)	—	5.85	9.65
	Oxygen (O_2)	15.14	—	—
	Nitrate (NO_3^-)	—	0.976	—

^aVolatile suspended solid (VSS), used to represent microbial biomass.

Modified from J. M. Ebeling, M. B. Timmons, and J. J. Bisogni, Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic removal of ammonia–nitrogen in aquaculture systems, *Aquaculture*, 257 (1) (2006) 346–358. <https://doi.org/10.1016/j.aquaculture.2006.03.019>.

Nitrification is the process that transforms highly toxic ammonia ($\text{NH}_3\text{--N}$) to nitrite ($\text{NO}_2\text{--N}$), and finally, nitrate ($\text{NO}_3\text{--N}$), performed by diverse groups of chemoautotrophic bacteria. The nitrogen removal capability of each microbial group is different based on their metabolic characteristics. This can be summarized by the stoichiometry of metabolism as presented in Table 6.1, with the intake and output of metabolic products of microorganisms to convert 1.0 g ammonium nitrogen [17]. Although heterotrophic bacteria can remove nitrogen much faster than nitrifying bacteria, up to 10 times [18], the latter still plays a vital role in the nitrogen cycle in a biofloc system. From NH_4^+ to $\text{NO}_2\text{--N}$, the first oxidation step is usually associated with the *Nitrosomonas* group, while *Nitrobacter* bacteria accomplish the steps to the final product, $\text{NO}_3\text{--N}$. All three forms of nitrogen are present in a water body; however, the most toxic form is $\text{NH}_3\text{--N}$, followed by $\text{NO}_2\text{--N}$ and $\text{NO}_3\text{--N}$. Efficient nitrification can shift the nitrogen cycle toward more $\text{NO}_3\text{--N}$. However, despite being less toxic, a high concentration of $\text{NO}_3\text{--N}$ can cause trouble for oxygen uptake in fish [8] and can be removed by the uptake of microalgae or by the denitrifying process.

Zooplankton are usually found in abundance after establishing microalgae and bacteria [11]. This group includes protozoans, rotifers, amoeba, ciliates, and nematodes responsible for transferring nutrients to a higher trophic level and ultimately becoming live food for cultured animals [7]. Zooplankton foraging may shift the composition of microalgae, based on their food selection, for example, rotifers can reduce certain microalgal divisions in the water body [19]. They are also responsible for the alkalinity decline in water as the biofloc matures [12].

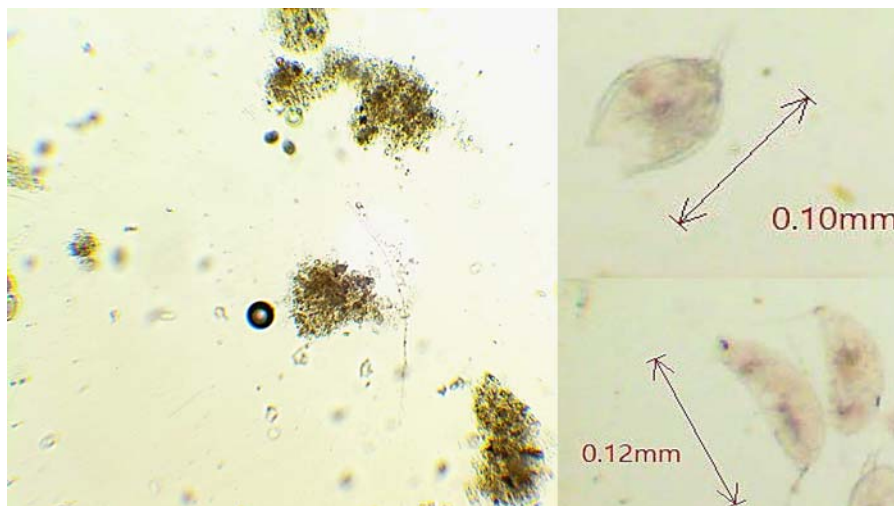
2.3 Biofloc formation

Biofloc is formed as an aggregate of tiny microorganisms composed mainly of heterotrophic bacteria, algae (chlorophytes, dinoflagellates, diatoms), fungi, ciliates, flagellates, rotifers, nematodes, metazoans, and detritus in a symbiotic manner (Fig. 6.5) [20]. The aggregation process of microorganisms is associated with extracellular polymeric substances (EPS), biosynthesized by bacteria. The expanding matrix of these substances, mainly composed of polysaccharides, proteins, and DNA [21], encapsulates unicellular cells or attracts free-living cells by their electron affinity [14,22]. Benthic diatom cells also possess this ability and are hypothesized as a potential contributor to the formation of biofloc in saline biofloc systems [23]. However, the electrical charge of microorganisms and organic particles can affect the stability of bioflocs, as cells with the same affinity tend to push each other away; thus, additional ions like calcium and aluminum are recommended for maintaining stable bioflocs [14].

3. Operational parameters to optimize biofloc systems

Environmental parameters such as DO, pH, alkalinity, temperature, and salinity are fundamental to ensuring the optimal growth of farmed aquatic animals. Changes in environmental parameters can substantially shift the production of microorganisms, and so it needs particular care to maintain these parameters within optimum ranges. This is more

FIGURE 6.5 Biofloc particles viewed under a microscope. Credit: K.R. Salin.



critical in biofloc systems as the activity of the microbial community can change the environment dramatically; thus, continuous monitoring is always required. Changes in microbial community alter floc characteristics affecting floc availability to the cultured animal and are evaluated by the sludge volume index (SVI), a valuable indicator tracking the changes occurring in an activated sludge process. Floc volume index (FVI) is another parameter that helps understand the biofloc system's stability. It represents the volume occupied by 1 g of floc—VSS (volatile suspended solids) computed as mL/g by measuring the floc settled in an Imhoff cone following 30 min of sedimentation and the VSS value. Some of the most important environmental parameters affecting the stability of a biofloc system are discussed in this section.

3.1 Temperature

The effect of temperature on bioflocs is complex as it impacts microbial activity and the floc structure. At a low temperature of 4°C, deflocculation occurs in activated sludge [24], while bulking of the sludge with a high SVI of over 500 mL/g was observed at high temperatures (30–35 °C) [25]. This is because the temperature directly affects the metabolism of microorganisms and so the microbial kinetics due to excessive production of EPS by microbes [22]. The extremity of temperature may bring adverse results. Increasing metabolic rates at higher temperatures result in enhanced biomass production, contributing to greater organic matter and total settleable solids with consequent higher SVI values. In the case of pink shrimp, *Farfantepenaeus brasiliensis*, better shrimp survival rates in a superintensive biofloc nursery were found at temperatures between 21 and 27 °C, although the total suspended solid (TSS) levels were higher than optimal (ranging from 855 to 963 mg/L), while the best growth performance was obtained at 27 °C [26]. The best water temperature range to achieve optimum SVI of about 200 mL/g to provide stable flocs was recommended between 20 and 25 °C [22]. However, consideration of temperature is often beyond the farm operator's capacity. So, the choice of the species and farming system is dependent on the prevailing weather conditions.

3.2 Dissolved oxygen

Adequate DO levels in biofloc ponds are essential for maintaining proper microbial cell metabolism and can influence floc composition and the structure of bioflocs. More compact and larger bioflocs appear to be obtained in high DO levels at 2.0–5.0 ppm with lower SVI values of about 100 mL/g, while low DO of 0.5–2.0 ppm results in loose and smaller bioflocs but with a higher SVI of 250 mL/g [27]. There seems to be a shift in microbial composition, from high floc-forming zoogloeal bacteria at high DO to the dominance of filamentous bacteria with greater oxygen affinity at low DO to outcompete the former, but with less capacity to aggregate organic matter. Although low DO conditions result in higher SVI, which is desirable for slow sinking flocs for animals to consume without accumulating in pond bottom, too large FVI may cause clogging in gills apart from the dramatic effects of low DO to cultured animals, such as reduced feeding [22].



FIGURE 6.6 Proper aeration also helps in sludge removal from the center of a biofloc pond; an empty pond (left) and a fully operational pond (right). *Credit: K.R. Salin.*

3.3 Mixing intensity

Mixing, mainly by aeration, keeps bioflocs suspended and maintains DO concentration in a water body. However, vigorous mixing may also break the biofloc particles structurally, rendering them smaller. Hence, an appropriate mixing intensity is essential and regulated at a reasonable level where the aggregation and breakage rates are balanced, and floc sizes are uniformly distributed in the pond (Fig. 6.6) [28]. Floc size can influence the nutritional value of biofloc; Ekasari et al. [29] observed that larger flocs ($>100\ \mu\text{m}$) contained a higher percentage of protein, but smaller flocs ($<48\ \mu\text{m}$) had better protein quality indicated by their more balanced amino acid profile. Floc size also determines the choice of biofloc for consumption by various farmed species, which means biofloc size must be in the forageable range for cultured animals to feed on [22]. Different aeration types may determine flocculation efficiency, for example, among propeller aspirator pump aerator, vertical pump aerator, and diffused air blower, air blower gives the best result in the aggregate and formation of bioflocs (Fig. 6.7) [30].

3.4 Alkalinity and pH

Alkalinity in a biofloc system may decline drastically due to the assimilation of bacteria, especially autotrophic bacteria that need inorganic carbon (carbonate and bicarbonate) as the energy source. In order to transform 1g of ammonia nitrogen, heterotrophic bacteria consume roughly 3.57 g alkalinity [17]. Low alkalinity can result in high pH fluctuations that impose stressful conditions for cultured animals, making them vulnerable to infectious diseases and stunted growth. Unfavorable pH can also affect the stability of bioflocs. For the normal development of heterotrophic and nitrifying bacteria, pH of 7.0–9.0 is recommended [31]. However, pH lower than 6.5 or higher than 9.5 can reduce the productivity of bioflocs in water [14]. Every cultured species can only grow well within a specific range of pH; for example, pH of 6.8–8.0 is suitable for most freshwater



FIGURE 6.7 Additional diffusers from air blowers are often required in a biofloc pond. *Credit: K.R. Salin.*

species, but pH lower than 7.0 can result in poor microbial proliferation [10]. Raising alkalinity can help maintain suitable pH while ensuring the growth of animals in a biofloc system [32]. Chemical additives to regulate alkalinity drop in bioflocs include sodium bicarbonate (NaHCO_3), sodium carbonate (Na_2CO_3), and calcium hydroxide ($\text{Ca}(\text{OH})_2$) [32–34]. Sodium bicarbonate is often suitable, while excess Ca^{2+} ions from chemical treatments can cause extreme hardness in water [33].

3.5 Carbon sources

Carbon sources can also influence the biofloc's behavior, reflected by the type and amount of storage polymers [35]. Complex carbon sources like rice bran, starch, and high cellulose carbohydrates have lower and delayed nitrogen removal efficiencies than soluble carbohydrates like sugars and molasses. However, complex carbohydrates may encourage higher microbial diversity in the pond [36] and produce bioflocs with higher protein content [37]. In other words, complex (insoluble) carbohydrates are more suitable to maintain bioflocs in the long term, while soluble carbohydrates can tackle spiked ammonia quicker [38]. There is considerable evidence that different carbon sources influence the microbial composition of bioflocs. For example, glucose and glycerol stimulate the growth of Proteobacteria and Bacteroidetes, while starch-based bioflocs encourage Cyanobacteria to bloom [39].

In addition, the price may also determine the selection of organic carbon sources for a biofloc system. Over time, besides common carbon sources such as glucose, acetate, and glycerol, more ingredients have been added to reduce price constraints, and also, there is a need to find more viable carbon sources for a biofloc system [40] to meet the desired production goals. Some alternatives have been researched recently, such as jaggery (a sugarcane product) [41], biochar [38], and tapioca [36,42], among others. It is best to use local or by-product carbon sources to reduce costs [22]. However, by-product carbon sources are generally high polymers (e.g., rice bran), which affect the timing of biofloc formation. When these sources are added to the pond, the decomposition process first occurs to reduce polymers to monomers—a simpler form that heterotrophic bacteria can use to assimilate ammonia nitrogen [43]. This process demands more oxygen consumption and can lower pH. This, however, can be overcome with fermentation to partially break down high polymers [44,45].

3.6 C:N ratio

The carbon to nitrogen ratio (C:N ratio) determines the stability and composition of the microbial community in a biofloc system that will impact the growth of farmed species. Usually, the ratio that falls between 10 and 20 may achieve the best growth [14]. However, at the start of the crop, more carbon is required to trigger bacterial growth, and the addition of carbon can be reduced once the bioflocs have 'matured' [14]. It is recommended that the C:N ratio be adjusted considering the microbial dynamics in the system.

The application of carbon sources to adjust the C:N ratio can be divided into two phases: (1) during the initiation and biofloc formation, a ratio of 12–20:1 is recommended, and (2) in the steady phase, a low C:N ratio, for example, 6:1 is favorable for rapid removal of ammonia nitrogen [10]. The first phase is aimed to increase heterotrophic bacterial biomass in the system; hence, high carbon is needed. High C:N ratio encourages accumulating more suspended solids until the bioflocs mature (around 30–50 days) when the floc levels reach 5 mL/L. During the formation phase, suspended solids must be monitored to avoid excessive application of carbon, which may result in high sludge, higher oxygen demand, and sometimes gill choking to fish or shrimp. The growth of bioflocs can be accelerated by restocking flocs from a previously matured system as they already have necessary microorganisms. After biofloc maturation, a lower carbon rate can be maintained (or stop carbon application completely). When total ammonia nitrogen (TAN) rises over 1 mg/L, C:N ratio of 6:1 is helpful to remove excess nitrogen. In this case, monosaccharides, and oligosaccharides such as molasses and sugars, can act faster to the response of heterotrophic bacteria to assimilate nitrogen.

Changing C:N ratio was reported beneficial for carp culture. A high C:N ratio of 19–23:1 was found to remove ammonia nitrogen during the first 4 weeks efficiently, but after that, high C:N had no significance in nitrogen removal compared to low C:N of 11–15:1 [46]. This indicates the need for a lower C:N ratio in mature bioflocs to reduce the cost and the risk of low DO in the pond. In rearing *Litopenaeus vannamei* juveniles, Xu et al. (2015) [34] found that C:N of 9:1 to 12:1 using molasses resulted in more microalgae and autotrophic bacteria while a high carbon input of 18:1 shifted more toward heterotrophic bacteria. It was also revealed that low C:N was more favorable in this case as microalgae and autotrophic bacteria supported shrimp growth better. In addition, using molasses as a carbon source, the African catfish, *Clarias gariepinus*, showed better growth at C:N = 20:1, while the ratio of 15:1 had higher nitrogen removal capacity [47]. Although more research on the desirable C:N ratios to suit specific production targets and species is needed, from currently available information, C:N ratios ranging from 10:1 to 15:1 appear safe for most biofloc systems for fish, while for shrimp production systems, lower C:N ratios are desirable.

4. Benefits of biofloc production systems

Biofloc systems have several advantages in reducing water consumption and improving water quality in an intensive culture model. Significant reduction in water exchange is possible while still maintaining stable water quality conditions, provided an appropriate management protocol is followed with continuous monitoring of various parameters. While the reduction of ammonia, nitrite, and nitrate is the key to improving water quality, the simultaneous reduction in alkalinity and increase in suspended solids become major challenges that can be overcome by the regular addition of chemicals like sodium bicarbonate or by the regulated supplementation of carbon sources like wheat flour. Bioflocs also provide supplementary nutrition for the farmed fish and shrimp, enabling lower feeding rates and feed conversion ratio (FCR), and potentially faster growth rates than conventional systems. The specific microbial communities in a biofloc system help keep the stress levels of fish low while imparting a natural inhibition and some degree of resistance against many pathogens. Farmed fish meat quality is also known to be affected by biofloc farming conditions. The multiple benefits of biofloc technology in optimum water quality management, providing supplementary nutrition, health management, and product quality are discussed in the following section.

4.1 Reduced water use, effluents, and improved water quality

One of the most significant benefits of a biofloc system in aquaculture production is its impact on maintaining optimum water quality parameters throughout the crop period. The multitude of phytoplankton, heterotrophic bacteria, and chemoautotrophic bacteria mutually play a central role in removing organic matter and nitrogen, the primary pollutants in the biofloc system [7,10]. However, this process has some constraints, and recent research aims to resolve the inherent limitations in biofloc systems, such as lowering alkalinity and high total suspended solids (TSS). Testing three carbon sources such as sugarcane molasses, tapioca flour, and wheat flour in a biofloc trial with white shrimp *L. vannamei*, Rajkumar et al. [37] found wheat flour to be superior in many aspects, including reducing ammonia and nitrate levels. However, alkalinity in wheat flour-supplemented treatment reduced faster, hence the need to supply NaHCO_3 . Although high biochemical oxygen demand (BODs) were observed in all biofloc-related treatments, DO remained suitable for the shrimp (at > 4 ppm). The use of wheat flour was also examined by Kim et al. [48], revealing that the addition of wheat flour helped maintain the pH and alkalinity at a stable level (even without providing sodium bicarbonate) while reducing TAN and nitrate-N, and it could even reverse the environmental deterioration in treatment with no previous addition of wheat flour. In culture conditions with adequate sunlight, microalgae, especially Cyanobacteria, proliferate, which is found to have some advantages. Biochar from Cyanobacteria has the potential to produce a biofloc system with less TSS (reduced to ~ 505 from ~ 665 mg/L compared with the use of glucose alone as a carbon source) while still retaining its unique ability of nitrogen removal from the system [38]. Combining glucose and biochar provides a synergistic effect that maintains a healthy microbial community through the fast- and slow-soluble carbohydrates. Another option to reduce the nuisance of excess TSS might be to integrate the biofloc production system with grazers such as mullets, as demonstrated by Holanda et al. [49] (see Section 5.1 in this chapter).

In a pond aquaculture system, water consumption in general (total water lost by evaporation, seepage, and exchange) is estimated to be $16.9 \text{ m}^3/\text{kg}$ of fish produced [3]. Limiting water exchange in a biofloc system could significantly reduce this with the daily exchange rate that can be kept as low as 1% (or even 0.5%), helping save a significant amount of water [4]. The water use in a biofloc system was examined in Nile tilapia, *Oreochromis niloticus* culture, which was substantially lowered by 186 times (from $23.93 \text{ m}^3/\text{kg}$ production to only $0.13 \text{ m}^3/\text{kg}$) compared to a conventional production system [50].

Many bacterial groups (Proteobacteria, Bacteroidetes, Actinobacteria, Planctomycetes, Verrucomicrobiota, etc.) have been extensively reported in rearing water and animal gut. The microbial composition is differentiated by complex factors such as carbon source, C:N ratio, stocking density, environmental parameters, and culture period (Table 6.2). Complex carbohydrates are often associated with higher bacterial diversity in biofloc systems [36,39,51]. Among common bacterial groups in the biofloc system, Proteobacteria is important in the nitrogen cycle and removing organic matter [55]. The abundance of gram-negative Proteobacteria in gut biota may also enhance the overall health of aquatic animals [56]. Actinobacteria are composed of many probiotic species, and high temperatures may result in the proliferation of this group [57]. Bacteroidetes abundance tends to increase with organic matter; thus, their role may be associated with organic degradation in aquaculture systems [58]. Some Flavobacteriaceae of Bacteroidetes phylum are pathogenic [59], especially in low water temperatures [48], while free-living genera may be beneficial in biofloc formation as they can secrete extracellular binding polymers [58]. Chloroflexi is more abundant in high temperatures [51], and they play actively in organic degradation [60]. However, Planctomycetes are more prevalent in lower temperatures [48]. Many genera of this group possess the ammonium oxidation ability, which contributes to nitrogen cycling in the

TABLE 6.2 Organic carbon sources affecting water quality and microbial composition in biofloc systems.

Parameters assessed	Cultured species	Water quality	Microbial community (phylum level)	References
Carbon source: Wheat flour, in relation with temperature and culture period	<i>Litopenaeus vannamei</i>	Positive effects on NH ₃ , NO ₂ , NO ₃ , and alkalinity. Wheat flour might recover deteriorated water even when added late during a crop, but this is associated with fluctuating pH and alkalinity.	Higher microbial diversity with wheat flour addition. Core bacterial phyla include <i>Proteobacteria</i> , <i>Planctomycetes</i> , <i>Actinobacteria</i> , <i>Bacteroidetes</i> , and <i>Verrucomicrobiota</i> . The difference of core phyla depended more on the culture period, followed by habitat (in rearing water or shrimp's intestine and stomach) and temperature over wheat flour treatment. <i>Proteobacteria</i> decreased largely during culture, while the rest increased. <i>Proteobacteria</i> and <i>Bacteroidetes</i> were most abundant in rearing water, while <i>Planctomycetes</i> and <i>Verrucomicrobiota</i> dominated intestinal microbiota, and the stomach was dominated with <i>Actinobacteria</i> phylum. Elevated temperature (>29 °C) led to the dominance of <i>Proteobacteria</i> and <i>Actinobacteria</i> , while <i>Bacteroidetes</i> were abundant at 25 °C.	[48]
Carbon source: Glucose, starch, and glycerol	No animal	Dissolved oxygen (DO) higher in treatment with starch, followed by glucose and glycerol. NH ₃ , NO ₂ , and NO ₃ are similar in glucose and glycerol treatments and much lower in starch treatment.	<i>Proteobacteria</i> and <i>Bacteroidetes</i> were dominant in all treatments, but in starch treatment, <i>Cyanobacteria</i> constituted a major proportion. Adding glucose and glycerol as carbon sources resulted in a greater microbial diversity than starch.	[39]
Carbon source: Biochar (B), glucose (G), and mixed use of glucose and biochar (GB)	<i>Oreochromis niloticus</i>	All treatments showed positive inorganic nitrogen control (highest efficacy in G); DO, pH, and alkalinity in suitable ranges. The inclusion of biochar (when alone or combined with glucose) reduced the total suspended solids.	Core phyla were <i>Actinobacteria</i> , <i>Proteobacteria</i> , <i>Chloroflexi</i> , <i>Firmicutes</i> , <i>Planctomycetes</i> , and <i>Bacteroidetes</i> . In bioflocs, <i>Actinobacteria</i> and <i>Proteobacteria</i> were the most dominant phyla in both G and GB, whereas <i>Chloroflexi</i> and <i>Proteobacteria</i> were most abundant in B. In the gut, the most dominant phyla featured a similar pattern. In a carbon source mixture, one source might play over another (especially soluble and non-soluble mixture) in shaping the microbial community, e.g., glucose in this study.	[38,51]
Carbon source: Tapioca flour, plant cellulose (carp excreta), and the mixture of both carbon sources, C:N ratio > 13:1	<i>Pelteobagrus vachelli</i>	TAN decreased significantly in biofloc systems. No significant difference among biofloc systems in pH, DO, chemical oxygen demand, nitrite, and nitrate. In the late stage of culture, pH decreased dramatically in resonance with decreased dissolved organic carbon.	Bacterial richness was similar in all biofloc systems, but higher diversity was associated with different biofloc structures, especially plant cellulose. Added cellulose provided the substrate for several types of bacteria to thrive. Thirteen phyla were found, but <i>Proteobacteria</i> and <i>Bacteroidetes</i> were the first and second most abundant phyla in all treatments. Subclass <i>Alphaproteobacteria</i> was more prevalent in cellulose-added treatment, whereas <i>Betaproteobacteria</i> was found predominantly in treatments with tapioca flour and the control group (no added carbon).	[36]

TABLE 6.2 Organic carbon sources affecting water quality and microbial composition in biofloc systems.—cont'd

Parameters assessed	Cultured species	Water quality	Microbial community (phylum level)	References
Stocking density—using brown sugar (C:N ratio of 15:1)	<i>L. vannamei</i>	NA	High (800/m ²) and low (400/m ²) stocking densities had higher diversity than medium (600/m ²) density. <i>Proteobacteria</i> was mostly dominant in both water and the gut. Besides <i>Proteobacteria</i> , <i>Actinobacteria</i> , and <i>Planctomycetes</i> were most prevalent in the gut, whereas <i>Bacteroidetes</i> were abundant in water. In rearing water, high stocking density treatment had more <i>Proteobacteria</i> , whereas <i>Actinobacteria</i> were abundant in low stocking density. Similar dynamics were observed in gut microbiota, indicating the influence of water and gut microbiota through the consumption of bioflocs.	[52]
Light and C:N ratio (carbon source molasses): Low C:N ratio with light condition (LCL), low C:N ratio with dark condition (LCD), high C:N ratio with light condition (HCL), and high C:N ratio with dark condition (HCD) <i>Low C:N treatments without additional carbon</i>	<i>L. vannamei</i>	TAN was not significantly different in all treatments; high NO ₂ and NO ₃ nitrogen in treatments with added carbon; without-light treatments displayed poorer water quality.	Bacterial richness increased continuously with added carbon, but diversity did not change. Phyla (over 1% prevalence): <i>Bacteroidetes</i> , <i>Proteobacteria</i> , <i>Cyanobacteria</i> , <i>Actinobacteria</i> , <i>Verrucomicrobiota</i> , <i>Gracilibacteria</i> , <i>Chloroflexi</i> , <i>Chlorobi</i> , and <i>Planctomycetes</i> . <i>Bacteroidetes</i> and <i>Proteobacteria</i> constituted over 74% of all treatments. When additional carbon was applied, <i>Bacteroidetes</i> tended to decrease at the end. <i>Cyanobacteria</i> were high in treatments with light. Biofloc system with light might have more beneficial bacteria than without light, which could trigger the growth of harmful bacteria; but light could also result in the blooming of toxic <i>Cyanobacteria</i> . Water quality affected the microbial composition.	[53]
C:N ratio: 15:1, 20:1, tapioca starch	<i>Carassius auratus gibelio</i>	Nitrogen removal was better with a higher C:N ratio.	<i>Proteobacteria</i> , <i>Deinococcus-Thermus</i> , <i>Bacteroidetes</i> , and <i>Planctomycetes</i> were dominant in C:N ratio of 15. In C:N ratio of 20:1, <i>Proteobacteria</i> , <i>Bacteroidetes</i> , and <i>Planctomycetes</i> were dominant. <i>Bacteroidetes</i> had a much higher proportion in C:N ratio of 20:1 than in 15:1. Mainly aerobic denitrifying bacteria might contribute to high nitrogen removal capacity in <i>Bacteroidetes</i> phyla.	[54]

water environment [55]. The above are just a few examples of the microbial function of different groups in biofloc systems. The central picture of microbial activity related to many parameters is largely unknown. Extensive research in this field may help optimize the efficacy of biofloc systems in the future.

4.2 Enhanced nutrition for cultured animals

It is estimated that 50%–70% of nitrogen is lost to a water body or built up in the pond's bottom in conventional farming systems. This high load of nutrients is a potential source of pollution in the culture system and outside environment. However, in the biofloc system, this high nutrient availability becomes useful if suitable microorganisms are included; they assimilate the

excess nutrients to store in their cell and finally aggregate into nutritious bioflocs that benefit the consuming animals. By this characteristic, the flocs usually are high in protein content, which provides excellent natural food for the animals and an alternative feed ingredient for supplementary feed in the future [61,62]. However, the biofloc's nutrients values differ based on floc sizes [29], and the type of organisms coexisting in the bioflocs due to the difference of carbon source and C:N ratio.

The presence of bioflocs in aquaculture systems provides continuously available feed supplements for farmed aquatic animals that can reduce the feeding rate and lower FCR. Fish and shrimp fed on bioflocs also showed a higher growth rate due to better digestibility and retention ratio of nitrogen [56,63]. Increased activity of digestive enzymes such as proteases, amylases, and lipases, which are essential in breaking down polymeric macromolecules to smaller molecules, aiding digestibility of fish and shrimp, was also demonstrated in fish and shrimp reared in the biofloc system, while these advantages are believed to be associated with better gut microbes [48,64,65]. Table 6.3 summarizes some of the recent findings on the effect of various carbon sources on improving nutrient efficiencies of the biofloc systems and growth performance of various species farmed.

TABLE 6.3 Organic carbon sources affecting water quality, nutritional quality, and animal growth performance in biofloc systems.

Carbon source(s)	Species	Water quality	Biofloc nutritional value	Growth and/or health benefits	References
Jaggery, C:N ratio = 12:1, 20:1	<i>Oreochromis niloticus</i>	pH and DO in suitable ranges. TAN, nitrite, and ammonia were removed faster with high jaggery addition. Higher total suspended solids with high jaggery input.	Crude protein (CP): 38.5%–51.9% Crude lipid (CL): 6.01%–6.67% Crude fiber (CF): 15.63%–17.1% Ash: 10.67%–11.73%	Better growth performance, higher survival rate, immunostimulation, and resistance to <i>Aeromonas hydrophila</i> .	[41]
Biochar (B), glucose (G), and mixed use of biochar and glucose	<i>O. niloticus</i>	Glucose reduced DO significantly compared to biochar but showed better nitrogen removal. Biochar reduced total suspended solids. Alkalinity was lower in biochar treatments.	CP: 25.9% CL: 1.83%	Similar growth and physiological parameters in all treatments. Biochar and glucose could compensate the disadvantages of both carbon sources (associated with low and quick response of non-soluble and soluble sources).	[38]
Sugarcane molasses, tapioca flour, and wheat flour	<i>Litopenaeus vannamei</i>	Lower DO in molasses treatment. All biofloc systems showed better nitrogen removal than non-biofloc ones (control). Nonsoluble sources had lower TAN, nitrite, and nitrate but higher suspended solids and chlorophyll a concentration.	Sugarcane molasses: CP: 45.98%, CL: 0.57%, CF: 12.92%, and ash: 22.53% Tapioca flour: CP: 52.03%, CL: 0.7%, CF: 15.25%, and ash: 14.88% Wheat flour: CP: 53.65%, CL: 0.92%, CF: 16.65%, and ash: 25.04%	Increased growth with biofloc system but no difference in survival rate. Shrimp grown with wheat flour treatment had the highest growth performance compared to other carbon sources. Biofloc consumption might enhance protein content in shrimp meat.	[37]
Maida flour, wheat flour, gram flour, millet flour, rice flour, corn flour, molasses, and multigrain flour	<i>L. vannamei</i>	Biofloc systems showed high control of TAN, nitrite-N, nitrate-N, and PO ₄ , but reduced alkalinity significantly (especially in molasses treatment). Chlorophyll, total suspended solids, floc volume, and turbidity were higher in millet, multigrain, and molasses treatments.	No proximate composition data provided. Millet flour, molasses, and multigrain flour possessed higher amount of essential amino acids.	Higher growth and survival with the addition of millet flour, molasses, and multigrain flour. Challenge test revealed that these carbon sources might upregulate immune genes, hence increasing the ability of shrimp to combat <i>Vibrio parahaemolyticus</i> .	[66]

TABLE 6.3 Organic carbon sources affecting water quality, nutritional quality, and animal growth performance in biofloc systems.—cont'd

Carbon source(s)	Species	Water quality	Biofloc nutritional value	Growth and/or health benefits	References
Wheat milling by-product and rice bran, C:N ratio = 15:1, feed designed with 20% and 30% protein content	<i>O. niloticus</i>	Significantly higher TAN, nitrite, and nitrate in biofloc systems than control (water exchange system).	NA	Improved growth performance and immunology of fish reared in biofloc system. Bioflocs might help lower protein content in the feed to 20% instead of the recommended level of 30%. Wheat milling by-product appeared a better carbon source for Nile tilapia.	[67]
Poly-β-hydroxybutyrate (PHB), polycaprolactone (PCL), and (to compare with) glucose	<i>O. niloticus</i>	Better maintenance of dissolved organic carbon and ammonium ratio (DOC:NH ₄) in PHB and PCL in glucose treatment. Both PHB and PCL had a similar nitrogen removal efficiency as glucose addition in biofloc system.	CP: 31%–39% for all treatments, significantly higher in glucose-added treatment CL: >5% and not significantly different in all treatments	Similar growth performance was observed in all treatments, but biofloc system with glucose supply had slightly better growth.	[68]

4.3 Biosecurity and disease prevention

Biofloc can potentially protect fish and shrimp from pathogens by either enhancing their immune response, which translates to better resistance to disease agents, or developing specific inhibition mechanisms to keep pathogens under control [7,40,69]. It has been found that besides nutrients in bioflocs, there are bioactive compounds such as chlorophylls, bromophenols, beta glucan, carotenoids, amino sugars, and antibacterial components with beneficial effects on animal health. Those substances, apart from having properties of growth promoters [62], may improve the physiological health of animals [70,71] and contribute to the enhanced immunity in cultured species [7,63]. Another substance found extensively in the biofloc system is poly-β-hydroxybutyrate (PHB), an intracellular polymer secreted by many bacteria as a form of energy and carbon reserve for their survival during adverse conditions [72]. PHB is believed to confer positive effects on disease prevention in biofloc systems against pathogenic bacteria such as *Vibrio* spp. [73–75]. Dry bioflocs can contain up to 16% of PHB, which is sufficient to protect animals from bacterial infection [76].

Biofloc systems also render lower stress levels that improve health and translate to the ability to resist diseases in aquatic animals. Recent research has found a systematic reduction of cortisol (stress hormone) and glucose level in fishes reared in biofloc systems, such as *Labeo rohita* [63], carp (*Cyprinus carpio*) [77,78], and tilapia [79,80]; the latter two reports indicate that the fish also gain some advantages to adapt to adverse environmental conditions.

The protection from diseases in fish and shrimp is often associated with the upregulation of immune-related genes such as prophenoloxidase, lysozyme, serine proteinase, tumor necrosis factor alpha, interleukin 1 beta, beta glucan binding protein, and paroxetine [41,66,67,81] as summarized in Table 6.4. The upregulation of these genes (and the increased concentration in serum of corresponding protein) are linked to the enhanced innate immunity of animals cultured in the biofloc system. Antioxidant enzymes such as superoxide dismutase and catalase, whose combined function is vital in protecting the cell from oxidative damage by reactive oxygen species, were found upregulated in many species reared in biofloc systems [66,81]. This evidence has substantiated the benefits of consuming bioflocs structurally composed of

TABLE 6.4 Regulation of immune genes in fish and shrimp grown in biofloc systems.

Species	Related gene/protein	Significant result with	References
<i>Oreochromis niloticus</i>	Upregulated gene expression of lysozyme (LYZ) C, tumor necrosis factor alpha, and interleukin 1 beta Increased concentration of superoxide dismutase (SOD), malondialdehyde, catalase (CAT), and glutathione	C:N ratio = 20:1	[41]
<i>O. niloticus</i>	Upregulated mRNA expression of cytoplasmic manganese superoxide dismutase, beta glucan binding protein, paroxetine, SOD, and CAT	Different carbon sources resulted in different mRNA expressions for immune protein	[66]
<i>O. niloticus</i>	Increased concentration of myeloperoxidase (MPO), LYZ, SOD, and CAT	Biofloc system showed higher values over conventional system	[67]
<i>O. niloticus</i> (GIFT strain)	Increased concentration of glutathione peroxidase and LYZ	Biofloc system showed higher values over conventional system	[56]
<i>Labeo rohita</i>	Increased concentration of mMPO and LYZ	Higher with culture time (>60 days)	[63]
<i>Litopenaeus vannamei</i>	Upregulated expression of RAs-related nuclear gene, serine proteinase (SP) gene, prophenoloxidase (proPO) activating enzyme gene, and crustin gene	Mostly higher with a higher C:N ratio (>10:1)	[66]
<i>Fenneropenaeus chinensis</i>	Upregulated mRNA expression of proPO, LYZ, and SP Decreased concentration of SOD and CAT	From 120% biofloc	[81]

bacterial elements such as peptidoglycan, lipopolysaccharides, and β -1,3-glucans, stimulating the immune system and producing the suite of enzymes in the nonspecific immune mechanism of the animals [29,66,81].

The ability to disrupt quorum sensing, the cell-to-cell communication of bacteria through molecular signals, is believed to be a feature of the biofloc system to keep pathogenic bacteria under control [22,40,69]. Quorum sensing bacteria use signal molecules called inducers to respond to cell population density and stimulate gene expression of the cell population, which results in changes in symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation, and biofilm formation [82]. Quorum sensing inhibitors are molecules that interfere with bacterial quorum sensing signals and are found in various probiotics strains of *Lactobacillus*, *Bifidobacterium*, and *Bacillus* [83]. The presence of natural probiotics strains [84] in the biofloc system may contribute a promising strategy to control bacterial pathogens in aquaculture [40,62].

4.4 Aquaculture product quality

Fish farmed in biofloc systems appear to improve the meat quality if specific rearing protocols are followed. Tilapia reared in 24 h of light had better meat quality with the highest body protein and lipid than the fish reared in 24 h darkness [85]. The preferred fillet quality by sensory evaluation was obtained in tilapia reared in the biofloc treatment with sugar as the carbon source [86]. However, the biofloc system also poses potential issues in the carcass quality of the reared fish. One major problem related to aquaculture product quality in the biofloc system is the off-flavor of fish, mainly observed in an outdoor production environment, such as that reported in channel catfish (*Ictalurus punctatus*) and hybrid tilapia [87]. Two substances of bacterial origin, viz. geosmin and 2-methylisoborneol (MIB), are responsible for causing 'earthy' and 'musty' off-flavors, respectively, found primarily on filamentous cyanobacteria and sometimes actinomycetes [88]. These microorganisms proliferate in the biofloc system, attach to bioflocs, and impart off-flavor substances to the fish feeding on biofloc particles, rendering low-quality fillets. In tilapia, bioaccumulation of geosmin and MIB can occur during either passive diffusion through the gills or grazing of biofilm formed in the biofloc system; thus, frequent cleaning may help reduce off-flavor substance uptake [87]. Purging can be an option to eliminate off-flavor substances from fish. However, the ultimate solution lies in preventing fish from access to off-flavor sources associated with the development of certain microorganisms [89].

5. Recent advances in biofloc research

While the benefits of biofloc systems to improve water quality are much more apparent, the nutritional and aquatic health benefits are far more complex than initially thought. A better understanding of the microbial communities and their interactions with water quality parameters and animal behavior is required to enhance the specific beneficial effects rendered by bioflocs. Polyculture and integrated multitrophic systems based on improved trophic efficiencies of compatible species cocultured in the same production systems have been beneficial. New research unraveling the intricacies of microbial interactions affecting flocs, their structure, and nutritional quality for efficient consumption for improved growth, immunity, and disease resistance of commercial species is emerging.

5.1 Better food utilization in biofloc-integrated systems

The provision of superior quality feed is vital in deciding the success of aquaculture. Efficient use of the various trophic levels and the food web improves feeding and benefits optimum growth of farmed animals. One of the major limitations in a biofloc system is the accumulation of suspended solids from the residues from feeds and added carbon sources, which ultimately degrades water quality and impacts the growth of culture species [90,91]. Hence, the costocking of filter-feeding fish within the biofloc system can control surplus sediments. Such integrated farming systems are increasingly becoming popular.

Recent research introducing the concept of polyculture or the integrated multitrophic aquaculture (IMTA) within a biofloc system has proven beneficial in better nutrient cycling and improved food consumption by multiple species present in the system. The IMTA facilitates trophic diversity of species in the system to use excess nutrients from a main cultured species into food for other species of lower trophic level [92]. The IMTA is usually applied as an integrated recirculating and biofloc system; one species can be raised in a biofloc system, and the water is then recirculated to another unit where filter feeders feed on excess bioflocs before the water returned to the biofloc system [93].

Applying the IMTA principle, Poli et al. [94] tested the efficiency of stocking tilapia (*O. niloticus*) in separate units with white shrimp (*L. vannamei*) culture. The results showed no adverse effects on the growth of either species, while the presence of tilapia contributed to reducing harmful inorganic nitrogen and phosphorus in the water. In addition, total productivity was enhanced by up to 31.2% compared with shrimp monoculture in the biofloc system [94]. As tilapia can live in brackish water, coupled with its ability in controlling suspended solid, a system of three species (white shrimp; red drum, *Sciaenops ocellatus*; and tilapia) was also designed with advantages of optimal growth and much lower TSS levels [90]. Similarly, when tilapia was replaced with a detritivorous species such as mullet (*Mugil liza*) in coculture in separate units with white shrimp, it significantly reduced the excess total suspended solids; however, shrimp growth was reduced when both species were cultured in a single unit [49]. More combinations of species in the biofloc system have been assessed, such as white shrimp and sea cucumber (*Holothuria inornata*) [95]; polyculture of three carp species (benthic feeder mirror carp [*C. carpio*], filter feeder silver carp [*Hypophthalmichthys molitrix*], and bighead carp [*Aristichthys nobilis*]), each species occupying their trophic niches so that nutrients were utilized more efficiently [46]; tilapia and freshwater prawn (*Macrobrachium rosenbergii*) [96]; etc. Such integrated biofloc farming systems with multiple species hold great promise for the future.

5.2 Unveiling the secrets behind microbial communities

Most of the beneficial effects of the biofloc systems are rendered by their microbial communities acting in unison to modulate water quality and enable nutrient cycling. The need for understanding microbial dynamics, particularly the roles and interactions of microorganisms, has been emphasized by many authors [10,69,97]. Advancements in molecular technology, such as metagenomics, have allowed us to visualize broader perspectives of the microbiome. This has shifted the focus, for example, how the carbon source alters microbial community to achieve the desired effects. Changes in microbial community with carbon source were confirmed in many studies. The transition from soluble carbohydrates (glucose) to an insoluble source (biochar) affected the microbial community when glucose was excluded entirely. For example, Actinobacteria and Proteobacteria dominating in treatments with glucose or both glucose and biochar were replaced by Chloroflexi and Proteobacteria when only biochar was used as a carbon source [51]. The addition of solid carbohydrates such as multigrain flour shifted the microbial community to higher heterotrophic bacteria [66], but the inclusion of starch as a carbon source favored the domination of Cyanobacteria [39,98].

Environmental fluctuations affecting microbial communities were also examined. It was reported that low temperature might reduce the density of Proteobacteria, which is essential in the nitrogen assimilation process; however, such an

environment also favored the Flavobacteriaceae group, usually associated with infectious diseases [48]. Light affects phytoplankton (algae and cyanobacteria), and some harmful bacteria in the genus *Leucothrix* develop only in dark conditions [53]. The microbial community responds differently to various factors, be it environmental factors, carbon source, or days of culture; the combined effects of these factors should be the basis of further research to enhance the proficiency of a biofloc system [48,53]. Mastering microbial community patterns can help predict the production more precisely [12].

6. Perspectives

Biofloc technology is recent in its application to common aquaculture systems, but a considerable volume of research on various aspects has been published thus far. Most of the research focus has been on water use and quality issues, given the significant impact of biofloc systems on the sustainable management of water resources. Major biotechnological breakthroughs are evolving, which help in a deeper understanding of the nutritional quality and benefits to aquatic animal health. The nutritional profiles of flocs formed by different carbon sources under different C:N ratios are being researched. Results from studies on the efficacy of biofloc as a feed ingredient in replacing fish meal are promising, although some of the known nutritional deficiencies of the flocs need to be compensated. The differential expression of genes responsible for immunity in finfish and shellfish is also under investigation, with encouraging results indicating that the biofloc system has a noticeable effect in modulating the immune system. This aspect needs further investigations to understand the specific immune responses of commercial species to major pathogens affecting aquaculture worldwide. This will also be a significant step toward reducing antimicrobial use and eliminating AMR, impacting human health and welfare.

7. Conclusions

Biofloc technology may serve as a future model for aquaculture, thanks to its benefits on the environment, stabilizing environmental parameters, enhancing feed efficiency with nutrients recycling, and maintaining animal health and well-being. Most of the initial focus was on improving water quality by eliminating nitrogenous metabolites from the culture system. Appropriate maneuvering of the husbandry conditions and the use of different carbon sources and C:N ratios have proven to modulate the structure and quality of microbial flocs playing a significant role in ensuring the fast growth of fish and shrimp. Recent biotechnological advances in characterizing the microbiome in water and various animal sources have revolutionized our understanding of the microbial dynamics for the successful operation of biofloc systems. Many new species are being characterized for their suitability for biofloc production system, and integration of multiple species effectively utilizing the various trophic niches has been found beneficial in improving growth and maintaining optimum water quality conditions. More research is needed to improve nutrient utilization, and the availability of data on microbiology may help design a more sustainable system in the future. Some future innovations will elucidate the molecular mechanisms underlying the beneficial effects of biofloc systems to inhibit pathogens and provide protective effects against diseases. A more robust understanding of the environment–microbe–animal interactions will help enhance the benefits of biofloc as an ecological aquaculture technology to meet the imminent global demand for sustainable food production.

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Monosex population in aquaculture

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1. Introduction

Aquaculture, the farming of aquatic species with technical and managerial interventions in the rearing process to increase production, is one of the fastest-growing food-producing sector contributing to half of the world's fish production. Fish and shellfish are the more prominent aquatic species used for aquaculture either for food or for ornamental purpose. Aquaculture has gained momentum with the advent of new species and innovative farming technologies to enhance fish production from a unit area. With easy access to land, water, and large number of species with their captive breeding, rearing, and grow-out technology available, and comparatively ease of operation, freshwater aquaculture is advancing faster than marine aquafarming.

Among the top 10 groups of farmed species, freshwater species account for more than half (57.5%) of the total fish production in terms of quantity [1]. Carps are on the top of aquaculture species group and are grown in over 92 countries contributing to one fourth (25.7%) of the world aquaculture production. "Tilapias and other cichlids" contribute to 5.25% of the world aquaculture production and are farmed in 127 countries, whereas farmed catfishes contribute 4.93% and are grown in 86 countries [1]. "Salmons, trouts, and smelts" (i.e., Salmoniformes) are the only migratory species on the top 10 lists, ranked at number nine in terms of quantity (3.5 million tonnes; 3.11% of all species quantity) and at third by value (USD 22 billion; 8.94% of all species value) [1].

A few aquaculture species of commercial importance have disadvantages such as precocious sexual maturation and prolific breeding nature (tilapias and common carp) in pond conditions leading to recruitment of additional fish resulting in overcrowding and impeding the growth of stocked fish. There are a few other species that exhibit sexual dimorphism, where one of the sexes has superior production traits (males exhibiting superior growth in tilapia and freshwater prawn) or higher market value.

Sex manipulation is one of the prioritized areas of aquaculture research in addressing the problems of precocious sexual maturation and sexual dimorphism in important aquaculture species. Understanding sexual differentiation, maturation, and reproduction is very critical to manage reproduction in captivity. The control over sex and reproduction has been the primary facilitating factor for large-scale commercial production in aquaculture species that have become global commodities [2]. Production of monosex populations is one such approach to tackle the issues of precocious sexual maturation, prolific breeding, and sexual dimorphism in aquaculture traits. The advantages of monosex population in aquaculture and different approaches to produce monosex populations, based on mode of sex determination and sexual differentiation in fish, and commercial production of monosex population with a few successful examples and status of application are discussed in this chapter.

2. Sex determination in fish

In many fish species, the sex is influenced by various environmental parameters that can interact with hormonal and genetic regulatory pathways to alter fish sexual development [3]. In few fish species, processes of sex determination and gonadal differentiation may partially overlap [4,5]. This is particularly so in teleost fish, where mechanisms of sexual development have complex pathways and are controlled either by genetic (G) or environmental (E) factors and sometimes interaction between the two (genetic $\times$ environment interactions, GxE) [6].

2.1 Genetic sex determination in fish

Genetic sex determination can be grouped into two broad categories: (1) chromosomal sex determination, where sex is governed by the inheritance of sex-related genes located on specific chromosomes (i.e., the sex chromosomes), and (2) polygenic sex determination, where sex-determining genes are distributed across the chromosomal structure. Chromosomal sex determination in fish may include both male (XX/XY) and female (ZZ/ZW) heterogamety. In most fish species, sex chromosomes are not clearly differentiated and are very insignificant with only around 7% exhibiting sex-associated heteromorphic nature [7,8]. This nonassociation with chromosomal differentiation may suggest high prevalence of polygenic sex determination in fish, but this has not been proved categorically. This may possibly be due to rapid evolutionary changes in sex determination process in fish and is an important consideration in domestication of farmed fish.

2.2 Role of epigenetics in sex determination in fish

The epigenetic mechanisms play an important role in sex determination and differentiation in fish [9]. Epigenetics is the study of changes in gene function that cannot be explained by changes in the DNA sequence [10]. These changes enable or inhibit the activity of transcriptional machinery on the DNA to regulate patterns of gene expression and are prone to be influenced by environmental stimuli, such as temperature [11]. In many fish species, differences between the sexes cannot be fully explained by differences in the genes alone, particularly in cases where sex ratio shifts occur in response to temperature, but also in serial and sequential hermaphrodites. Epigenetic modifications may be a key mechanism allowing for interactions between the environment and sex determination and differentiation processes in fish [9].

2.3 Environmental factors affecting sex differentiation

The environmental sex determination does not depend on sexual dimorphism at the genomic level because it is not genetic but influenced by environmental factors. The first report on the effect of environment temperature on sex differentiation was in the Atlantic silverside (*Menidia menidia*) [12]. However, in many fish species, it is commonly observed that application of external factors like thermal shock and pressure shock rarely produces complete monosex populations and is only a shift toward one of the sexes. In such cases, sex determination likely to be controlled both by genetic and environmental factors (GxE).

2.4 Sex reversal and the labile period

The mode and timing of treatments that can induce sexual reversal in fish vary from species to species. There is a discrete time period during development, called as the labile period, during which the gonad undergoes differentiation toward a particular sex (male or female). It is very important to identify this period for giving treatments, failing which treatments become ineffective after this period of differentiation and once the gonads have become established and sex is stabilized. Information on the specific factors that influence sexual development, as well as knowledge of the timing of gonadal development and the labile period, allows for the most accurately timed and therefore effective treatments in a given species. A better understanding of sex determination and differentiation in aquaculture species is very crucial in achieving the targeted goals.

2.4.1 Monosex populations and aquaculture

Monosex population refers to either sex (all male or all females) in a population. Production of monosex population is a much sought-after approach in aquaculture. A few major benefits of using monosex population include the following: (1) one of the sexes have superior production traits, (2) avoiding early reproduction, (3) reduction of sexual/territorial behavior, (4) reduction of differential weight at harvest, and (5) environmental impact resulting from escapes into wild [13]. The application of monosex technology opens the window for environmental-friendly applications and additional aquaculture species both in fish and shellfish [14]. Depending on the species, the use of monosex may have many advantages as shown in Table 7.1. It is evident that the choice of male or female monosex is totally determined by the species under consideration. Reduction in variation in size at harvest is a highly desirable feature in aquaculture. It reduces the need for grading of fish with consequent savings in labor and reduction of stress on the fish cultured.

TABLE 7.1 Potential benefits of monosex fish in aquaculture.

Beneficial aquaculture traits	Preferred sex for monosex production
Higher growth rate of one sex	Male/female
Preventing large energy diversion into gonadal development	Male
Production of uneconomic recruits	Male
Courtship behavior	Male/female
Reducing aggressive behavior	Female
Greater uniformity of size at harvest	Male/female
Avoiding undesirable impacts of sexual maturation on appearance and flesh quality	Male/female
Reducing unfavourable environmental impact through escapes	Male

3. Methods for production of monosex population

There are a number of approaches for production of monosex population in fish. Although the principle of monosex production is the same, application varies from species to species and is species specific. This holds good for all approaches, specially to genetic methods. The general methods available for production of monosex population are as follows:

1. Manual sorting
2. Hybridization or crossbreeding
3. Hormonal sex reversal
4. Genetic or chromosome set manipulation—gynogenesis and androgenesis
5. Production of all-male population

3.1 Manual sorting

This approach involves manual or mechanical sorting of individual fish at early stage of sex differentiation sex wise based on gonadal development showing sexual dimorphism. This approach is not only very laborious but also not very accurate and dependable. Furthermore, the sorting process is also stressful to fish and practically not feasible in commercial aquaculture.

3.2 Hybridization

Hybridization or crossbreeding between fish species was one of the first techniques employed for producing monosex population. The phenomenon of all-male or nearly all-male hybrids has been observed in the sunfishes, but the best-known examples are in the tilapias. The production of nearly all-male hybrids has been reported in sunfishes. Although a number of intergeneric hybrids have been produced between different species of carps, none of the carps were monosex and most of the F1 carp hybrids were fertile and did not show any inclination toward particular sex [15–17]. Successful examples of monosex hybrids are reported in tilapia species, and hence most of the research on fish hybridization as a potential tool for production of monosex hybrids has been carried out in tilapia. Many tilapia hybrids by a majority are males. This success created significant interest for mass production of all-male progeny to prevent the serious problem of unwanted reproduction in aquaculture [13].

3.3 Direct hormonal sex reversal

Among different techniques available, the administration of hormone treatment to sexually undifferentiated fish to produce monosex population in fish has been in practice in aquaculture and has been proved to be successful to a large extent under controlled environment in a wide range of aquaculture species. The information available indicates sex reversal, using over 31 different steroids in over 50 species representing at least 15 different families. For sex reversal to male, as many as 16 natural or synthetic androgens have been used to reverse sex in approximately 35 different species. The most commonly

applied hormone to produce male population is 17 α -methyltestosterone (MT), which has been tested in over 25 species within Salmonidae, Cichlidae, Cyprinidae, Anabantidae, Poeciliidae, and Cyprinodontidae [18].

The mode of administration of hormones is through either supplementary feeds or immersion techniques. The most common practice is hormone administration through supplementary diets. The hormone is dissolved in alcohol prior to mixing with feed. This method is relatively easy to apply but has few drawbacks. The chances of hormone getting degraded while storing are more. Furthermore, possible lack of uniformity in spread of hormone in the feed may result in variability in dose. The overdose of some hormones can cause sterility or paradoxical feminization following aromatization of androgens to estrogens. Immersion techniques have also been applied successfully and appear to be more effective in temperate species.

The sex reversal to produce large numbers of desired phenotypes in ornamental fish production is more common. In food fishes, it is mostly in tilapia that sex hormonal reversal is routinely used in commercial production of male progeny. There have been a number of successful sex reversal in several tilapia species, and commercially applicable protocols have been standardized. However, the success depends on how rigorously the protocols are followed in commercial sex reversal. Any failure to follow the guidelines meticulously in feeding quantity, timings, and frequency can result in failure in obtaining complete sex reversal to males as seen in many commercial hatcheries. Checking the rate of sex reversal for each batch using simple techniques is required to assess the success as the results may vary for different batch of fish and treatments.

There are concerns on the safety of using commercial steroid hormones with regard to safety of persons handling hormones in large quantities and to the consumer using this hormone-treated fish beside the possible impact on the environment of the hormone-treated water on soil and biota. Currently, this issue is not addressed seriously but needs to be adequately addressed as there are a large number of commercial hatcheries using steroid hormones like MT in large quantities and these need to be responsibly addressed.

3.4 Sex manipulation and all-male technology (genetically male tilapia)

This approach involves the combination of hormonal sex reversal to produce neomales or neofemales and use these sex-reversed neomales or neofemales with normal male or female species to produce all-male or all-female population over a period of generations through selection. This approach is safe from the consumer point of view as these do not contain any hormones. It has been demonstrated that a breeding program combining hormonal feminization and progeny testing can result in the production of novel YY male genotypes, which sire all- or nearly all-male progeny in nilotic tilapia [18,19], and this technology is in practice in many developed and developing countries.

3.5 Chromosome set manipulation or genetic manipulation

3.5.1 Gynogenesis

Gynogenesis is a gene manipulation technology to produce all-female fish. It is a form of asexual reproduction that requires the presence of sperm to activate the fertilization of eggs without contributing its DNA to the progeny. This method is used for producing fish in which all the genetic material is inherited from only female parent, obtained from fertilizing the irradiated sperm, and the offsprings are called gynogenic females. Gynogenesis is induced by denaturing DNA of the milt by ultraviolet (UV) or gamma rays and activating the eggs with irradiated milt. The diploid is restored by giving thermal or hydrostatic pressure shocks. It can be done in two ways. (1) By suppressing metaphase II in the second meiotic division, thereby preventing the extrusion of second polar body called meiotic gynogenesis, and the offsprings are called meiotic gynogens. For inducing meiotic gynogenesis, the shock treatment should be given immediately after fertilization. (2) By blocking the metaphase I cell division: the process is called mitotic gynogenesis and to induce mitotic gynogenesis, the shock treatment should be given after 20–30 min after fertilization to block the first cleavage. The timing of shock may vary slightly from species to species.

3.5.2 Androgenesis

Androgenesis is just the reverse of gynogenesis, where the method of producing all the genetic information is derived from male parent and the mitochondrial DNA is still maternally derived. Androgenesis is induced by UV irradiation of the egg to inactivate chromosomal DNA followed by fertilization with normal sperm. This results in a haploid zygote containing a single chromosome set. The normal diploid state is restored by applying pressure or temperature shock at the first cleavage. Approaches like production of gynogenic females or androgenic males or transgenic populations have created little impact

in aquaculture and are not used in commercial aquaculture. These clonal lines are more useful in research specially in immunological studies or in generating homozygous lines for breeding programs.

4. Current status of monosex production technology in commercially important aquaculture species

The carps followed by tilapia, catfishes, crustaceans, and salmonids are the topmost important groups of fishes grown across the globe and contribute significantly to world aquaculture production. Application of monosex production techniques in these groups is discussed with the advantages, the disadvantages, and future of use of monosex population in aquaculture.

4.1 Carps

Carps are in the topmost groups of farmed species both in developing and developed countries. Among carps, Indian major carps, Chinese carps, and common carp are the more dominant and commonly used species in a variety of production systems. Except common carp, all other carps sexually mature at the end of second year and do not breed naturally in static water bodies and also rarely exhibit sexual dimorphism in growth or any other aquaculture traits. However, the common carp is known to mature early (in the first year) and reproduce in grow-out production ponds specially in tropical region leading to overpopulation in the pond impeding the growth of fish, resulting in small size at harvest and fetching low market price. Therefore, research on sex manipulation to produce either monosex or sterile fish to control early sexual maturation and unwanted reproduction in ponds is focused more on common carp compared to other carp species. The techniques used to produce monosex populations include hormonal sex reversal and chromosome set manipulation.

Precocious maturation and undesired reproduction have been identified as potential constraints on growth of common carp in aquaculture and culture-based fisheries. Carps exhibit sexual dimorphism in growth, with female fish generally weighing more than male fish of the same age [20]. Therefore, most of the studies focused on production of all-female common carp progeny for aquaculture purpose. Different approaches like hormonal sex reversal and sex reversal with progeny testing to find neomales and use these neomales to produce all-female progeny are the most commonly used approaches. The most suitable techniques for production of monosex population are direct hormonal sex reversal and combination of sex reversal and use of neomales to produce all-female progeny [21]. Commonly used hormones for inducing sex reversal and/or sterility in common carps are MT, 17 α -methyl-di-hydroxy-testosterone (MDHT) for masculinization, and 17 β -estradiol for feminization in most of the studies [22]. Fingerlings of common carp treated with tamoxifen and letrozole (200 mg/kg feed) brought about 82.5% masculinization with increased level of testosterone [23]. Fry of different age and size groups treated with MT at 100 mg/kg yielded only partial sex reversal (77.14% male), while those treated with MDHT, in a series of trials, at 50 and 100 mg/kg feed resulted in complete masculinization (100% male) in “small” size 50-day-old common carp. The hormone-treated fish crossed with normal females produced all-female or predominantly female progeny (Table 7.2). The 20-day-old common carp fry treated with different dosages of androgen at different doses for 50 days resulted in significantly high yield of males, with females accounting to only 10%–16% of the population. The progeny testing of sex-reversed males with normal females yielded 72%–88% of female progeny and suggested the possibility of producing female-dominated population. Sex reversal using synthetic male steroid hormone as an alternative safe technique of fish sex reversal to oral administration of hormone resulted in significantly high percentage of male population (95%) at hormone concentrations of 450 and 600 μ g/L for an immersion time of 72 h [24].

The success of sex reversal may vary with each hormone and the dosage used, mode of treatment, age, and size at treatment appear to determine the rate of sex reversal in common carp. This could be the reason why there is variation between the rate of sex reversal in different species, though able to get near to full range but not consistent. Most of the studies on hormonal sex reversal in common carp indicate the varying percentage of sex reversal but not 100% sex reversal except the report of MDHT resulting in complete sex reversal in common carp.

Gynogenesis and androgenesis are useful techniques for the rapid production of inbred strains of fish [25]. The development of techniques for the production of gynogenetic, androgenetic, polyploid, and monosex progenies in common carp (*C. carpio* Linnaeus) is well described. Mitotic gynogenesis was used for production of clones in common carp. In androgenetic progenies of common carp, YY males were identified, which after crossing with normal females (XX) produced all-male progenies. All-female populations of common carp were produced on a large scale by crossing normal females (XX) with hormonally sex-reversed males (XX). Culture of all-male progenies increased the production marginally by 7%–8% [26]. The published information on the benefits and performance of gynogenetic and/or androgenetic population

TABLE 7.2 Techniques available for monosex production with merits and demerits.

Technique	Commercially applied	Advantages	Disadvantages
Sorting	Rarely	Relatively easy	Not reliable, labor intensive
Hormonal sex reversal	Yes	High percentages of preferred sex	Relative expensive Hormonal residues Worker protection Needs high level of control Public perception
Interspecific hybridization	Yes	Easy to produce	Possible environmental pollution through escapes Brood stock contamination
Androgenesis/ gynogenesis	No	Suitable for species with homogametic males/ females	Not easily applied for commercial production Not consistent
All-male technology	Yes	Suitable for species with heterogametic males	Care needed in selection and maintenance of brood stock
Transgenic	No	May be technically feasible	May not be practical because of consumer perception on transgenic products

Source: J.A. Beardmore, G. C. Mair, R. I. Lewis, Monosex male production in finfish as exemplified by tilapia: applications, problems, and prospects, in: Reproductive Biotechnology in Finfish Aquaculture. Elsevier, 2001, pp. 283–301.

in aquaculture is very scant, and the available reports do not give encouraging results. Although it is technically possible to produce gynogenic and androgenic individuals in common carp, their production on commercial scale may not be economical and beneficial to commercial farming as they do not result in improved performance in growth or any other aquaculture traits. This is the probable reason why the use of monosex populations in commercial carp farming is very insignificant.

4.2 Tilapia

Tilapias and other cichlids contribute to 5.25% of the world aquaculture production and are farmed in 127 countries [1]. Tilapia is one of the most consumer-preferred farmed fishes in the world and is the most farmed fish in varying production systems next to cyprinids, contributing a production of 6.6 million tonnes in 2019 to aquaculture production [27]. The most commonly used species in aquaculture across the globe is the Nile tilapia (*Oreochromis niloticus*), and genetically improved strain, genetically improved farmed tilapia strain, developed by WorldFish (earlier the International Center for Living Aquatic Resources Management), which is known for its faster growth rates and adaptation to varying environmental conditions and suitable for different aquaculture systems from pond-based aquaculture to cage farming and latest innovative feed-based intensive aquaculture technologies like circulatory aquaculture system, biofloc, and aquaponics [28]. The bulk (80%) of the production comes from a single species, the Nile tilapia (*O. niloticus*) [29–31]. However, the main disadvantage of *Tilapia* spp. is precocious sexual maturation and prolific breeding in pond conditions, resulting in large induction of small-sized fish affecting the growth of the already stocked fish in the system. Tilapia also exhibits sexual dimorphism in growth, with males growing significantly faster than females; hence, males are preferred to females for profitable aquaculture. Hence, benefits of monosex tilapia apply to all production systems—pond culture, cage farming, and feed-based intensive circulatory aquaculture systems.

As a global aquaculture species, tilapia is attracting more focus on research to develop reliable and adaptable techniques for all-male tilapia population to address the problem of early sexual maturation and to take advantage of faster growth rate of males. A number of techniques right from manual sorting of sexes at early stages, production of interspecific hybrids, hormonal and environmental sex manipulation, to genetic manipulation of sex have been attempted to produce all-male tilapia population. Hormonal sex reversal and combination of sex reversal and progeny testing to produce all-male tilapia have resulted in reliable level of all-male tilapia. The attempts to produce all-male tilapia date back to 1970s. Although encouraging results were obtained, the reliable techniques to produce all-male progeny in tilapia emerged only during mid-1980. Development of captive breeding and rearing techniques to collect tilapia eggs and larvae proved to be the basis for hormonal sex reversal to produce nearly complete (99%) sex-reversed male tilapia using MT [32,33].

4.2.1 Direct hormonal sex reversal

The principle behind hormonal treatment is based on the fact that the level of sex hormones (androgens and estrogens) present in the sexually undifferentiated stages in early life cycle (up to 2 weeks after hatching) of tilapia is equal [33]. Hence, increasing the level of one of the hormones that is originally present will change the fish to either male or female depending on the type (male or female) of hormone administered. Accordingly, if the tilapia larvae are fed with feed incorporated with male hormone, viz. MT, the fish will develop into functional male but will carry the female genotype (XX). The hormone augmentation through dietary supply and immersion technique are the two techniques available for hormonal sex reversal. However, sex reversal through feed incorporated with MT is most commonly used and is an effective method for all-male tilapia production even today [34,35]. The list of hormones that have been researched and worked upon on different species are mentioned in Tables 7.3 and 7.4.

The recommended dosage of MT for sex reversal in tilapia is between 30 and 60 mg/kg of feed for a total of 25–30 days depending on the targeted outcome [36], and the percentage of obtaining male will range between 85% and 98% [37]. This technique can yield up to 100% females if the dosage and time and period of hormone treatment are standardized and the male hormone is commercially available [38].

4.2.2 Genetically male tilapia technology

The common methods used for producing all-male tilapia, mainly direct hormonal masculinization, are not giving consistent results. Successful adoption of these techniques is seriously limited by a number of factors involved in their effectiveness like the hormone source and age and size of the fish. The technique of production of monosex males, YY genetically male tilapia (GMT) technology in Nile tilapia, is an example of a genetic technology for the production of monosex males and is adopted by the aquaculture industry in many countries [13]. The production of all YY supermale

TABLE 7.3 Summary of sex ratios in the control and 17 α -methyl-di-hydroxy-testosterone (MDHT)—treated common carp.

Treatment	Hormone dose (mg/kg feed)	Mean weight of the fish (g)	Period of treatment (days)	Survival (%)	Sex ratio (%)		
					Male	Female	Intersex
Control	Hormone-free feed	0.148	50	86.00	68.00	22.00	00.00
MDHT 1	50	0.052	50	42.33	100.00	00.00	00.00
MDHT 2	50	0.240	50	94.66	84.00	11.00	14.00
MDHT 3	100	0.051	50	56.33	100.00	00.00	00.00
MDHT 4	100	0.240	50	94.00	69.33	25.33	05.33

Source: M. Kumar, H. M. P. Kumar, N. Reddy, Y. Basavaraju. Sex reversal in common carp, *Cyprinus carpio* using 17 α – methyl – di – hydroxyl – testosterone, Proc. Zool. Soc. India. 9 (2) (2010) 1–9.

TABLE 7.4 Reported hormones used for the production of monosex population in tilapia species and their response.

Species	Hormone used	Treatment duration (days)	Male %
<i>Oreochromis niloticus</i>	Fadrozole	30	92.50–96.00
<i>O. niloticus</i>	17 α -ET	25–28	91.00–99.40
<i>O. niloticus</i>	17 α -MT	21	99.00
<i>Oreochromis mossambicus</i>	17 α -ET	18	100.00
<i>O. mossambicus</i>	17 α -MT	18	98.00
<i>Oreochromis aureus</i>	17 α -MT	42	100.00
<i>O. aureus</i>	17 α -ET	25–28	83.00–97.00

ET: ethynyltestosterone; MT: methyltestosterone.

Source: A. Bardhan, S. K. Sau, S. Khatua, M. Bera, B. N. Paul. A review on the production and culture techniques of monosex Tilapia, Int. J. Curr. Microbiol. Appl. Sci. 10 (1) (2021) 565–577.

technology is based on the principle of genetic manipulation of predominantly mono proposed as a technology for the mass production of all-male progeny for culture and is well described [39]. This approach focused on a breeding program on the hypothesis that while ultimate control of the sexual phenotype might depend on an unknown number of genetic and environmental variables, the major switching factors must be of an XX/XY type as postulated. This program is time consuming, largely due to the very considerable amount of progeny testing involved and, to some extent, the need to optimize sex reversal procedure for generating brood stock [40]. The steps involved in the development of YY all-male technology are depicted in Fig. 7.1. This technology has shown that novel YY male genotypes are viable to produce all- or nearly all-male (XY) progeny in crosses with normal XX females.

However, this technology is not widely adopted in many Asian countries including India, even though the technology has been adopted by many entrepreneurs in many countries in other parts of the world. This could probably be due to dominance of carps in the Asian region, specially so in South Asian region. With the advent and adoption of innovative and intensive feed-based aquaculture technology, for which carp species are not ideally suited, tilapia is bound to be preferred as it is well suited for these production systems [40].

The GMT populations are fast growing and can attain marketable yields under all environments in less time than mixed-sex tilapia (MST) and sex-reversed tilapia (SRT). The GMT had higher yields than both MST (+58.8%) and SRT (+31.03%). Furthermore, there was no recruitment in GMT pond populations, whereas recruits were present in MST and SRT populations. GMT produced from novel YY males in this strain has considerable potential for increasing yields from tilapia culture [40]. The genetics-based monosex technology developed for tilapia can also be applied to produce monosex females in appropriate situations. This technology has already been used to produce monosex females in silver barb in Thailand [41].

4.3 Monosex in crustaceans

Another important group of aquaculture species where sexual dimorphism displayed is crustaceans, particularly in freshwater prawns. The prominent species which is farmed on commercial scale is freshwater giant prawn, *Macrobrachium rosenbergii*. Size dimorphism between males and females and differential growth is the key advantage for farming of monosex population over mixed-sex population. This is particularly true in case of freshwater prawn, *M. rosenbergii*, where both the sexes have advantages. Differential growth and dominance of larger individuals characterize *Macrobrachium* population. It is normally believed that males grow faster than females and will be more beneficial. Farming of monosex (generally all male) population is the recent practice adopted in large-scale farming of freshwater prawn, in order to increase yield and individual weight at harvest [42]. However, the large-sized males show aggressive and territorial behavior and dominant males hinder the growth of smaller males and females. Therefore, all-female monoculture could be a better option as females are less aggressive and show relatively homogenous growth pattern. The all-female population showed better performance than the mixed-sex population both in intensive and extensive farming practices. The survival rate and the feed conversion was better in all-female cultures, and all-female populations exhibited significantly higher size uniformity [43].

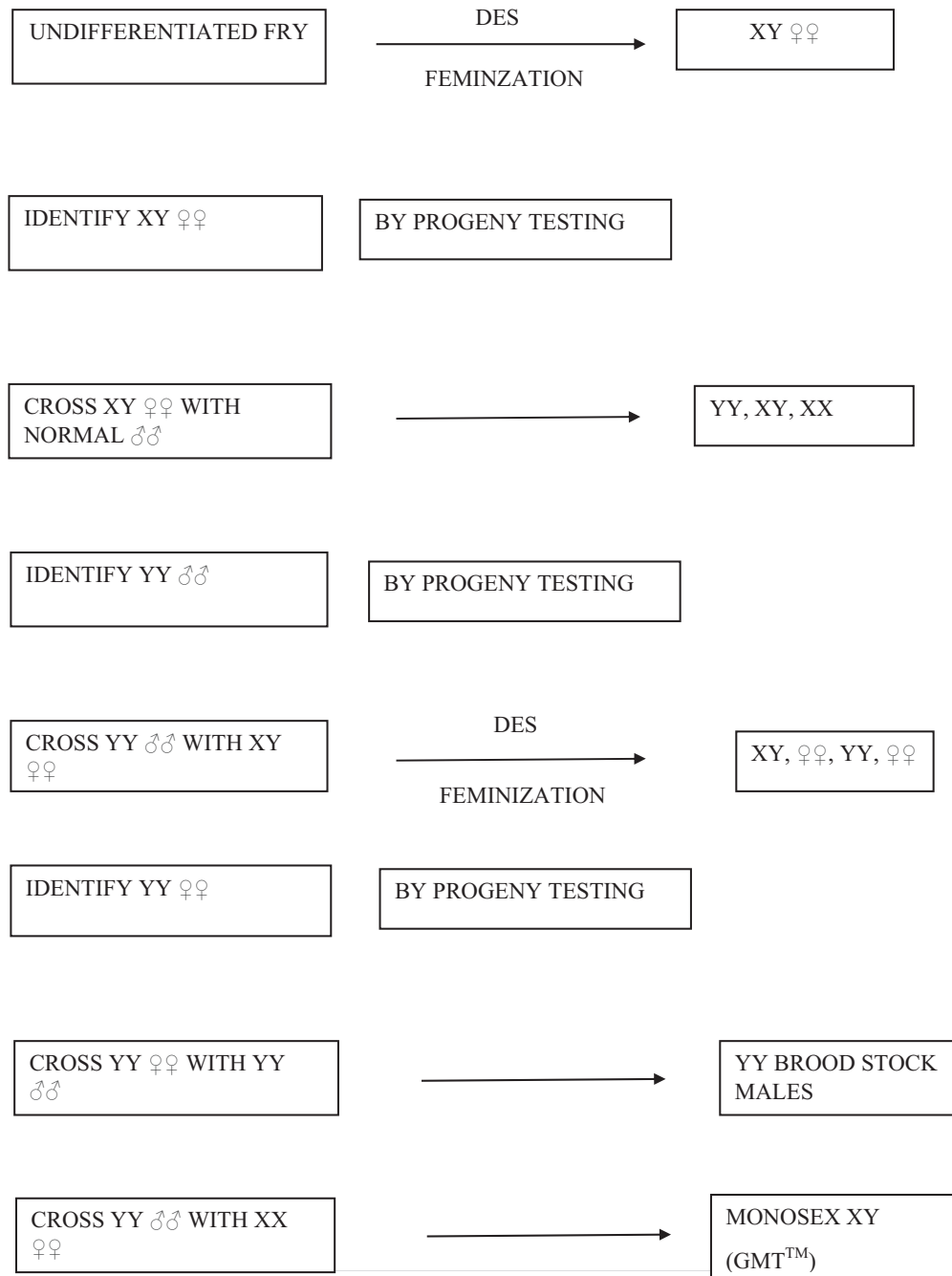


FIGURE 7.1 Illustration of mass production of YY male brood stock in Nile tilapia. Source: J.A. Beardmore, G. C. Mair, R. I. Lewis, *Monosex male production in finfish as exemplified by tilapia: applications, problems, and prospects*, in: *Reproductive Biotechnology in Finfish Aquaculture*. Elsevier, 2001, pp. 283–301.

In *M. rosenbergii*, the monosex population is also produced using the technique of sex reversal and developing of neomales or neofemales. This progeny is used to develop as brood stock and when crossed with normal males/females will produce all monosex population of desired sex. *M. rosenbergii*, like most decapod species, is assumed to have the W/Z model of sex inheritance in which males are homogametic (ZZ genotype) and females are heterogametic (WZ genotype) [42]. The sexual differentiation in prawn and development of male primary and secondary sexual characteristics are determined by androgenic gland (AG). Removal of the AG (antrectomy) from male prawn in the initial development stage will transform the male into a fully functioning neofemale. These neofemales can mate with normal males to produce all-

male prawn progeny, which can be used for commercial prawn farming and to improve production efficiency and profitability [44]. The biotechnology for all-female population in the economically important prawn (*M. rosenbergii*) involves WW males AG cells transplantation which caused full sex reversal of WW females to functional males. AG cells transplantation caused full sex reversal of WW females to functional males. Crossing the WW males with WW females will yield all-female progeny lacking the Z chromosome. Similarly, developing different genotype combinations (ZZ, WZ, and WW) to retain either male or female phenotypes, monosex populations of either gender can be produced [45].

4.4 Other finfish species

Other potential candidates for male monosex culture are salmonids and some of the catfish species. Monosex production in salmonids was popular for a while in the initial phase, but it is not so relevant today. Catfish species such as *Heterobranchius longifilis* are reported to show faster growth in females, whereas in *Ictalurus punctatus* males are faster growing. Growth performance of monosex females, monosex males, and mixed sexes showed harvest weight to be significantly greater with monosex males than monosex females [46]. However, except *Tilapia* and *Macrobrachium* spp. to certain extent, no other groups have created any impact in commercial farming.

5. Conclusion

The monosex populations have considerable potential in aquaculture of fin and shellfish when control of undesirable reproduction in production systems is one of the major constraints and/or to take advantage of the species that exhibit sexual dimorphism in important aquaculture. However, the list is limited to a few species like tilapia, common carp, and freshwater prawn. Most commonly used techniques in most cases are either direct sex reversal or production of genetically monosex population using the combination of sex reversal, progeny testing, and selection program. The direct hormonal approach has several disadvantages like inconsistency in producing desired level and no standards for use of hormones. There are also concerns on the safety of using steroids from safety of persons handling hormones in large quantities to the consumer using this hormone-treated fish beside the possible impact on the environment of the hormone-treated water on soil and biota. Currently, this issue is not addressed seriously but needs to be adequately addressed as there are a large number of commercial hatcheries using steroid hormones like MT in large quantities and these need to be responsibly addressed. Biotechnology to produce monosex population needs careful maintenance of brood stock and proper selection program. Another major problem associated with this technology is the lack of markers, and these markers when developed will have greater utility. The larger issues like safety and biosecurity in using these techniques need to be addressed to make the technology technically feasible, easily adoptable, environment friendly, and safe for consumption.

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Applications of cell lines and cell culture systems of finfish and shellfishes to attain sustainability in aquaculture

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1. Introduction

Sustainability in aquaculture can only be brought out through precision in the all-round processes, which include farm preparation, brood stock development, maturation and spawning, nursery rearing, feed formulation and feeding, culture environment quality maintenance, and timely harvest. At every stage in the culture cycle, the system is under threat of collapse due to various reasons, and farmers are ever under the pressures of uncertainties. Precision aquaculture addresses all those issues and tries to make the life of the farmer comfortable.

In the ambit of precision aquaculture, cell lines and cell culture systems have pivotal roles to play. This chapter examines the areas where it can find application and describes how can they be integrated to the system management, making it foolproof.

2. Isolation and study of viral pathogens

Host specificity of the viral pathogens dictates the requirement of specific cell lines having receptor sites for the particular pathogen. Accordingly, the susceptibility of the fish cell lines to the virus varies with species as well as the tissue from where the cell line is derived.

Because most of the viruses maintain very high levels of tropism, the situation demands the development of species- and tissue-specific cell lines from all the cultured species. Goswami et al. [1] summarized the most commonly used fish cell lines, which form a ready reckoner to choose an appropriate cell line for the isolation and study of known viruses. Meanwhile, it is over and again realized by experienced virologists that detection and isolation of an unknown virus may not be accomplished through an established cell line as during the in vitro transformation, the cells might have modified the receptor sites, making them undetected by the virus concerned. Under such situations, primary cell culture from the specific organ of specific fish species is the best option. Therefore, it is imperative to have the capability to maintain established fish cell lines and apply them for virus isolation. Besides, one should have the capability to develop primary cell cultures from any species of fish on demand to accomplish the study of fish viruses as well as to effect a genuine isolation of unknown viruses. This capability must be part of a fish virology laboratory and must be able to be customized.

3. Cytopathic effects

During the viral multiplication in the infected cell, the cell undergoes characteristic biochemical and morphological changes, called cytopathic effects (CPE), having said the responsible virus cytopathogenic. The degree of visible damage to cells caused by viral infections varies with the type of virus, type of host cell, multiplicity of infection (MOI), and other factors. Some viruses cause very little or no CPE in cells of their natural host. In such instances, their presence can be detected visually only by hemadsorption or interference, in which infected cell culture showing no CPE inhibits the

replication of another virus subsequently introduced into the cultures or in situ by viral antigen (fluorescent antibody) or by way of nucleic acid detection (polymerase chain reaction). However, some viruses cause complete and rapid destruction of the cell monolayer after infection. It is of high-level practical application that the CPE caused by some of cytotoxic viruses may be sufficiently characteristic to allow provisional identification of unknown viruses [2].

Erica and Carol [3] have explained the methodology for detection of CPE in cell lines that can be readily adopted in a fish virology laboratory.

It has to be borne in mind that a given virus may produce different CPE in different host cell types and therefore it is imperative to use the same cell type consistently for a specific virus in a laboratory by a technician. Invariably control and infected cells should always be observed to distinguish normal cell changes that occur as the cells age, from that of CPE.

With respect to identification of known viruses, the rate of CPE appearance is also a characteristic that can be used. The thumb rule is that a virus is considered slow if CPE appears after 4–5 days in culture inoculated at low multiplicity of infection (MoI). It has to be understood that at a high MoI, all CPE can occur rapidly. Accordingly, the rate of CPE appearance should be based on the lowest MoI that produces CPE.

The CPE are generally categorized as follows: (1) total destruction: all cells in the monolayer rapidly shrink, become dense (pyknosis), and detach from the plate within 72 h; (2) subtotal destruction: consists of detachment (death) of some but not all the cells in the monolayer; (3) focal degeneration: production of localized areas (foci) of infection due to direct cell-to-cell transfer of virus rather than diffusion through the extracellular medium; cells become enlarged, rounded, and retractile and get detached from the surface leaving cleared areas surrounded by roundup cells as the infection spreads concentrically to be observed under low-power objective; (4) foamy degeneration (vacuolization): due to the production of large or numerous cytoplasmic vacuoles, difficult to visualize without staining with Giemsa; and (5) cell fusion (syncytium or polykaryon formation): involves the formation of the plasma membranes of four or more cells to produce an enlarged cell with four or more nuclei, readily seen by Giemsa staining. Cell fusion has to be distinguished from cell clumping or clustering in which plasma membrane remains distinct. Inclusion bodies are areas of altered staining in cells, not generally seen in live cells. The type of inclusion body may vary from single or multiple, large or small, round or irregularly shaped, intracellular or intracytoplasmic, to eosinophilic (pink staining) or basophilic (blue–purple staining). In some cases, chromatin margination may also be present and cause a thin stained ring to form around the edge of the nucleus. There could be two reasons for the formation of inclusion bodies: in one instance, inclusion bodies indicate areas of the cell where viral protein or nucleic acid is being synthesized or where virions are being assembled, but in some cases, no virus is present and inclusion bodies indicate areas of viral scarring.

4. Primary isolation of viruses

In aquatic animal health management, primary isolation of viral pathogen from diseased fish is always a challenge. If one has to make a genuine isolation of the viral etiology, several requirements have to be met with such as the viability of the pathogen, susceptibility of the employed cell line to the virus, conspicuous CPE, and potential to passage to fresh bottles. However, in several occasions, the fishes arrive the laboratory in a putrefied state and are apparently unacceptable for proceeding with the operation. Nevertheless, having the viral particles within a cell, they are very much protected irrespective of the type of nucleic acid (RNA or DNA viruses), the tissue can be used for attempting to isolate the pathogen linking with clinical signs of the disease, external, internal and behavioral. If looking for a known virus, based on the clinical signs, PCR on the tissue would greatly benefit.

5. Method of extraction of virus from tissue

The method adopted for extracting virus from the affected tissue has greater significance to get the virus isolated. Once the samples are obtained from the field, they must be transported to the laboratory in chilled ice, dissected and the organs are stored at -20°C for short term and -80°C for long term, thawed only once, that too for macerating the tissue with glass wool in the respective tissue culture medium at aseptic conditions using a tissue homogenizer at 4°C (in an ice bath), centrifuged at 10,000 rpm at 4°C , filter sterilized using polyvinylidene difluoride membrane having 0.2- μm pore size, inoculated into cell lines in log phase of growth, and looked for characteristic CPE.

6. The absolute requirements

The situation warrants the technicians involved well trained having adequate knowledge base on the disease of fishes by and large, besides having the laboratory well set for the purpose with primers for PCR, positive controls, the required cell

lines in liquid nitrogen, media, antibiotics and stains. Living cell culture with molecular tools such as PCR and serological techniques such as ELISA would benefit a great deal to the identification of the virus isolated. Meanwhile, the search for novel viral etiology must be accompanied with histopathology and electron microscopy. Hence, to get maximum benefits from the fish cell lines available around in the management of viral pathogens in aquaculture, the absolute requirements are as follows: (1) well-informed manpower having holistic knowledge and mindset to approach the problem, (2) a standard animal cell culture laboratory, (3) molecular biology laboratory, (4) serology laboratory, (5) histopathology laboratory, (6) access to electron microscope facility, and (7) bioassay systems with biosecurity.

7. Fish cell lines for the study of intracellular bacterial pathogens

Application of fish cell line in the isolation and study of intracellular bacterial pathogens is noteworthy. Bacterial fish pathogens such as *Rickettsia* spp. and *Renibacterium salmoninarum* have been isolated from fish using cell lines [4,5]. *Piscirickettsia salmonis* is the first Gram-negative intracellular bacterial pathogen isolated from fish using the CHSE-214 cell line [6,7]. Through fulfillment of Koch's postulates, it was confirmed as the causative agent of the disease in Coho salmon [8]. Ortiz-Severín et al. [9] cultured *P. salmonis* in two cell lines derived from Atlantic salmon kidney, salmon head kidney (SHK-1) and anterior salmon kidney (ASK). Gentamycin was added to a final concentration of 50 µg/mL to kill extracellular bacteria. Kebbi-Beghdadi et al. [10] investigated the permissivity of two fish cell lines, EPC-175 (fathead minnow) and RTG-2 (rainbow trout), to three chlamydia-related bacteria: *Waddlia chondrophila*, *Parachlamydia acanthamoebae*, and *Estrella lausannensis*. Menanteau-Ledouble et al. [11] investigated the invasion and replication of *Yersinia ruckeri* in fish cell cultures (CHSE-214, ASK, and SHK).

The above description strongly suggests the existence of diverse intracellular bacterial pathogens in fishes and usefulness of cell lines in their study aimed at integrated health management at field level.

8. Fish cell lines for virus vaccine preparation

Literature suggested that 54.9% bacterial pathogens, 22.6% viruses, 3.1 5% mycotic agents, and 19.4% parasitic agents are responsible for periodical disease outbreaks in fish culture [12]. The Office International des Epizooties (OIE) has listed certain important diseases caused by DNA viruses such as epizootic hematopoietic necrosis (EHN), koi herpesvirus disease (KHVD), and red sea bream iridovirus disease (RSID) and RNA viruses such as infectious hematopoietic necrosis virus (IHNV), infectious salmon anemia virus (ISAV), spring viremia of carp (SVC), and viral hemorrhagic septicemia (VHS) causing major catastrophes in aquaculture [13–16]. In the control of viral pathogens, vaccination is the only option at the right life stage. Several kinds of vaccines have been described in aquaculture, of which the most popular and less expensive is conventional or traditional vaccines, which are inactivated whole viruses. These viruses have to be cultivated in specific cell lines, extracted, and inactivated with β-propiolactone, binary ethylenimine, formaldehyde, or in high temperature, of which the BPL inactivation was found to provide maximum efficacy as reported in IHNV by Anderson et al. [17] and Tang et al. [18]. However, a few fish viruses are not easily propagated in cell cultures such as lymphocystis disease virus of Iridoviridae family. Hence, development of inactivated vaccine for such viruses still remains a constraint [19]. This situation demands increased attenuation to either screen the existing cell lines for the susceptibility of the important viruses or generate novel cell line for the purpose. Among the three categories of vaccines such as inactivated whole vaccines, subunit recombinant vaccines, and DNA vaccines, the inactivated vaccine if applied along with appropriate adjuvants will be the most suitable one for aquaculture application through oral route.

9. Fish cell lines for developing antiviral chemotherapy

In the management of viral pathogens in aquaculture, an essential requirement is a strategic development of antiviral compounds for prophylactic and therapeutic application. Other than using fish—animal models for screening such compounds especially from natural bioresources, cell line—virus complexes are excellent tools. While doing so, two modes of screening can be undertaken, one for understanding virucidal property in vitro, which would have a killing effect on the virions, and the other for interfering with the viral multiplication cycle within the cell. While employing cell line for screening antiviral compounds, molecular tools to assess the viral gene expression through cDNA route, cellular health through trypan blue exclusion assay, MTT assay, and bromodeoxyuridine incorporation assay are recommended. Besides, the inhibition of virus multiplication can also be assessed through passing them to new cell lines and assessing infectivity in vitro and also in vivo by introducing them to animal models.

10. Fish nutrition and metabolism with respect to feed formulation

Cell lines from the respective fish serve as an excellent *in vitro* model to study nutrient absorption and assimilation. Specifically, to attain this target, intestinal cell culture systems need to be developed. The primary cultures of adipocytes or hepatocytes and myoblasts are significantly used to study the molecular mechanisms related to fish nutrition [1,20,21]. Rainbow trout hepatoma-derived cell line has been used to address nutrition-related queries based on major pathways such as macroautophagy (autophagy), general control nonderepressible 2, and mechanistic target of rapamycin pathway that regulate cell homeostasis through amino acids to study the nutrient-sensing signaling [1,22,23]. Likewise, RTH-149 cells have been used for studying the starvation-induced autophagy kinetics [1,22]. Several fish cell lines have been used as *in vitro* models to study elongation and denaturation of different polyunsaturated fatty acids (PUFA), which are useful for understanding the proinflammatory mechanisms underlying the relationship between dietary PUFA and cardiac lesions in salmon [1,24]. The above events suggest the requirement of more such cell lines useful for employing in fish nutrition studies as well as designing precise experiments incorporating specific such cell lines in lieu of animal models. Requirement of cell line–based nutritional assay becomes very much important when functional feed and feed ingredients are considered, suggesting invigorated development of fish cell line technology in support of fish nutrition.

11. Aquaculture environment quality assessment for sustainable aquaculture production

Environmental quality is an integral part of the monitoring, which is of primary concern. Days are gone when animal models have been used for testing the environmental quality due to various constraints involved and the animal-to-animal variation to a specific situation besides ethical issue. Fish cell lines have been adopted as an *in vitro* tool for ecotoxicological evaluation of chemicals by many international regulatory bodies in Europe and the Food and Drug Administration in the United States [1].

12. Embryonic stem cell and transgenesis

Since the establishment of first embryonic stem (ES) cells in mice, stem cells have been the focus of active research because of their enormous potential for basic research, medicine, and animal biotechnology. Thirty years of long history of fish stem cell culture has been narrated through several reviews depicting the systematic development achieved in the discipline [25–27].

Among the stem cell cultures, germ cells including primordial germ cells (PGCs) and gonadal germ stem cell spermatogonia in the testes and oogonia in the ovary have attracted considerable interest in the development of germ line–competent stem cell cultures. PGCs solely colonize the gonad for egg or sperm production and hold promise to develop into pluripotent stem cell cultures. Since the identification of zebrafish *vasa* as a molecular marker of fish germ cells [28], *vasa* promoter has been successfully used for the generation of transgenic lines in several fish species including medaka [27] and trout [29]. Future work is needed to determine whether fish PGCs can give rise to stable cell cultures and retain the ability for germ line transmission [30]. Fish stem cells have the potential for use in gene targeting, germ cell transplantation, and semiclone by nuclear transfer [31]. These procedures along with CRISPR-based genome editing will pave the way for augmented strain improvement programs to enhance fish yield, disease resistance, and environmental resilience.

ES cell–mediated transgene introduction has many advantages over conventional methods. It ensures stable site-specific integration/deletion of transgene, *in vitro* selection of clones with desired genotypes, germ line transmission of the transgene into progenies, and economic feasibility. Another attractive point is that when introduced into host embryo (of same or different genera), the ES cells can participate in normal development and contribute to several tissues of the host, including cells of the germ line. In addition, ES cells are becoming important candidates for the cryoconservation of biodiversity. Furthermore, Blastula cell transplantation (BCT) can achieve a high efficiency of fertility restoration for the experimental analysis of these biological parameters towards BCT-mediated surrogate production of high yielding, genetically improved fishes for conservation of biodiversity.

13. Organoid culture of fish pituitary and tissue engineering

The pituitary gland, a major endocrine gland, controls growth, development, and the functioning of the other endocrine glands. It produces gonadotropic hormones that are essential for the maturation and spawning of fish. These gonadotropins

include follicle-stimulating hormone and luteinizing hormone, which are secreted and correlated with the cycles of gonadal maturity. Other than hypophysation, commercial hormones such as human chorionic gonadotropin (hCG) (hCG alone or in combination with fish pituitary [hCG 70% + PG 30%]), luteinizing hormone–releasing hormone (LHRH), progesterone (17 α -hydroxyprogesterone and 17 α -hydroxy-20 β -dehydroprogesterone), antiestrogens (clomiphene citrate and tamoxifen), and Ovaprim (Linpe method) (LHRH-A and the drug domperidone) are used in fish breeding with varying level of success. If the pituitary gland can be maintained in an organoid culture mode, it gives an avenue to produce the hormones in vitro and regulate the production by way of tissue engineering. In this context, it is to be brought to attention that organoids, spheroids, and the study of 3D cell culture models show great potential in having an organoid pituitary culture in place, which opens up great opportunities in fish endocrinology, breeding, and regulated seed production.

14. Crustacean cell lines

Crustacean cell line development has a long and arduous history of 35 years. It remained elusive due to the neglect on “know your animal” [31] philosophy, as the successful history of insect cell lines started from the in-depth knowledge gained on the insect biochemistry with which an appropriate and exclusive insect cell culture medium could be developed [32,33]. Meanwhile, there has been successful development of primary cell cultures from lymphoid organ, hemocytes [34–36], and heart tissue [37] of crustaceans with proven susceptibility to viruses. Despite the modification of commercially available medium based on hemolymph analysis [38,39], an exclusive medium for the growth and development of shrimp cells in vitro had not been accomplished. Even though Wyatt et al. [32] were not totally successful, their contribution was essential to Grace’s ultimate success in the development of Grace’s insect cell culture medium [40,41], which resulted in the development of over 500 insect cell lines [31,42]. Such a scientific temper could be imbibed in the shrimp cell culture research for successful development of a continuous cell line with the development of a medium exclusive to shrimp cell culture by Jayesh et al. [43,44]. The next attempt was to induce in vitro transformation, which included identification of a putative promoter system to construct transformation and transduction vectors specific to shrimp (baculoviral expression system) [45,46], and the use of oncogenes happened to be another option proposed by Jayesh et al. [47] and Anoop et al. [48]. Quite significantly, the first shrimp–insect hybrid cell line (*PmLyO-Sf9*) was brought out by I. S. Bright Singh and his coworkers, which could support white spot syndrome virus, but without the formation of virions [49,50]. Subsequently, it was found to be susceptible to infectious hypodermal and hematopoietic necrosis virus, monodon baculovirus, and hepatopancreatic parvovirus (I. S. Bright Singh, personal communication). Moreover, transgenic expression of oncogene and telomerase reverse transcriptase might also lead to a successful outcome of valid shrimp cell lines [43,47]. To sum up, crustacean cell line development still remains a gray area demanding concerted efforts from all the angles as suggested above in support of sustainable aquaculture. Meanwhile, *PmLyO-Sf9* would serve as an in vitro model to solve several problems in shrimp culture confronted with viral involvement.

15. Molluscan cell lines

Despite the attempts that started since the 1960s, no validated cell line of marine molluscs could be made available. Considering the significance of marine bivalve aquaculture to the world economy, the prevailing viral threats, and the rapid advancements in molluscan virology, the requirement of a molluscan cell line is indispensable. There could be various factors hindering the establishment of a molluscan cell line, and it is essential to solve each one of them to bring about a successful outcome. The crucial aspects to be considered are proper decontamination procedures, choice of tissue, and the formulation of an appropriate cell culture medium to optimize the growth and division of the molluscan cells in vitro. The appropriate intervention of gene transfer and cell hybridization techniques may also be relied upon to bring about successful outcome.

16. Conclusion

Cell lines and cell culture systems are powerful tools for the detection and isolation of viral pathogens, study of the viral multiplication cycle, development of antiviral chemotherapy, study of intracellular bacterial pathogens, vaccine production, study on fish nutrition and metabolism with respect to feed formulation, aquaculture environment quality assessment, development of transgenic fish, and in vitro production of hormones through organoid culture. Their involvement contributes to precision aquaculture paving the way for sustainability. But the cardinal requirement is the appropriate integration of the tools to make it functional in the appropriate place on time to solve specific problems. In this scenario, the human resource who would use the tools is of paramount importance as their knowledge base and skill contribute

substantially to the application of cell culture and cell lines appropriately. The educational system must provide sufficient space in the curriculum to impart the required skills to utilize the cell culture systems appropriately and effectively.

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An ocean of opportunity: challenges and opportunities for developing cultivated seafood

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The following is an excerpt from the Good Food Institute's (GFI) report, *An Ocean of Opportunity*:

Across the globe, there is an urgent need for new approaches to complement existing efforts to meet increasing global demand for seafood. The development and widespread commercialization of cultivated seafood, which is produced by cultivating cells from marine animals, is a promising approach for alleviating pressure on both wild fisheries and aquaculture systems, particularly if pursued in coordination with the right enabling policy frameworks. Cultivated meat exhibits fundamentally higher efficiencies than cycling caloric value through animals, and it offers the unique opportunity to level the trophic playing field within seafood production. In other words, the raw materials and resources to create cultivated versions of a top predator like tuna are essentially the same as those required for or cultivated versions of species at the bottom of the food chain.

Accelerating the development and commercialization of scalable cultivated seafood products that compete on taste, price, accessibility, and nutritional quality with their ocean-derived counterparts should comprise a core component of global strategies to maintain the vitality and, ultimately, the survival of our oceans.

The cultivated meat production process relies on providing animal cells with molecular and environmental cues that govern developmental organization of the skeletal musculature, adipose, and connective tissues. Regardless of species of origin, the fundamental biological requirements of these cell types are largely similar across species, as developmental pathways are conserved by evolution in organisms ranging from annelids to vertebrates. Thus, the general process workflow for cultivated meat production should look similar for marine animals as for more established mammalian or avian systems. Modifications to the general process and optimization for each final product will be required because the repertoire of growth factors and the precise nutrient requirements of different cells will vary slightly.

1. Unique advantages and challenges

The mission to create cultivated seafood products exhibits unique advantages and challenges relative to cultivated meat from terrestrial farmed species. The most notable challenge is that cells from fish and other aquatic animals are not routinely cultured in most research labs, so protocols optimized for these cell types are not readily available in most cases. Additionally, resources such as sophisticated genome annotations for seafood-relevant species are limited compared to common laboratory species (e.g., mouse, rat, fly) or even common livestock species (e.g., cow, pig, chicken), and species-specific reagents like validated antibodies are not generally commercially available for most aquatic animals. Thus, this

¹Cultivated meat has become one of the industry-preferred terms for products made from animal cell culture as a neutral term that is conducive to conversations with the conventional meat industry and with regulatory agencies. Other terms include clean meat—as a nod to the environmental benefits (akin to clean energy) as well as the reduced risk of bacterial contamination—and cultured meat and in vitro meat have historically been used as well. The terms cultivated meat and cultivated seafood are used throughout this report.

field lacks established protocols and a rich scientific literature from which to draw, thereby requiring significant up-front investment in basic R&D by companies entering this space.

Cultivated seafood exhibits several potential advantages over mammalian or avian cell culture. Cells grown in culture perform best when growing conditions mimic the natural environmental conditions for that particular animal. In contrast to mammalian cell culture, which is typically conducted at 37°C, fish cell culture can be performed at appreciably lower temperatures of 4–24°C for saltwater and 15–37°C for freshwater species [1]. Many fish species also undergo muscle hyperplasia as juveniles, leading to rapid expansion in muscle cell number and biomass. This rapid growth ability may offer higher yield of skeletal muscle tissue in a shorter time when translated to a cultivated meat production environment. Additionally, many fish and crustaceans retain high expression of the enzyme telomerase in multiple tissue types, which may enable long-term proliferative capacity or facilitate the establishment of immortalized cell lines for research use and, ultimately, commercial cultivated seafood production [2–5].

Cultivated seafood may also pose both advantages and unique challenges regarding developing tissues that mirror the structural patterns found in fish muscle. Meat in finfish exhibits a simple structure relative to muscle tissues found in many terrestrial animals, which tend to form complex and stochastic vasculature and marbling. This suggests that scaffold fabrication to encourage differentiation into muscle and fat in defined patterns may be more straightforward for cultivated fish than for meats like beef or pork. At the same time, the more regular fish structure may make any differences in cultivated fish more obvious, necessitating finer control over the structure. Scaffolds for fish will also need to recapitulate the properties of fish connective tissue, such as its lower melting temperature compared to that of terrestrial meat [6].

Much of the appeal for pursuing cultivated seafood relates to consumer issues around how seafood is consumed as well as market considerations, as many types of seafood command very high prices per pound. The meat of aquatic animals, such as oysters and tuna steaks, is more likely to be consumed raw or minimally cooked compared to meat from other animals. As previously noted, aquatic animals carry a variety of bacteria, viruses, and parasites that frequently cause foodborne illness.

Given that raw consumption increases the likelihood of foodborne illness and these raw dishes tend to command fairly high price points from consumers, these items may present an ideal entry market for introducing high-end, contaminant-free, cultivated seafood products prior to introduction of mainstream seafood products, which may take longer to achieve price parity at scale. Lastly, many consumers place a premium on fresh seafood that has not been frozen [7], but product loss due to spoilage is a major concern within the seafood industry. Because cultivated seafood will be produced in aseptic cultivators, product shelf life may be dramatically improved without having to resort to freezing.

1.1 Industry needs

While cultivated seafood products will ultimately be produced and supplied through the private sector, the underlying technologies and their path toward commercialization will require a robust innovation ecosystem. Given that very little funding outside of a few companies' research and development (R&D) budgets has been expended in this area and that the estimated *total* global R&D expenditure to date across all forms of cultivated seafood is on the order of about \$100 million, this industry exhibits tremendous potential to benefit from concerted public and private resource allocation. To accelerate the process from early product development through to widespread market adoption, activities must be coordinated across startup companies, multiple sectors of established industries, private and public funders and investors, governments, trade associations, and academic and other research institutions. All of these entities—and any individual who envisions a future with sustainable oceans of abundance—should consider this a call to action to contribute to the development and growth of the cultivated seafood industry.

GFI's full report on alternative seafood, *An Ocean of Opportunity*, can be found at <https://gfi.org/resource/an-ocean-of-opportunity/>.

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Recent advances in fish disease diagnosis, therapeutics, and vaccine development

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1. Introduction

There has been significant growth in fish and fisheries worldwide in last 50 years. Aquaculture is currently one of the fastest-growing food sectors in the world, with the majority being finfish production. Aquaculture is an important industry globally as it remains one of the significant alternatives of animal protein source supplies for humans [1]. Aquaculture has shown steady pace of growth, being the fastest food-production sector, globally. Global fish production has been estimated to be about 179 million tonnes in 2018, with a total value of USD 401 billion, out of which 82 million tonnes, valued at USD 250 billion, came from aquaculture production [2]. The total world fish production is expected to reach 196 million tons (Mt) by the year 2025, where aquaculture is estimated to surpass the total production of capture fisheries [3]. By 2025, aquaculture is expected to contribute approximately 57% of fish consumption worldwide [4,5]. The majority of growth will take place in developing countries, where freshwater species is expected to become more important [3].

India is the third largest fish producing country and the second largest aquaculture fish producer in the world. India contributes about 7% to the global fish production. The country is also home to more than 10% of the global fish biodiversity and is one of the 17-mega biodiversity rich countries [6]. The rapid growth of aquaculture has also been the source of multiple interventions to enhance production. The progression of this industry is being dampened by the increasing rate of fish mortality, mainly the outbreak of infectious diseases. Besides these, international trade in live aquatic animals and their products have been the major reason associated with new epizootics [7]. The cultured animals are being exposed to various environmental stresses, often the cause of disease induction, emergence, and spread of newer pathogens [8]. Induction of fish disease in culture systems is evident only when stressful condition dominates, thereby suppressing the immune status of fish [9,10].

Without a strategic approach to curb infectious diseases, the increasing demands of the aquaculture industry may not be sustainable and hence contributing to the overfishing of wild fish. Improved rapid diagnostics is crucial for the sustainable future for aquaculture. There is need for innovative biotechnological interventions to develop suitable field level diagnostic methods for rapid and accurate diagnosis of fish pathogens and develop suitable preventive and therapeutics in aquaculture disease management. Molecular techniques can be used to solve that type of problems and increase sensitivity and specificity of pathogen detection [11]. This chapter reviews development and application of modern biotechnological tools in disease diagnosis and sustainable development of aquaculture.

2. Disease problems in fish culture

Disease outbreaks are recognized as a significant constraint to aquaculture production and trade, affecting both the economic development and socioeconomic revenue of the sector in many countries in the world. The current trend in

aquaculture development is toward increased intensification and commercialization of aquatic production. Like other farming sectors, the likelihood of major disease problems increases as aquaculture activities intensify and expand. Disease issues are of great concern in aquaculture production. Production costs are increased through investment lost in dead cultured animals, cost of treatment, and decreased quality and quantity of yields [12]. Thus, the aquaculture industry has been overwhelmed with its share of diseases and problems caused by viruses, bacteria, fungi, parasites, and other undiagnosed and emerging pathogens. Disease is now a primary constraint to the culture of many aquatic species, impeding both economic and social development in many countries [12,13].

Outbreaks of disease are greatly influenced by the susceptibility of the hosts, the virulence of the pathogens, and adverse environmental conditions. Intensive and semi-intensive farming practices favor disease occurrence, due to high stocking densities, increased stress to cultured stocks, and inadequate water exchange [12,14]. A wide variety of parasites and pathogens can and do infect fish. Most disease agents are naturally present in low numbers and normally do not cause problems. The natural defense mechanisms of fish (i.e., undamaged skin, mucus covering the skin, and various components of the immune system) keep disease agents in check. However, when fish already crowded in culture operations are further stressed (e.g., by low dissolved oxygen, nutritionally inadequate feeds, excessive handling) their natural disease defense systems may be weakened and the ability of the fish to protect itself against infectious diseases may be reduced. Thus, fish become more susceptible to various infectious organisms such as protozoa, helminths, crustaceans, bacteria, fungus, and virus [15–17]. Disease-induced catastrophic mortalities are frequently the result of, and response to, a stressful experience. Most disease problems can be avoided with proper management. List of some common infectious diseases reported in Indian aquaculture have been presented in Table 10.1. These microorganisms are essentially opportunistic pathogens which invade the tissues of a fish host rendered susceptible to infection by stress factors [12,13]. Common bacterial pathogens, *Aeromonas* species, *Aeromonas salmonicida*, *Vibrio* spp., *Edwardsiella ictaluri*, *Edwardsiella tarda*, *Streptococcus* spp., and other related gram-positive cocci, cause disease in aquaculture species [18]. Gram-negative bacteria

TABLE 10.1 Common diseases recorded from cultivable freshwater fish species in India.

Sl. No	Disease condition	Symptoms	Involvement of pathogens
A. Bacterial disease			
1.	Aeromoniasis or motile <i>Aeromonas</i> septicemia (red disease/ <i>Aeromonas</i> septicemia/Dropsy)	Hemorrhagic and ulcerative lesions on skin fins, and head; exophthalmia; dropsy; erosions; septicemia	<i>Aeromonas hydrophila</i> , <i>Aeromonas veronii</i> bv. Sobria, <i>Aeromonas sobria</i>
2.	Tail rot and fin rot	Erosions, discoloration, and disintegration of fins and tails	<i>A. hydrophila</i> , <i>Pseudomonas</i> spp., <i>Cytophaga</i> spp., <i>Haemophilus</i>
3.	Columnaris disease	Hemorrhagic and ulcerative lesions on fins, head, and back, which may look yellow to orange due to bacterial growth and pigmentation	<i>Flavobacterium columnare</i> (<i>Flexibacter/Cytophaga columnaris</i>)
4.	Edwardsiellosis or <i>Edwardsiella</i> septicemia	Ulcerative abscesses in internal organs; hemorrhagic ulcers on skin, fins, and body; rectal protrusion	<i>Edwardsiella tarda</i>
5.	Pseudomoniasis/ <i>Pseudomonas</i> septicemia	Hemorrhagic lesions on skin, fins, and tail	<i>Pseudomonas</i> sp. <i>Pseudomonas fluorescens</i> <i>Pseudomonas anguilliseptica</i>
6.	Bacterial gill disease	Gasping, lethargic, gills look discolored with trapped materials, secondary fungal infection	<i>Flavobacterium branchiophilum</i> , <i>Cytophaga</i> spp., <i>Flexibacter</i> spp.
7.	Enteric red mouth disease (ERM)	Hemorrhagic lesions on skin around mouth, fins, and tail; internal hemorrhages	<i>Yersinia ruckeri</i>
8.	Eye disease	Cataract of eyes, affect cornea, eyeball gets putrefied	<i>Aeromonas liquefaciens</i> , <i>Staphylococcus aureus</i> , <i>Streptococcus</i> sp.
9.	Vibriosis	Ulcerative abscesses in internal organs, hemorrhagic ulcers on skin, fins, and body	<i>Vibrio anguillarum</i> , <i>Vibrio parahaemolyticus</i> , <i>Vibrio alginolyticus</i>

TABLE 10.1 Common diseases recorded from cultivable freshwater fish species in India.—cont'd

Sl. No	Disease condition	Symptoms	Involvement of pathogens
B. Fungal diseases			
1.	Saprolegniasis (Fish molds/cotton wool disease)	Fish become weak, ulceration of skin with hemorrhages, cotton wool growth on ulcers with gray patches	<i>Saprolegnia parasitica</i> , <i>Saprolegnia diclina</i> , <i>Achlya</i> sp., <i>Aphanomyces</i> spp.
2.	Branchiomycosis (gill rot disease)	Redness of gills, which later become grayish-white, necrosis of gill filaments	<i>Branchiomyces demigrans</i> , <i>Branchiomyces sanguinis</i>
3.	Epizootic ulcerative syndrome (EUS)	Fish become lethargic, redness of skin, ulcerative patches	<i>Aphanomyces invadans</i> sp. (fungus), <i>A. hydrophila</i> , <i>A. sobria</i>
C. Parasitic diseases			
1.	Argulosis (carp lice)	Skin lesions with secondary bacterial infections, hemorrhagic spots and ulcers observed	<i>Argulus</i> spp.
2.	Gyrodactylosis (Skin fluke)	Destroy the skin, gills with clumps of white masses, frequently associated with secondary infections	<i>Gyrodactylus</i> spp.
3.	Dactylogyrosis (gill fluke)	Mostly affects gills, destroying the gill filaments, loss of reddish color, gills with clumps of white masses	<i>Dactylogyrus</i>
4.	Lernaeasis (Anchor worm)	Irritation and scratching of body noted. There may be focal hemorrhages and ulcers	<i>Lernaea</i> sp.
5.	Trichodiniasis	Whitish cysts observed on skin, fins, and gills. Disc-shaped, spherical cysts can be observed in whitish material under microscope	<i>Trichodina</i> sp.
6.	Ichthyophthiriasis (ich/white spot disease)	Whitish cysts or pustules of 1 mm diameter, mostly observed on skin, fins, and gills	<i>Ichthyophthirius multifiliis</i>
7.	Myxosporidiasis	Parasite produces cysts on different parts of body, internal organs, and gill filaments. Fish becomes weak, falling of scales	<i>Myxosporidium</i> spp.

Adopted from S.S. Mishra, R. Das, M. Dhiman, P. Choudhary, J. Debbarma, S.N. Sahoo, A. Barua, B.S. Giri, R. Ramesh, K. Ananda, C.K. Mishra, P. Swain, Present status of fish disease management in freshwater aquaculture in India: state-of-the-art-review, J. Aquac. Fish. 1 (2017) 1: 004.

commonly known to be pathogenic to fish, like *Aeromonas* spp., *Flavobacterium* spp., *Pseudomonas* spp., and *Shewanella putrefaciens*, are replaced by other species, which until now have not been known to be virulent or even conditionally pathogenic to fish. Nowadays, among these other species *Acinetobacter* spp., *Plesiomonas shigelloides*, *Sphingomonas paucimobilis*, and *Stenotrophomonas maltophilia* are the most frequently isolated from fish exhibiting clinical signs of disease [19]. Meanwhile, in Central Europe, infections caused by *Aeromonas* spp. are the most common among bacterial fish diseases, causing motile *Aeromonas* septicemia (MAS), motile *Aeromonas* infection (MAI), and furunculosis. Infections caused by *Flavobacterium* spp. are also very often observed [19]. There are reports of *Vibrio* hemorrhagic septicemia or vibriosis has a widespread infection in cultured groupers in Brunei Darussalam, Malaysia, Taiwan, Indonesia, Kuwait, Thailand, Singapore, and the Philippines [1,20]. The causative pathogens of vibriosis are from Gram-negative bacteria of *Vibrio* species, which include *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Vibrio vulnificus*, *Vibrio carchariae*, *Vibrio anguillarum*, *Vibrio ordalii*, *Vibrio harveyi*, *Vibrio mimicus*, and many more from the family of *Vibrionaceae* [21]. Again cases of furunculosis infection due to *A. salmonicida*, a Gram-negative, nonmotile rod bacterium affecting mainly salmonid species, trout, charr, and grayling, have been commonly reported [22]. However, these diseases are not of significance in Indian aquaculture.

Contrary to bacterial diseases, occurrence of parasitic infestations is of great concern to fish farmers. The production from culture system is hampered by the infestation of various fish parasites [23]. Fish parasites multiply rapidly under favorable conditions, thereby affecting the health of fishes, often leading to high mortality. Parasites interfere with nutrition of hosts, disrupt metabolism and secretory functions of alimentary canal, and damage nervous system [24]. Different parasites like protozoan ciliates (*Ichthyophthirius* sp., *Trichodina* sp.), monogenetic trematodes (*Dactylogyrus* sp., *Gyrodactylus* sp.), larger crustacean ectoparasites (viz. *Lernae* spp., *Argulus* spp.), and the freshwater louse (*Ergasilus*) cause substantial economic loss in fish culture system in India [16,17], [23].

3. Disease diagnostic techniques

Pathogen identification is a key area in disease diagnosis and management. In case of disease outbreaks, quick diagnosis and rapid removal of infected fish are necessary for the successful implementation of effective control and health management strategies in aquaculture [25]. Classical, immunological, and molecular methods have been routinely and extensively used to address this area. A variety of methods are available to detect pathogens both in fish and in the aquatic environment. These include traditional diagnostic methods and a diverse range of immunological and molecular methods. However, a combination of methods is often required for a definitive diagnosis of disease [26,27]. Traditional methods involve first culturing and isolating the pathogen from infected tissue and then identifying the organism involved. Histology, histopathology, biochemical tests, microscopy, and electron microscopy are also routinely used in disease diagnosis. OIE [28] and FAO/NACA [29] have recommended the use of three levels of diagnostics for emerging and reportable fish and shellfish diseases. All three levels of diagnostic specialization are necessary for confirmation of newly or rarely encountered disease. The different levels of disease diagnosis which can be undertaken when investigating a disease situation are as follows:

- a. Level I: Farm/Production site observations, record-keeping, and health management. This is important, as this forms the basis for triggering the other diagnostic levels (II and III).
- b. Level II: It includes the specializations of parasitology, histopathology, bacteriology, and mycology, which require moderate capital and training investment.
- c. Level III: It comprises the types of advanced diagnostic specialization, which requires significant capital and training investment. Isolation of the causative bacteria, fungus, and virus, biochemical characterization of pathogens, immunoassays (enzyme-linked immunosorbent assays or fluorescent, antibody technique), transmission electron microscopy, molecular diagnostics like polymerase chain reaction (PCR) and nucleic acid assays using specific probes, etc., come in this category.

Different diagnostic techniques used in pathogen identification and disease diagnosis in aquaculture have been presented in Table 10.2.

3.1 Serological techniques

The study of antigen–antibody reactions in vitro is called serology and is important in clinical diagnostic microbiology. Immunodiagnostic tests use an antigen–antibody reaction to detect and identify a specific antigen or antibody associated with a disease-causing organism. Rapid detection and identification of microorganisms without culture was made possible by the development of immunoassays, which are based on the specific binding between an antigen and its corresponding antibody. The assays either detect the presence of specific antibodies raised in response to an antigen or detect the substance with antigenic properties itself [30]. Reactions of antigens and antibodies are highly specific. An antigen will react only with antibodies elicited by itself or by a closely related antigen. Because of the high specificity, reactions between antigens and antibodies are suitable for identifying one by using the other. The most important point about serologic methods is that they are useful for determining whether animals have been previously exposed to a disease agent; however, they provide little information regarding the presence of a current infection in an animal or population [31]. The immunoassays can be grouped into several categories according to the type of detection systems used: (1) colorimetric, (2) radiometric, (3) chemiluminescent, or (4) fluorescent [32]. The technique is being used in many applications in bacteriology and is available in many different formats, such as enzyme immunoassays, immunofluorescence assays, latex agglutination assays, line immunoassays, and lateral-flow immunoassays [33]. Monoclonal antibodies provide ideal standardized reagents for use in these methods, and many such products are now commercially available [26].

TABLE 10.2 Commonly used diagnostic techniques in aquatic animal disease diagnosis.

Visualization techniques	Serological techniques	Molecular techniques
(i) Gross examination, wet preparation, cytology, and histology	(i) Agglutination assays: latex agglutination assay	(i) Polymerase chain reaction (PCR) (a) Conventional PCR, nested PCR, multiplex PCR (b) Reverse transcriptase PCR (c) Real-time PCR (qPCR) (d) Loop-mediated isothermal amplification (LAMP)
(ii) Special histological/cytological techniques	(ii) Immunofluorescent assays: Immunofluorescent antibody (IFA) testing	(ii) Restriction fragment length polymorphism (RFLP)/DNA fingerprinting
(iii) Electron microscopy (a) Scanning electron microscopy (SEM) (b) Transmission electron microscopy (TEM)	(iii) Enzyme-linked immunosorbent assay (ELISA)/Dot-ELISA	(iii) Microarray analysis
(iv) Culture techniques (a) Bacterial culture (b) Virus culture (c) Fungal culture (d) Protozoal culture	(iv) Western blotting technique	(iv) In situ hybridization
	(v) Lateral-flow diffusion assay	(v) Omics technology (proteomics)
	(vi) Plaque reduction neutralization test	(vi) Nanotechnology and nanosensors

3.1.1 Agglutination test and latex agglutination test

Agglutination reactions are among the most easily performed of immunological tests. Their usefulness in presumptive identification of bacteria has long been recognized and for bacterial fish pathogens has been reconfirmed and documented [34]. With as few as a dozen antisera, it is possible to confirm the identity of the majority of known bacterial fish pathogens. Whole-cell agglutination using polyclonal antisera has been used extensively for identifying cultures with accuracy depending on the specificity of the antiserum. A culture is mixed with antiserum in the presence of saline on a microscope slide and a positive result indicated by clumping of the bacteria observed within 2 minutes. Agglutination test has been widely used in detecting bacterial fish pathogens belonging to the genera *Vibrio*, *Pasteurella*, *Aeromonas*, *Yersinia*, *Edwardsiella*, and *Pseudomonas* [30].

In latex agglutination, antisera/antibodies were absorbed onto latex particles, which is known as sensitized latex. Pure or mixed cultures or pathological material containing pathogens are mixed with the reagent on a glass or plastic surface. A positive reaction as indicated by clumping of the latex particles develops within 2–3 min. The latex agglutination test is used to check for certain antibodies or antigens in a variety of bodily fluids including saliva, urine, cerebrospinal fluid, or blood [34,35].

3.1.2 Immunofluorescent assays

These assays are used to detect antigens of specific disease agents inside infected tissues or cells and allow for visualization of the antigen in the cell when it binds to a fluorescent-labeled antibody. Searching for bacteria in fish tissue is easier than defining viral infections by fluorescent antibody test (FAT). The FAT makes use of the feature that some dyes (fluorescein isothiocyanate, rhodamine isothiocyanate, Texas red) fluoresce when viewed under ultraviolet (UV) light. Immunofluorescence can be detected either by direct or by indirect procedures and occurs when both the antigen and antibody are present [30,34]. Direct test (detects antigen, i.e., infection) requires an antigen-specific antibody labeled with a fluorochrome (fluorescein, rhodamine). Indirect test (detects antibody, i.e., exposure) requires a fluorescent labeled antiserum to an agent-specific immunoglobulin. Advantage of this method is that it is very sensitive and rapid method for detecting and identifying disease agents in smears, tissues, or cultures, but the disadvantages are that it requires development of fluorescent-labeled antibody or antiserum [26]. Results can be subjective, causing false-positive or false-negative results. Cross-reaction with another antigen may occur.

3.1.3 Enzyme-linked immunosorbent assay

ELISA is one of the most powerful of all immunochemical techniques. It employs a wide range of methods to detect and quantitate antigens or antibodies and to study the structure of antigens. This technique makes use of antibodies to which enzymes have been covalently bound such that the enzyme's catalytic properties and the antibody's specificity are unaltered. Typical linked enzymes include peroxidase, alkaline phosphatase, and beta-galactosidase, all of which catalyze reactions whose products are colored and can be measured in very low amounts using ELISA reader [34]. There are many variations on the ways the immunoassays can be performed. Basic ELISA designs include indirect, direct, and competitive ELISA, but these ELISA designs can be combined. In indirect ELISA, antigen of interest is purified, and the presence or concentration of antibodies against it is detected or measured. In direct or antigen capture ELISA, the presence or concentration of the antigen of interest is detected or measured. In competitive ELISA, inhibition-type assay, the presence or concentration of antibody is detected or measured. In sandwich ELISA, the presence or concentration of antibodies is detected or measured.

Dot-ELISA is an extensively used immunological tool in research as well as analytical/diagnostic laboratories. In sandwich Dot-ELISA, the antigen is sandwiched directly between two antibodies which react with two different epitopes on the same antigen. Here one of the antibodies is immobilized onto a solid support and the second antibody is linked to an enzyme. Antigen in the test sample first reacts with the immobilized antibody and then with the second enzyme-linked antibody. The amount of enzyme-linked antibody bound is assayed by incubating the strip with an appropriate chromogenic substrate, which is converted to a colored, insoluble product. The latter precipitates onto the strip in the area of enzyme activity, hence the name Dot-ELISA [35]. The enzyme activity is indicated by intensity of the spot, which is directly proportional to the antigen concentration.

ELISA test has the advantage of high throughput and can be automated. This assay is particularly useful for detecting and quantifying pathogens during clinical disease but is less useful for subclinical infections due to the sensitivity limits of the assay [26]. ELISA test has been used to detect many bacterial diseases like *A. salmonicida* in fish, *V. parahaemolyticus*, and *V. harveyi* in penaeid shrimp and viral diseases like viral hemorrhagic septicemia virus (VHSV) and striped jack nervous necrosis virus in fish [30]. These immunoassays are of significant use for detection of exotic pathogens of cultured finfish, e.g., channel catfish virus (CCV), infectious hematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV), VHSV, viral nervous necrosis virus (VNN), and bacterial kidney disease (BKD).

3.1.4 Western blotting

Western blotting is a rapid and sensitive assay for the detection and characterization of proteins. Western blot and dot blot are methods not routinely used in fish diagnostics, but their application can be useful in certain situations. The technique allows one to identify particular proteins by utilizing the specificity inherent in antigen–antibody recognition [30]. Proteins are revealed as discrete bands by running sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a nitrocellulose membrane. When serum is applied, antibodies in the serum bind to the proteins on the membrane. In this technique, capture antigens such as proteins are electrotransferred to a nitrocellulose membrane. If target antibodies are present in the specimen, they will bind to the antigens present on the nitrocellulose strips. Visualization of the antibodies bound to antigen is accomplished using a series of reactions with goat antihuman IgG conjugated with biotin, avidin conjugated with HRP, and the HRP substrate. The bands corresponding to the antigens will be seen on the nitrocellulose strip [32]. These immunodiagnostic tests are currently used to screen and/or confirm diagnosis of many fish diseases [30]. The advantage of this technique is that it is most specific viral diagnostic test available, as it provides a full antibody profile against the disease agent. However, the disadvantages are that reagents, such as monoclonal or polyclonal antibodies, are necessary to run the assay.

3.1.5 Lateral-flow diffusion assay

This method has been a recent development of serological test, designed more for the antigen-specific immunoassay than for antibody detection. It uses colloidal gold, carbon, paramagnetic, or color latex beads to create a visible line in the capture zone where there is a nitrocellulose or nylon membrane. Lateral-flow assays have been available on the commercial market. The assays are typically designed on nitrocellulose or nylon membranes contained within a plastic or cardboard housing. In the antibody detection format, a capture antigen is bound to the membrane, and a second labeled antibody is placed on a sample application pad. As the sample migrates down the membrane by capillary action, antibody present in the sample binds to the labeled antigen and is captured as the complex passes. Colloidal gold, carbon, paramagnetic, or colored latex beads are commonly used particles that create a visible line in the capture zone of the assay membrane for a positive result. These assays are simple to use, require minimal training, and require no special storage conditions. In most

cases, the manufacturer provides simple instructions that include pictures of positive and negative results. This technique has the potential for future use once validated, particularly for detecting pre-exposure of fish to viral pathogens [26].

3.2 Molecular diagnostics for fish diseases

The application of biotechnology in aquaculture has enabled the development and improvement of a wide range of immunodiagnostic and molecular technologies, and reagents and commercial kits have become more generally available. Recently, method development has increased exponentially as techniques used for clinical and veterinary medicine are adapted and optimized for use in aquaculture. Fish disease diagnosis has progressed from the traditional approaches of isolation and phenotypic characterization of the pathogen to the modern methods of molecular biology [27,34]. Traditional diagnostic methods tend to be expensive, labor intensive, and time-consuming and might not lead to a specific diagnosis, even when compared with histological evidence. Sometimes, immunological methods are unable to detect the pathogen because the amount of pathogen present in the sample is below the sensitivity threshold of the assay, or the antigens on the pathogen have been altered and no longer recognized by the antibody [26]. Nucleic acid-based diagnostics gradually are replacing or complementing culture based, biochemical, and immunological in routine microbiology laboratories [33]. The main function of all diagnostic bacteriology laboratories is the detection and identification of microorganisms in a variety of samples of freshwater, brackish water, and marine origin. Again, the identification and characterization of unculturable and slow-growing pathogens is easier to detect using such molecular methods [7,36]. In addition to high sensitivity and the possibility of rapid diagnostics, the main advantage of molecular methods is to determine the uncultivated infectious agents. Deoxyribonucleic acid (DNA) amplification allows identifying pathogenic microorganisms at very small quantities even in the minimum sample volume [33]. Molecular diagnostics are the basic routine techniques to be followed in every laboratory nowadays. Such methods are essential for any research-based or clinical-diagnostic-affiliated purposes with utilization of molecular elements of the cell. With an objective to improve with the ongoing research and facilitate the future of diagnostics, repetitive modifications have been necessarily employed. Invention and availability of advanced automated instruments, approach toward disease diagnosis has taken another leap. Molecular methods of diagnostic enable the determination of infection in latent or acute phases. These methods allow showing the differences between pathogens with similar antigenic structures. Recent improvements in detection technologies have paved the way for the development of multiparameter assays using macro arrays or microarray, while the introduction of closed-tube real-time PCR systems has resulted in the development of rapid microbial diagnostics with a reduced contamination risk. Molecular techniques which have the major significance are mainly PCR-based molecular diagnostic methods including PCR, real-time polymerase chain reaction (RT-PCR), multiplex polymerase chain reaction (multiplex PCR), etc. [27,37]. The use of these molecular technologies is not restricted to detection and identification of microbial pathogens but also can be used for genotyping, allowing one to determine antibiotics resistance or to perform microbial fingerprinting [26,33].

3.2.1 Polymerase chain reaction

The introduction of nucleic acid amplification technologies that enable the multiplication of a few target molecules to a detectable level has provided new tools for rapid, specific, and sensitive detection, identification, and resistance testing of microorganisms starting from sample material without culturing. The PCR is the technique most used in research or diagnostic laboratories. The PCR technique was an important and revolutionary method developed by Kary Mullis in the 1980s. It is an efficient and cost-effective way to copy or amplify small segments of DNA. One of the fundamental uses of PCR is detection of pathogens with specificity in amplifying a particular stretch of sequence. Many variations of this PCR assay exist, including a two-step or a nested PCR or nested reverse transcriptase PCR (RT-PCR), random amplified polymorphic DNA, reverse cross-blot PCR, real-time PCR, and an RT-enzyme hybridization assay [26]. PCR has wide scope of applications, including analysis of ancient DNA from fossils, DNA fingerprinting, mapping the human genome, and detection of microorganisms present in low numbers in water, food, soil, or other systems [30,38].

3.2.1.1 Conventional polymerase chain reaction

In 1983, the PCR was discovered by Kary Mullis [39]. PCR resembles an in vitro and elementary form of DNA replication, a physiological process used by all living cells to duplicate their genetic material before cell division [37]. Theoretically, the increase in the amount of product after each round will be geometric [40]. In every cycle of PCR, approximately the amount of DNA increased to double, as well as it is repeating in the following cycle, and a new strand of DNA subsequently acts as a template for replication. This results in an exponential increase in the number of targeted segments of DNA during PCR. A total of 25–40 PCR cycles are carried out, depending on the expected yield of the PCR products.

PCR method has been valuable for detecting specific pathogens that are difficult to culture in vitro or require a long cultivation period, and it is significantly more rapid in providing results compared to culture [37]. The assay has also been used in the detection of *Vibrio penaeicida* in shrimp and *A. salmonicida* subspecies *salmonicida* [38]. The basic requirement of PCR is to design specific primer sequence for target gene of importance that can be able to characterize and differentiate specific strain/species of organism or animals. Hence work on recognizing specific target genes must be carried out for different fish pathogens to fully exploit the potential application of PCR technology in diagnosis.

Nested and heminested PCRs are designed to increase the sensitivity of PCR by directly reamplifying the product from a primary PCR with a second PCR. Nested PCR primers are ones that are internal to the first primer pair. The larger fragment produced by the first round of PCR is used as the template for the second PCR [38]. Nested PCR can also be performed with one of the first primer pair and a single nested primer. In heminested PCR, the second round of PCR uses one of the first-round primers and one new, internal primer. The amplicon from the second round of PCR is shorter than that of the first [32]. With this approach, the sensitivity and specificity of DNA amplification might be greatly improved [37,38]. The advantage of nested PCR is that the sensitivity and specificity of both DNA and RNA amplification can be dramatically increased by using this method. The specificity is particularly enhanced because this technique almost always eliminates any spurious nonspecific amplification products. Disadvantages of nested PCR include extra time and cost associated with two rounds of PCR and the increased risk of contamination incurred during transfer of first-round amplification products to a second tube [30,32,38].

3.2.1.2 Multiplex PCR

Multiplex PCR (mPCR) alludes to utilize polymerase chain response to intensify a few diverse DNA arrangements at the same time [37]. In multiplex PCR, two or more primer sets planned for the expansion of diverse targets are included within the same PCR reaction [9]. This technique intensifies DNA in tests utilizing numerous primers and a temperature-mediated DNA polymerase. The preliminary plan for all primer sets should be optimized so that all primer sets can work at the same annealing temperature amid PCR [32,41]. The multiplex PCR is a widespread molecular technique utilized for the amplification of multiple targets in just one PCR experiment. Multiplex PCR has been to be a valuable method for identification of viruses, bacteria, fungi, and parasites [14]. mPCR assays are already examined and studied to detect the fish pathogenic bacteria and viruses, such as *Aeromonas hydrophila*, *E. tarda*, and *Photobacterium damsela*, which can be detected in a single run of PCR by using multiple sets of primers [37]. Fish pathogenic viruses like IPNV, IHNV, and VHSV can be identified by multiplex PCR [42]. Multiplex PCR amplification for simultaneous detection of fish herpesvirus (CyHV-1, CyHV-2, and CyHV-3) has been developed [43]. Another, multiplex RT-PCR assay has been developed for the simultaneous detection of four rainbow trout viruses (VHSV, IHNV, IPNV, and sleeping disease virus) in a single assay [44]. Altink [45] used an mPCR for the simultaneous detection of four major fish pathogens: *Flavobacterium psychrophilum*, *Lactococcus garvieae*, *Pseudomonas aeruginosa*, and *Pseudomonas putida*. However, there are some disadvantages of mPCR. The self-inhibition among different sets of primers can be a major drawback. Hence primer design and compatibility is important in case of mPCR.

3.2.1.3 Reverse transcriptase PCR

Taq polymerase does not work on RNA samples, so PCR cannot be used to directly amplify RNA molecules. Because DNA polymerase requires a double-stranded DNA template, RNA must be transcribed into complementary cDNA prior to PCR by the enzyme reverse transcriptase because *Taq* has limited reverse transcriptase activity. This method of RNA amplification is called reverse transcriptase PCR (RT-PCR). Two reverse transcriptase enzymes commonly used in RT-PCR are Moloney murine leukemia virus reverse transcriptase and Avian myeloblastosis virus reverse transcriptase. Both enzymes have the same fundamental activities but differ in some characteristics, including temperature and pH optima. These enzymes are available in preoptimized RT-PCR kits. One-tube RT-PCR incorporates both the reverse transcriptase enzyme and a thermostable DNA polymerase in a single tube for synthesis and amplification of the target RNA sequence. This is the preferred procedure for routine analysis. Commercial RT-PCR kits are available, and alternatively, reagent mixes can be prepared also from separate component parts [32]. There are several different kinds of primers that can be used to make cDNAs: oligo-dT will prime cDNA synthesis on all polyadenylated RNAs, random-primed cDNA synthesis gives a broad range of cDNAs and is not limited to polyadenylated RNAs, and lastly, oligonucleotide primers complementary to the RNA(s) of interest may be used to synthesize highly specific cDNAs [30]. RT-PCR is commonly used for detection of RNA shrimp viruses, which have caused havoc in aquaculture. Different researchers have used RT-PCR for detection of Tilapia Lake Virus in fish [46,47] and the test was found to be very sensitive and specific.

3.2.1.4 Real-time PCR

In the area of molecular diagnostics, real-time PCR—based assays have acquired great application in diagnostic laboratories. A real-time PCR, additionally recognized as quantitative polymerase chain reaction (qPCR), is a laboratory technique of molecular biology based totally on the PCR. It observes the amplification of a targeted DNA molecule in the course of the PCR, in real time and not at its end, as in conventional PCR [37]. qPCR or real-time PCR is the most sensitive molecular method to quantify viral load and estimate the degree of infection of a particular virus. It has provided the ultimate way of counting virions per cycle of amplification of DNA. This has mediated the idea of correlation between the expected clinical signs of a virus depending on its count in the host cell, thus being able to make possible conclusions and observation. Real-time PCR reduces time for nucleic acid assays by eliminating post-PCR result detection.

The introduction of homogeneous or real-time PCR assays based on solution phase hybridization methods, which combine fluorescent labeled probes with amplification technologies, has contributed strongly to the acceptance of molecular assays in the microbiology laboratory. Real-time PCR is carried out in a thermal cycler with the ability to illuminate every sample with a beam of light of at least one particular wavelength and realize the fluorescence emitted by using the excited fluorophore. Several different probe technologies that are compatible with different real-time thermocycling instruments currently are available. Most of these technologies are based on the principle of fluorescence resonance energy transfer. This process occurs when two fluorophores are in close proximity to each other and the energy from an excited donor moiety is transferred to an acceptor moiety. The PCR technique usually consists of a sequence of temperature changes that are repeated 25–50 times. The recommended annealing temperature and the duration for qPCR used for every cycle depend on a variety of parameters and factors, such as the concentration of divalent ions, the enzyme used to synthesize the DNA, deoxyribonucleotides (dNTPs) in the reaction, and the annealing temperature of the primers [37].

The so-called hydrolysis probes make use of the 5' to 3' exonuclease activity of *Taq* DNA Polymerase. An oligonucleotide probe is labeled with a fluorescent reporter dye at the 5' end and a fluorescent quencher dye at the 3' end. Upon hybridization of the probe molecule to the target DNA during amplification, the 5' nuclease activity of the enzyme cleaves the probe, separating the reporter dye from the quencher dye, resulting in a measurable increase in fluorescence. PCR thermal cycling results in an exponential amplification of the target DNA and, subsequently, of fluorescence intensity. This technology is also known as the TaqMan system, and many applications in bacteriology are described in literature or can be purchased as commercial kits for use on real-time instruments from Roche Molecular Diagnostics (Pleasanton, CA) or Applied Biosystems (Foster City, CA). Although TaqMan probes, molecular beacons, and hybridization probes are used most widely in real-time amplification, several alternative probe or primer systems have been developed, for instance, Scorpions (DxS Ltd., Manchester, UK) or minor groove binding probes (Epoch Biosciences, Bothell, WA). The TaqMan probe is monitored with a blue light-emitting diode as an excitation source and two photodiode detectors with band-pass filters centered at 530 and 590 nm wavelength. Molecular beacons are stem-loop (hairpin) shaped hybridization probes with a fluorescent dye and a quencher dye on the opposite extremities brought to their proximity by the complementary stem. The commonly used fluorescent dyes are FAM, TAMRA, TET, and ROX paired with a quenching dye, typically DABCYL. Molecular beacons are suitable for mutation analysis and single-nucleotide polymorphism detection when specific mutations are known [32].

In initial cycles the fluorescence is too low to be distinguishable from the background. However, the point at which the fluorescence intensity increases above the detectable level corresponding proportionally to the initial number of template DNA molecules is called the quantification cycle (C_q). This allows determination of the absolute quantity of target DNA in the sample according to a calibration curve constructed of serially diluted standard samples with known concentrations or copy numbers [48]. Fluorescence detection is considered to be more sensitive than immunological or radioactive isotope detection [32]. qPCR offers additional speed, sensitivity, and specificity over traditional PCR methods, and its application in multiplex format increases the number of pathogens that can be analyzed in the same reaction, thus reducing costs [9].

An advantage of real-time PCR is that it is not influenced by nonspecific amplification. This assay required less amount of the template material. The major advantage over the other PCR technique is the quantification and it quantifies the template DNA or RNA present in the sample [37]. By this method, amplification can be monitored in real-time. This assay required less amount of the template material. It needs no post-PCR processing. However, the disadvantage of real-time PCR is that it will only indicate the presence of antigenic material during infection and will not indicate if a host was infected. It needs specialized biocontainment laboratories, operated by highly trained technicians, which makes it an expensive test and difficult to scale. Real-time PCR (RT-PCR) kits are not available for all kinds of genes and disorders and the technical and standardized protocols are limited. Furthermore, higher expertise and technical skills are required for developing an RT-PCR assay [37].

3.2.1.5 Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) is a DNA amplification technique introduced in the year 2000, which was developed by Ref. [49]. This LAMP amplifies DNA with high specificity, efficiency, and rapidity under isothermal conditions [37]. The LAMP reaction uses a DNA polymerase with high strand displacement activity and pairs of specially designed inner and outer primers. This technique depends on autocycling strand displacement in DNA synthesis in the presence of *Bst* DNA polymerase under isothermal conditions. In this method, six different primers are specially designed to recognize eight distinct regions on a target gene, with amplification only occurring if all the primers bind and form a product. Unlike PCR, LAMP is carried out at a constant temperature range of 60–65°C eliminating the need of thermal cycler which makes it a portable tool.

At the three prime terminal, each of the inner primers has a sequence that is complementary to one chain of the amplification region and identical to the inner region of the same chain at the five prime terminal. Using the aforementioned stem-loop areas as a stage, DNA polymerase-mediated strand displacement synthesis repeats the elongation events in a sequential manner [14,49]. This approach works on the basis of producing a large number of DNA amplification products with a mutually compatible sequence and an alternating, repeating structure [35,37]. The reaction is carried out at isothermal condition, as the denaturation of strands takes place by strand displacement. In the initial stages of LAMP reaction all four primers are involved; however, in the later cycling reaction only the inner primers are used for strand displacement DNA synthesis. The LAMP reaction is initiated by an inner primer containing sequences of sense and antisense strands of the target DNA. This is followed by the release of a single-stranded DNA through the priming by an outer primer. This single-stranded DNA will serve as a template for DNA synthesis primed by the second inner and outer primers that can hybridize at the other end of the target [49]. This process will result in the formation of a stem-loop DNA structure. In the subsequent step of LAMP cycling, one inner primer will hybridize to the loop on the product and initiate strand displacement DNA synthesis which will result in the original stem-loop DNA and a new stem-loop. Cycling continues for a period of approximately 1 h and results in the accumulation of 10^9 copies of the target [35].

Some commercial LAMP kits use an enzyme substrate system to visualize the reaction on a membrane [26]. The amplification of the target gene is completed within 1 h at temperatures ranging from 60 to 65°C [49,50]. A large amount of product is formed, due to the strand displacement activity of *Bst* polymerase enzyme. Because of this property, identification of a positive reaction does not require any special processing or electrophoresis [50,51]. Different types of LAMP include proofreading LAMP (PR-LAMP), reverse transcription LAMP (RT-LAMP), *in situ* LAMP, allele-specific LAMP (AS-LAMP), real-time LAMP, and peptide nucleic acid LAMP (PNA-LAMP). Soliman and El-Matbouli [52] used one-step RT-LAMP assay for detection of VHSV. A set of six primers were designed, based on the G-protein sequence of the VHSV serotypes. The complete LAMP procedure can be performed in 90 min, and as it is carried out under isothermal conditions, it can be performed without the use of a thermocycler, making it suitable as a field test if a small oven is available.

LAMP has been used for the detection of bacterial, viral, fungal, and parasitic diseases in both animals and plants. In aquaculture, LAMP-based detection of pathogens of fish and shellfish like *E. tarda*, *E. ictaluri*, *Nocardia seriolae*, white spot syndrome virus, and IHNV has been reported [53,54]. A number of LAMP kits are now available on the market for aquaculture. An advantage of LAMP is that this technique is very easy and simple method to execute as soon as the suitable primers are prepared, requiring a DNA polymerase, four primers, and a regular laboratory water bath or heat block for reaction. Moreover, it is extremely precise for the target sequence and amplifies DNA with high efficiency producing 10^9 copies of target sequence less than an hour. Another benefit of LAMP is that it may efficiently amplify RNA sequences when used in conjunction with reverse transcription [37].

3.2.2 Restriction fragment length polymorphism/DNA fingerprinting

Restriction fragment length polymorphism (RFLP) is a commonly used technique that can be used for genotyping for nearly all organisms, including plants, animals, and humans. RFLP is widely used in genetic and genomic research, such as genome mapping and gene identification [55]. The availability of a variety of restriction endonuclease enzymes that cleave DNA at specific sites has made it possible to identify the presence of polymorphic regions in the isolated fragments. Such RFLP results owing to a variation in the number of tandem repeats (VNTR) of a short DNA segment. These VNTR sequences can uniquely specify an individual and, as such, are used in DNA fingerprinting and in paternity testing [56]. RFLP, as a molecular marker, is specific to a single clone/restriction enzyme combination. RFLP utilizes restriction endonuclease digestion to identify DNA sequence polymorphisms in genes or DNA regions of interest. When investigating families for inheritance of an RFLP, aliquots of genomic DNA from individual family members are digested to completion with the restriction enzyme known to generate the polymorphism of interest. After size fractionation on an agarose gel,

DNA is transferred to a membrane by capillary action in a high-salt buffer. The gel is first treated with NaOH to denature DNA, and after neutralization, the gel is placed between buffer-soaked filter paper and a sheet of membrane. Labeled probe is hybridized overnight to the Southern blot. The blot is washed under conditions designed to remove all nonspecifically adherent probe and exposed to X-ray film. Identified fragment sizes differ among individuals and can be traced from generation to generation [57]. An RFLP probe is a labeled DNA sequence that hybridizes with one or more fragments of the digested DNA sample after they were separated by gel electrophoresis, thus revealing a unique blotting pattern characteristic to a specific genotype at a specific locus. Short, single- or low-copy genomic DNA or cDNA clones are typically used as RFLP probes. The RFLP probes are frequently used in genome mapping and in variation analysis (genotyping, forensics, paternity tests, hereditary disease diagnostics, etc.). Certain criteria need to be fulfilled, however, for RFLP to be useful as a genetic disease marker, such as its closeness to the disease gene. Materials and methodology for detecting RFLP are reviewed with the current emphasis on amplification procedures utilizing the PCR [56]. The advantage of RFLP is that it is a highly specific, reliable, and repeatable method but the method is more time-consuming (10–14 days to produce results).

3.2.3 Microarrays

A microarray is used to detect gene expression. It assesses the expression or activity of a specific gene of an organism by quantifying the amount of mRNA the gene produces. Microarray analysis is an assay for the detection of gene expression and not commonly for the detection of infectious agents. Microarray analysis is a modification of conventional RT-PCR, in which multiple RT-PCR reactions, each for a different target sequence, can be run simultaneously. The principles of microarrays are therefore the same as for RT-PCR. In this technique a labeled probe molecule is hybridized to target DNA or RNA attached to a membrane. The probe is attached to the support substrate, usually a nonporous solid surface, and the labeled DNA or RNA then hybridized to the probe [14]. A microarray typically is used to evaluate the response of genes to a stimulus. The effect of an agent on the activity of several hundred genes of an organism can be evaluated.

The major advantage of this approach is that the sample can be screened with thousands of probes simultaneously. This technology allows, in one assay, for simultaneous assessment of the expression rate of thousands of genes in a particular sample. Gene expression profiling using DNA microarrays holds great promise for the future of molecular diagnostics. In diagnostics, microarrays have been shown to be a valuable method for identification and diagnosis of fish diseases caused by viruses, bacteria, fungi, and protozoa in one step [14,32]. This technique helps in simultaneous evaluation of the activity of several hundred genes of an organism but the disadvantage of this technique is that the function of most of these genes, especially of aquatic animals, is unknown. Microarrays may provide a better option for large-scale diagnostic testing and can survey a sample for a multitude of sequences simultaneously. Since most of the pathogens' genetic sequences are available in the GenBank, oligonucleotide probes complementary to all pathogens can be made and inserted into microarray so that a variety of microbes could be detected by a single microarray chip. As a result, microarray-based technology is potentially well suited for identifying fish pathogens in fish populations. Warsen et al. [58] developed a DNA microarray suitable for simultaneous detection and discrimination between multiple bacterial species based on 16S ribosomal DNA (rDNA) polymorphisms using glass slides. Similarly, Cao et al. [59] developed a microarray for the simultaneous detection and identification of diverse putative pathogens often associated with fishery products by targeting specific genes of *Listeria monocytogenes*, *Salmonella*, *Shigella*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *Yersinia enterocolitica* and the 16S–23S rRNA gene internal transcribed spacer (ITS) region of *Proteus mirabilis* and *Proteus vulgaris*. The DNA microarray method was found to be specific, sensitive, and reliable and has several advantages over traditional methods of bacterial culture and antiserum agglutination assays.

Microarrays are already proving valuable for assaying gene expression. A large number of probes on an array can reveal which genes are expressed or are present in the sample. This type of array would be particularly useful in studies of pathogens, where the presence of certain genes or gene products indicates whether the organism is pathogenic or not. Set-up cost for the use of DNA microarrays is high. The use of microarrays is well established in research projects for gene expression studies, but their use in diagnostic applications for microbiology is still in its infancy. Diagnostics companies also are developing DNA chips as illustrated by the recent introduction of an Affymetrix array Food-Expert-ID (Bio-Merieux, Marcy l'Etoile, France).

3.2.4 Hybridization techniques

Fluorescent in situ hybridization (FISH) is an attractive method for the rapid detection and identification of bacteria or fungi directly from slide smears. This technology has the speed and ease of use of conventional staining methods combined

with the specificity of molecular methods. Hybridization with fluorescent labeled probes that target rRNA is performed on smears, and results are observed by fluorescence microscopy. The assay is based on a direct hybridization of labeled (e.g., isotopes, fluorophores) oligonucleotide probes with specific DNA/RNA in sample. The oligonucleotide probes used to identify bacteria are usually short DNA molecules, between 15 and 25 nucleotides long. The shorter the probe, the lower the probe can tolerate mismatches. A variety of labeling and detection systems are available for nucleic acid probes. Radioisotopes (^{35}S , ^{32}P , or ^{125}I) earlier had great demand and use in labeling the DNA probes but health safety has been a great issue. Hence, other labeling methods have become increasingly popular. These include labeling with a variety of haptens such as biotin or digoxigenin and detection by antibody binding coupled with fluorescent, chemiluminescent, or colorimetric detection methods. The use of fluorescent labeled probe is ideal as it usually produces strong signal and less background [32].

An interesting new development is the use of peptide nucleic acid (PNA) probes in FISH applications. PNA molecules have a neutral peptide like backbone unlike the negatively charged sugar phosphate backbone present in DNA. This unique property allows PNA probes to bind strongly and rapidly to their target sequence. This bond is resistant to nucleases and proteases, and the hydrophobic nature of the molecule increases its ability to enter the organism through the hydrophobic cell wall. PNA-FISH can be used for rapid species identification of positive blood cultures and also is being applied to investigate the bacterial diversity in environmental samples. FISH method is used to label cells or chromosomes according to the sequences of nucleic acids contained within them. The process works by taking fluorescently labeled pieces of DNA or RNA called probes that are around 20 nucleotides in length. The probes are incubated in the presence of cells under appropriate conditions to permit specific hybridization of probe to target nucleic acid. Cell types that contain ribosomes with complementary RNA sequences become labeled by the binding of the fluorescent probe in situ. These labeled cells can then be visualized by flow cytometric or fluorescence microscopy [11,35].

Colony hybridization is another hybridization-based method using labeled polynucleotide probes complementary to a unique sequence of DNA of the suspected pathogen. The method is used in human medicine on pathogens isolated from clinical samples by culture. The resulting bacterial colonies are lysed, and the DNA is denatured and fixed onto an inert support, which is then subsequently hybridized with the DNA probe. The method allows specific pathogen DNA to be detected in mixed cultures of bacteria and there is no need to isolate the pathogen DNA before performing the assay. However, the method is time-consuming and the method can only be performed on samples where it has been possible to culture the microorganism [26]. Probes can be designed on different phylogenetic levels, specific for domain, phylum, family, genus, or species [32]. If hybridization is performed on actual tissue sections, cells, or isolated chromosomes in order to detect the site where the DNA or RNA is located, it is said to be done “in situ.” By contrast, “in vitro” hybridization takes place in a test tube or other apparatus and is used to determine sequence similarity of two nucleotide segments [14].

A very important factor that influences the sensitivity and specificity of probe hybridization is hybridization stringency. Stringency is increased by increasing temperature and formamide concentration or lowering salt concentration. Most commercial hybridization assays have been optimized; however, each laboratory may still need to work out its own hybridization/washing conditions [32].

3.2.5 Nanotechnology and nanosensors

The term nano, derived from the Greek word for *dwarf*, is usually combined with a noun to form words such as nanometer, nanorobot, and nanotechnology. Nanotechnology is broadly defined as systems or devices related to the features of nanometer scale (one billionth of a meter). In the last two decades, nanoscience and nanotechnology have seen a plethora of new developments in almost every field of science and technology, especially in biology and medicine. The small dimensions of this technology have led to the use of nanoarrays and nanochips as test platforms. The recent advancements in nanotechnology have led to the development of nanoparticle-based facile assays for specific detection of the bioanalytes of clinical interest [14]. Nanotechnology presents a great opportunity to develop fast, accurate, and cost-effective diagnostics for the detection of pathogenic infectious agents. The properties observed in nanomaterials are different from those observed in the bulk (micron-size) material due to their small size (1–100 nm) and large surface area, resulting in enhanced surface reactivity, quantum confinement effects, enhanced electrical conductivity, and enhanced magnetic properties, among others. Most importantly, modifications of the nanostructures' surface can alter dramatically some of their properties. Hence, a single binding event can be potentially recorded. Because of these phenomena, multiple nanostructures have been engineered to detect particular molecular targets in bidiagnostic applications, including pathogen detection [60]. The surface functionalization of nanomaterials by biomolecules has led to the development of new interdisciplinary research areas like biomedical nanotechnology, nanomedicine, diagnostic devices, theranostics, contrast

agents, nanobiosensors, and targeted drug delivery vehicles. Functionalities can be added to nanomaterials by interfacing them with biological molecules or structures [61].

One advantage of this technology is the potential to analyze a sample for an array of infectious agents on a single chip. Applications include the identification of specific strains or serotypes of disease agents or the differentiation of diseases caused by different viruses but with similar clinical signs [14]. Another facet of nanotechnology is the use of nanoparticles to label antibodies. The labeled antibodies can then be used in various assays to identify specific pathogens, molecules, or structures. Example of nanoparticle technology includes the use of gold nanoparticles (GNPs), nanobarcodes, quantum dots, and nanoparticle probes. GNPs with unique optical properties and high surface area are being extensively used for facile detection of bioanalytes of interest in the samples. The colloidal solution of GNPs exhibits intense red and blue/purple colors depending on the size, shape, and degree of aggregation of nanoparticles [14,61].

The need for rapid detection and characterization of pathogenic microorganisms has led to the evolution of the biosensor. Nanotechnology has tremendous potential to enhance the performance of biosensors. A biosensor consists of two components: a molecular recognition element and a transducer. The recognition element or bioreceptor is a molecule that recognizes and specifically interacts with a target analyte. The transducer converts the interaction into a measurable signal for quantification. In a biosensor, the bioreceptor is designed to interact with the specific analyte of interest to produce an effect measurable by the transducer. High selectivity for the analyte among a matrix of other chemical or biological components is a key requirement of the bioreceptor. While the type of biomolecule used can vary widely, biosensors can be classified according to common types of bioreceptor interactions involving: antibody/antigen, enzymes, nucleic acids/DNA, cellular structures/cells, or biomimetic materials [14]. The use of molecular self-assembly and GNPs plus EIS detection rendered a detection limit of 30 virus particles/mL for adenovirus 5 and 100 cells/mL for *Escherichia coli* 0157:H7 [62]. The GNP sensor surface could be self-assembled and regenerated on the electrode at least 30 times without losing analytical performance. The combination of nanotechnology and EIS is an attractive and powerful concept for future chemical and biological sensors research and integration into lab-on-a-chip devices for field deployable sensors [62].

Lateral-flow biosensors (LFBs) are diagnostic devices based on single use paper carrier material, where dry reagents are activated by applying any fluid sample. LFBs are important for diagnostic purposes since they are affordable, sensitive, specific, user-friendly, rapid, robust, and equipment-free. They are an ideal platform for single use at point of care, where a positive/negative signal is sufficient and results usually come within 20 min [63]. A few lateral-flow assays have been proposed for detection of PCR-amplified *Leishmania*-specific DNA, including the OligoC-Test PCR-oligochromatographic test. A GNP-based LFB was developed for *Leishmania* DNA detection. The biosensor allows visual detection of amplification products without special instrumentation. The detection limit was 100 fmol of target DNA, corresponding to 3 parasites/ μ L of sample. Results were visible within 20 min and amplification products were specifically detected on infected dogs. The assay is a step forward to the development of effective tools for human and veterinary medicine pathogens detection [63].

For detection of *Salmonella* in water samples, a heterostructured silicon/gold nanorod array is fabricated by the glancing angle deposition (or GLAD) thin film method and functionalized it with anti-*Salmonella* antibodies and organic dye molecules. Due to the high aspect ratio nature of the silicon nanorods, dye molecules attached to the silicon nanorods produce an enhanced fluorescence upon capture and detection of *Salmonella*. This novel nanobiosensor could have broad appeal to the food industry, food safety inspection agencies, government agencies overseeing food safety, and researchers focused on safety and biosecurity research. In addition to abovementioned nanobiosensors, quartz crystal microbalance (QCM)-based nanoimmunosensors can also be developed and modified with gold/silver nanoparticles for the detection of *Salmonella* using piezoelectric QCM [64]. Biosensors are frequently coupled to sophisticated instrumentation to produce highly specific analytical tools, most of which are still in use only for research and development due to the high cost of the instrumentation, the high cost of individual sample analysis, and the need for highly trained personnel to oversee the testing.

3.2.6 Omics techniques: proteomics in disease diagnosis

Omics techniques are becoming a reality that offers a vast array of benefits to the aquaculture industry. The use of high-throughput omics approaches is becoming a powerful multidisciplinary tool for life science research, including fish disease studies. Omics approaches to study DNA variations (genomics), gene expressions at mRNA level (transcriptomics), protein expression (proteomics), and metabolite concentration (metabolomics) are essential for investigating the interplay between fish immune systems and pathogen infections [1]. The emergent of these omics approaches widely used in fish disease studies facilitates understanding various fish disease mechanisms and discovers different biomarkers of both the disease virulence and the host defense mechanism. These high-throughput technologies combined with bioinformatics can generate large amounts of data to speed up the identification of potential biomarkers for various diagnostic and therapeutic

developments [1]. Genes, mRNAs, proteins, metabolites, and other molecules are types of potential molecular biomarkers that have been identified in numerous fish disease studies [65]. These biomarkers can outline and detect the differences between multiple factors that cause an organism to respond a certain way to an infection. Apart from that, biomarkers are used to detect the early stage of an infection and are used to interconnect the various mechanisms that relate to other factors with disease infection [66].

Proteomics techniques are one of those new tools and one of the most interesting approaches for health management, epidemiology, and fish disease research [67]. Proteomics can be an important complement to more classical approaches like pathogen identification, disease symptomatology, and histopathological analysis to achieve a good disease diagnosis in aquaculture. Proteomics refers to the methodology that addresses the study of the entire complement of proteins expressed in a specific state of an organism or a cell population. In proteomics, regardless the complexity of the analyzed protein mixtures that can range from hundreds, to several thousands of proteins, the major goal is the accurate identification of the highest number of proteins as possible in those mixtures [68,69]. Proteomics-based approaches can therefore offer unique insights into fish cellular regulation in response to pathogens and during disease progression, besides enabling fast and sensitive pathogen detection and identification [69].

In gel-based approaches, proteins are first separated by one (1-DE)- or two-dimensional gel electrophoresis (2-DE) and then identified by mass spectrometry, whereas in gel-free approaches (or MS-based) protein mixtures remain in solution prior to protein identification. In each case, protein samples may be digested to peptides by a sequence-specific enzyme, typically trypsin, in a so-called peptide-based “bottom-up” proteomics approach, to distinguish it from the analysis of entire proteins in “top-down” proteomics. Peptide samples can then be separated and analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), usually employing electrospray ionization as the method to convert the peptides to gas phase ions for MS analysis. Alternatively, peptide samples can be analyzed by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MALDI-TOF MS). This technology is increasingly used in diagnostic identification of microorganisms. MALDI-TOF MS generates a characteristic spectrum, called a peptide mass fingerprint, formed as a result of the presence of up to 2000 proteins found in a unique pattern in each organism. This approach identifies species in a microbial community and even differentiates strains based on molecular signatures (e.g., rRNA) [70]. The method of choice will always depend on the main research objective, costs, and expertise, with MALDI-TOF MS-based strategies being most suited for microbial identification and diagnosis, as rapid, sensitive, and economical in terms of both labor and costs. The diagnostic accuracy with MALDI-TOF MS against established methods of identifying microbes is high and well established [70]. On the other hand, LC-MS/MS is most suited for large-scale, systematic characterization of proteomes, e.g., involved in host–pathogen interactions, allowing multiplex sample analysis and quantitation [69].

Elucidation of the mechanisms by either genomic, proteomic, or metabolomics would benefit the idea of the development of a novel vaccine or immunostimulant method to boost fish immune response from bacterial invasions, which simultaneously reduce or solve the fish disease problems [1]. The transcriptomic approach has been extensively applied in many fish disease studies such as in grass carp (*Ctenopharyngodon idellus*), rainbow trout (*Oncorhynchus mykiss*), tilapia (*Oreochromis niloticus*), pacific cod (*Gadus microcephalus*), orange-spotted grouper (*Epinephelus coioides*), brown-marble grouper (*Epinephelus fuscoguttatus*), and others [65]. These studies have enabled the discovery of many immune-related genes and their involvement in immune-specific pathways that can eventually guide disease diagnosis and prevention [71]. Transcriptomics approach not only provides an insight into the response of the fish toward different types of infection at the transcriptome level but also helps in revealing the complexity of the host’s defense mechanism against infection by the identification of potential key resistance genes that is important to improve the immunity of fish for disease resistance [1,65].

Similar to the other omics, metabolomics has continued to grow rapidly and is considered a prominent tool that predicts and explains the complex phenotypes in a diverse biological system. It acts as a complementary platform and is often used in accordance with transcriptomics and proteomics since metabolomics is a downstream result of gene and protein expression. Metabolomics utilizes the high-throughput screening of various samples using instruments to assess any chemical risk [1]. The roles played by metabolites are again emphasized in a metabolomics analysis conducted by Ref. [72] to profile the metabolites in the kidneys of Atlantic salmon infected with *A. salmonicida*. It was found that the metabolites extracted from the infected samples have an altered profile compared to the control ones.

4. Preventive and control measures

Effective disease control is paramount within aquatic farming systems to stop the spread of infectious pathogens. Managing the fish disease is the most crucial task for the farmers to alleviate fish losses. This has proven to be a challenge as several pathogens are responsible for increasing the mortality of farmed fishes. Implementation of an effective health management program consisting of well-organized management and husbandry practices, efficient biosecurity and hygiene

measures, and improved resistance to disease through vaccination can all help to reduce and control disease at farm sites. However, disease monitoring in the form of surveillance and rapid diagnosis is also an important part of this process to allow appropriate action to be readily taken when pathogens are best detected and diseases are diagnosed, before they become a significant problem for the farmer. In addition to screening diseased animals, monitoring should include the screening of apparently healthy fish so that subclinical infections can be detected and used as an early warning signal of an imminent disease problem (OIE, World Organisation for Animal Health, 2008). The detection and characterization of pathogens in the environment, between harvesting and restocking, are also useful for reducing disease outbreaks [26]. The principles of optimal fish husbandry are: (i) proper stocking densities, (ii) proper feeding, and (iii) water quality management and hygiene. The most common error in fish husbandry is overstocking. This leads to problems such as fish to fish aggression, increased fish and feed wastes, and ease of disease spread [73]. Recent developments have seen many effective preventive methods to contain diseases that aim to enhance fish immunity. These include vaccination and natural products possessing immunostimulatory properties [1].

Biosecurity is an essential component of aquaculture operations given the serious risks of infectious disease transmission through the frequent movement of live fish and shellfish between countries, regions, and farms. Biosecurity is about protecting a farm, region, or country from the threats of infectious diseases, in particular exotic pathogens. Some practical examples are quarantining fish before inspection, removal of sick fish as soon as detected, frequent changes of water, and never using fish trash for feed. As part of sustainable and cost-effective disease surveillance, prevention and early response to disease outbreaks to support both export trade and border protection of imported aquatic animals and commodities, and a robust health testing, inspection and certification program is required [73].

5. Therapeutics in fish disease management

Although improvement of water quality, nutrition, and other general husbandry factors may be enough to improve the health of a population, chemotherapeutics are often required to ameliorate disease outbreaks [18]. The role of medications in the management of fish disease outbreaks is well recognized as an important aspect of aquaculture operations and animal welfare. While chemotherapy will perhaps remain one of the main strategies for controlling transmissible diseases in the foreseeable future, especially in finfish, there is increasing recognition of its limitations in terms of aquaculture situations, host species, and effectiveness against certain pathogen groups. Antibiotics are being a choice of therapeutics for even viral outbreaks with secondary bacterial infections. The different antibiotics and antimicrobials commonly used in aquaculture disease management are presented in Table 10.3.

In China, the farmers are aware of the problems associated with antibiotic use in aquaculture and they have been practicing water quality management using probiotics and disinfectants [74]. In some cases, rather than providing a solution, chemotherapy may complicate health management by triggering toxicity, resistance, residues, and occasionally, public health and environmental consequences [28]. Thus it is advisable to be taken under advisement of a veterinarian. A veterinarian is required to ensure that the medicines used present no risk to human health. Also, veterinarians can guide farmers in the use of other more appropriate drugs which when properly used have no effect on human health. In addition if the fish are to be sold for human consumption the matter of drug residues and using the correct withdrawal procedure has to be addressed. The prescribing veterinarian should make the farmer aware of the main principles of medication. These are: (i) correct selection of medicine, (ii) correct dosage calculations, (iii) correct method of application, (iv) test batch treatment, (v) observation and emergency measures, (vi) posttreatment management, and (vii) correct drug withdrawal time [73].

Food and Drug Administration (FDA's) Center for Veterinary Medicine has identified a number of "low regulatory priority aquaculture drugs" These compounds have undergone review by the FDA and have been determined to be new animal drugs of low regulatory priority [75]. European Union, USFDA, and Japan have notified zero-level drug residues of selected antibiotics, viz. chloramphenicol, furazolidone, nalidixic acid, neomycine, oxolinic acid (quinoline compound), oxytetracycline, tetracycline, and sulfamethoxazole/trimethoprim (sulfonamide) in the imported shrimp [76]. The details of drugs, chemicals, and antibiotics used in Indian aquaculture have been previously reviewed [77]. Similarly, antibiotics are a prevention method used to treat bacterial infections. Antibiotic use in aquaculture may have less than desirable impacts on the environment and human health. This would involve the development and transfer of resistance gene of aquatic bacteria to fish pathogens and other aquatic bacteria. Apart from that, the accumulation of residual antibiotics in aquaculture products and the possibilities in the ways it could affect microbial biodiversity are also a concern [1]. USFDA-approved drugs and chemicals permitted for use in aquaculture along with other drugs and chemicals being used in Indian aquaculture have been presented in Table 10.4.

TABLE 10.3 Use of different Antibiotic preparations in disease management in freshwater aquaculture in India.

Sl. No.	Name	Composition	Dose used by the farmers
1.	Vettrim; Basix	Trimethoprim IP—2% w/w Sulfamethoxazole—10% w/w	20–30 mg/kg body weight per day in divided doses in feed for 7–10 days
2.	Cephalex	Cephalexin oral	5–10 mg/kg fish feed for 5 days
3.	Cizole tab	Ciprofloxacin + tinidazole	30–35 mg/kg fish feed for 5 days
4.	Lixen powder	Cephalexin IP—7.5% w/w	5–10 mg/kg fish feed 3–5 g/kg prawn feed
5.	MeriQuin/Enrocin/Renoflox/Floxidin/Enrofloxacin/KCP-ENRO (vet)	Enrofloxacin—10% w/v, oral	For preventive purpose use 2.5–5.0 mg per kg body weight. 1 mL of solution in 2–4 L of water For curative purpose use 5–10 mg per kg of body weight. 1 mL of solution in 1–2 L of water
6.	Hydrodox	Doxycycline	5mg/kg fish feed for 5 days
7.	Oxymycin; Oxytetracycline	Oxytetracycline HCl IP—5.5% w/w	75–80 mg/kg of feed for 7–10 days
8.	Erythrovet	Each gram contains erythromycin thiocyanate Eq. to 100 mg of Erythromycin	20 mg/kg body weight for 3–5 days

TABLE 10.4 USFDA-approved aquaculture drugs [75].

	Drug	Commercial name	Indications	Approved species
1.	Chorionic gonadotropin	Chorulon	For improving spawning function in male and female brood finfish (21 CFR 522.1081)	Brood finfish
2.	Formalin	Formaldehyde solution	For the control of protozoa and monogenetic trematodes, and on the eggs of salmon, trout, and pike (esocids) for control of fungi of the family saprolegniaceae (21 CFR 529.1030)	Finfish Finfish eggs Penaeid shrimp-Salmon, trout, catfish, largemouth bass, and bluegill
3.	Florfenicol	Aquaflor type a medicated article	For the control of mortality due to enteric septicemia of catfish. The tolerance for florfenicol amine (the marker residue) in muscle (the target tissue) is 1 ppm (21 CFR 556.283)	Channel catfish Salmonids
4.	Tricaine methanesulfonate	Tricaine-SMS-222	It may not be used within 21 days of harvesting fish for food. The drug should be limited to hatchery or laboratory use (21 CFR 529.2503)	Ictaluridae (catfish), Salmonidae (salmon and trout), Esocidae (pike), and Percidae (perch)
5.	Oxytetracycline dihydrate	Terramycin 200	For feed use in salmonids, 21 days; catfish, 21 days; lobster, 30 days (21 CFR 558.450). Oxytetracycline tolerance in the flesh is 2.0 ppm (21 CFR 556.500)	Catfish, salmonids, lobster

TABLE 10.4 USFDA-approved aquaculture drugs [75].—cont'd

	Drug	Commercial name	Indications	Approved species
6.	Oxytetracycline hydrochloride	Oxymarine, Terramycin 343, Phennoxy 343, tetroxy aquatic	For feed use in salmonids, 21 days; catfish, 21 days; lobster, 30 days (21 CFR 558.450). Oxytetracycline tolerance in the flesh is 2.0 ppm (21 CFR 556.500)	Finfish fry and fingerlings
7.	Hydrogen peroxide			Fish eggsSalmonidsFreshwater-reared coolwater finfishChannel catfish
8.	Sulfamerazine	Sulfamerazine	It may not be used within 21 days of harvest (21 CFR 558.582). Note: this product is currently not marketed	Trout (rainbow, brook, brown)
9.	Sulfadimethoxine/orometrim	Romet-30	Withdrawal times are: salmonids, 42 days; catfish, 3 days (21 CFR 558.575)	Catfish salmonids (trout and salmon)
10.	Chloramine-T	HALAMID aqua		Freshwater-reared salmonids, wall-eye, freshwater-reared warmwater finfish

US FDA, 2022, Approved Aquaculture Drugs, U.S. Food and Drug Administration, updated 03/18/2022, <https://www.fda.gov/animal-veterinary/aquaculture/approved-aquaculture-drugs>.

6. Vaccines for fish diseases

Vaccination is an established, proven, and cost-effective method for controlling certain infectious diseases in cultured animals. Vaccinating fish constitutes only a part of the overall fish health management program, and suitable animal husbandry remains the capital attitude for progress and success in aquaculture. Vaccination is becoming an increasingly important part of aquaculture, since it is considered a cost-effective method of controlling different threatening diseases. The term vaccination strategy has been defined to include the decision as to which diseases to vaccinate against, as well as the vaccine type, vaccination method, the timing of vaccination, and the use of revaccination. The purpose of vaccination is to provide an individual with resistance to a disease without having to undergo a potentially risky infection. Vaccines work by inducing protective immune response which by virtue of the memory cells persists for a long period of time, though the precise longevity varies. Natural exposure acts as a booster to immunity produced by vaccination. In the absence of natural exposure, booster vaccination is necessary to maintain the level of immunity. Vaccines reduce the severity of disease, minimize the loss due to disease, enhance the production potential, reduce the need for antibiotic use, leave no residues in the product, and do not induce pathogen resistance [7]. Different attempts by researchers to develop effective vaccines against pathogenic bacteria have been carried out but good protection levels are not usually achieved. First-generation vaccines are vaccines that are composed of whole pathogens. Live vaccines or attenuated pathogens can stimulate both cellular and humoral immune responses and these two vaccines are mostly popular in application [1].

7. General principles of fish vaccination

Vaccines are preparations of antigens derived from pathogenic organisms, rendered nonpathogenic by various means, which will stimulate the immune system in such a way as to increase the resistance to disease from subsequent infection by a pathogen. An optimal vaccine must be able to induce innate mechanisms, a sufficient antibody response, induce T-cell response(s), and generate specific immune memory in the host fish species. The vaccines are recommended only for healthy fish. It is advisable not to vaccinate sick, diseased, or stressed fish. It is better that the fish be deprived of food prior to vaccination as they suffer less handling stress and respond better to anesthetics. The smaller the fish size and the higher the water temperature, the smaller is the fasting interval required for vaccination of the stock. The primary considerations for developing a vaccine included various factors like: (i) safety, (ii) cost-effectiveness, (iii) long-term protection, (iv) serotypic or genetic variation of the pathogen, (v) time/age when fish most susceptible to disease, (vi) species, (vii) route of administration, and (viii) method of vaccine preparation [78].

7.1 Types of vaccine

Modern vaccines can be classified as killed/inactivated whole-cell vaccine (adjuvanted or subunit), live-attenuated, DNA, synthetic peptide, recombinant vector, genetically modified, and subunit vaccines. Whole organism vaccines showed a better advantage than other types of vaccines. However, most of the vaccines do not completely prevent disease.

7.1.1 Inactivated or killed vaccines

The inactivated or killed vaccine could be formulated with whole inactivated cell or specific components (bacterins) fractioned from the pathogenic bacteria by physical, chemical, or biological processes. These are three types: (i) whole-cell killed vaccines, (ii) fractional inactivated vaccines, and (iii) multivalent or polyvalent vaccines. Whole-cell killed bacterial vaccines are prepared by killing the infectious agent and using it as an antigen to induce an immune response. These vaccines are produced by growing the pathogen bacterium in the appropriate media; these bacteria will be killed afterward with a chemical substance, generally formalin. For fish vaccination, the killed bacteria will be used as either the whole bacterial cell or a defined fraction of the inactivated cells. Inactivated pathogenic bacteria still preserve the original antigenic characteristics of the live bacteria since their basic shape and structure have not been altered [79]. Most of the applicability of bacterial vaccines in aquaculture have been recorded as usage with inactivated vaccines obtained from a broth culture of specific strains exposed to subsequent formalin inactivation. Different inactivating agents variably affect the efficacy of inactivated vaccine and duration of protective immunity produced post-vaccination [80].

Most of the commercial vaccines currently used in aquaculture are killed vaccine types. For viral pathogens, the development of killed vaccines has been too costly and experienced to be less than satisfactory [81]. The fractional inactivated vaccine could be a protein-based vaccine (toxoid or subunit) or polysaccharide-based vaccine (pure or conjugate). In fractional vaccines, the pathogenic bacteria are generally formalin killed and only antigenic components are extracted and used for vaccination [80]. There is an injectable formalin-inactivated vaccine available to control the red sea bream iridoviral disease, which is a significant cause of mortality among cultured marine fish. This vaccine is commercially available in Japan as “Formalin-inactivated Iridovirus streptococcosis-vibriosis combined vaccine” for use in marine fish [82]. Nevertheless, long-lasting protection by the inactivated vaccines can only be obtained when adjuvants are adjuncted to elevate the immunological strength and presentation of the vaccine formulation [83]. Oil-based adjuvants in salmonid vaccine formulation was introduced only early in 1990s, which resulted in significant reduction of outbreaks of infectious diseases. The high efficacy and long duration of protection that the water-in-oil emulsion adjuvants induce have been essential for the growth of salmonid aquaculture [80,84].

In Japan, the use of injection vaccines is now widespread among maricultured animals, proved effective against bacterial (e.g., *L. garvieae* infection of yellowtail) and viral infections (e.g., Iridoviral infection of red sea bream), and has changed the patterns of disease occurrences [8]. Some of the important bacterial diseases against fish vaccines that are commercially available include *A. salmonicida*, *Vibrio salmonicida*, *V. anguillarum*, *Yersinia ruckeri*, *Renibacterium salmoninarum*, *F. psychrophilum*, *Flavobacterium columnare*, *Piscirickettsia salmonis*, *L. garvieae*, *Streptococcus iniae*, *P. damsela* subsp. *piscicida*, and *E. ictaluri* [85].

Subunit vaccines represent a new generation of vaccines that utilize components of bacteria, parasites, or viruses to induce adaptive immunity against them. These components, often referred as antigens, are usually specific proteins or synthetic peptides [79]. These subunit vaccines take advantage of using only antigenic components for vaccination and there is no risk of pathogenicity to the host, as these do not multiply or replicate in the host cell or nontarget species [86]. But the disadvantage of using subunit vaccine is their ability to stimulate a potent immune response weaker than killed or live whole-cell preparations, due to the presence of limited number antigenic epitopes.

A polyvalent vaccine protects simultaneously against the majority of the diseases to which a particular fish species is susceptible; this type of vaccine must cover all the main serotypes of each pathogen existing in a particular geographical area. Polyvalent vaccines, for salmonids incorporating different *Vibrio* species and *A. salmonicida* as antigens, are also available [87]. A number of commercial *V. anguillarum* vaccines have been developed for use mainly by bath or injection; the majority of them includes in their formulations only O1, or mixture of serotypes O1 and O2a. Different polyvalent oil-adjuvanted vaccines, including different combinations of *V. anguillarum* with other pathogens, such as *V. ordalii*, *V. salmonicida*, *A. salmonicida*, *Moritella viscosa*, and IPNV, are also available on the market to be used for salmonids by the intraperitoneal route [88]. During the manufacturing of polyvalent vaccines, either bulk lots of monovalent antigens are produced and then combined as a step during the manufacture, or the antigens are produced, stored separately, and mixed before administration [79]. A polyvalent *Pasteurella/Vibriosis* vaccine called ICTHIOVAC VR/PD containing four antigens (*P. damsela* subsp. *piscicida*, *Listonella anguillarum* serotype O1, *L. anguillarum* serotype O2a, *L. anguillarum* serotype O2b) has been commercialized. Another fish vaccine is commercialized under the name AQUAVAC VIBRIO and

cover both of biotype I and biotype II strains of the bacteria *V. anguillarum*. These vaccines, delivered by oral or immersion routes, are shown to be very effective [79]. Polyvalent vaccines demonstrated significant efficiency over the monovalent ones; however, care must be taken in the formulation of polyvalent vaccines because the problem of antigen competition can occur, especially, when these vaccines are administered by injection [88].

Vaccine commercially available for enteric red mouth disease (*Yersiniosis*) is a formalin-inactivated whole-cell cultures of *Y. Ruckeri* serovar I, Biotype 1 (Hagerman strain), found to be very effective [88]. Again one aqueous live vaccine against BKD caused by *R. salmoninarum* developed by Novartis has been licensed under the name of “Renogen” for BKD prevention [88]. To control enteric septicemia of catfish, the first commercial bacterins for *E. ictaluri* were licensed to be used by immersion or oral routes, but killed vaccines have not been very successful [88]. Recently, an attenuated O-antigen-deficient *E. ictaluri* strain has been developed which was safe and provided high long-lasting acquired immunity following a single bath immersion in 9–14 days old channel catfish without booster vaccination [89]. This modified live *E. ictaluri* vaccine has been produced since 2000, by Intervet Inc., under the trade name AQUAVAC-ESCO, and constitutes the first licensed bacterial live vaccine in aquaculture formulated with an attenuated pathogenic strain [88].

Inactivated vaccines always require multiple doses. In general, the first dose does not produce protective immunity, but ‘primes’ the immune system. In this case the immune response is mainly humoral but the titer of antibody diminishes along time [90]. For fishes that should be cultured for a long time, a second booster dose must be injected after 3–4 weeks of first dose to develop a protective immune response. For better and long-term immunological response, adjuvants are usually mixed with inactivated vaccine, which will improve the presentation of the antigen to the fish immune cells and improve the duration of protection [91]. Although many furunculosis bacterins have been used in salmonids by injection, immersion, or the oral route, their efficacy has been questioned because of the lack of repetitive results and/or the short protection period [87]. The best results in terms of protection have been reported in salmonids with the mineral oil-adjuvanted vaccines but it has impacted weight gain. To avoid these problems, nonmineral oil-adjuvanted vaccines have been recently developed and marketed [88].

The advantages of these vaccines are: (i) they are easy to design, (ii) stable in storage, and (iii) less expensive and there are no virulence issues. Preparation strategy of these vaccines is that they most of the time target the outer surface of microorganisms or inner parts without avoiding the ability to replicate when administered to the host. IHNV, *A. salmonicida*, and *V. salmonicida* are some diseases that can be prevented by killed vaccines. These vaccines have major market in salmon, trout with expanding market in sea bass, sea bream, tilapia, turbot, halibut, yellowtail, cod, etc. [78]. Most vaccines commercialized are whole-cell killed vaccines. However, considering prevalent of various diseases in aquaculture and for ease of administration, no efforts are being made to develop multivalent inactivated vaccines for fish diseases.

7.1.2 Attenuated vaccines

Live-attenuated vaccine is made of live bacteria that have lost the virulence propriety after treatment and no longer have the properties that cause significant fish disease [79]. These are prepared by repeated laboratory passage and physical and chemical attenuation of the organisms. The attenuation of the pathogenic bacteria could be achieved by chemical treatment, molecular modification of the virulence gene like gene depletion or point mutations on more than one gene, as well as with repeated culturing by a serial passage using tissue culture media to lose their virulence without killing them [92]. In the vaccinated fish, live attenuated bacteria must replicate but they usually do not cause disease. The immune response to a live-attenuated vaccine is theoretically the same response produced by a natural infection. Vaccination with an attenuated vaccine is a simulation model of an infection, and if vaccinated fish could spread the vaccine strain, a distribution of the antigen in the population would happen over a prolonged period of time. These vaccines have the advantage that they stimulate the cellular immunity significantly. Also, they are capable of stimulating the humoral and mucosal immunity [79,80].

Swain et al. [93] developed two rough attenuated variants of *A. hydrophila* isolates by continuous passage over a period of 8 years in brain–heart infusion agar. They observed the immunogenic potential of these variants remained unaltered and used as vaccine candidates for fish immunization to control MAS disease. The vaccine is now being commercialized through Agrinnovate India Ltd., New Delhi. Live-attenuated vaccine against BKD, enteric septicemia of catfish disease, and columnaris disease have been licensed for marketing in the United States [94], and one viral vaccine for Koi herpesvirus for carp has been licensed in Israel [95]. Similarly, attenuated viral vaccine against spring viremia of carp virus is also used in China via immersion route [82]. Laboratory studies have shown the effectiveness of live vaccines in fish. They induce mucosal, cellular, and humoral immunity. Attenuated organism replicates in the target host without any clinical signs. Safety is the major concern of using modified live vaccines. Several research groups have suggested that

practical administration of attenuated or avirulent forms of the virus could be undesirable because the residual virulence in targeted species could spread the virulence in nontarget species [80]. Regarding the management eases, live-attenuated vaccines need to be stored at low temperatures to remain viable. Thus, adequate storage should be strongly considered in case of transport and supply of vaccines across long distances [79].

8. Recent advances in vaccine developments

Over the last few years, gene sequences of bacterial, viral, and metazoan genomes, combined with the knowledge on gene functions and derived proteins, have evolved novel methods for fish vaccination. Advent of biotechnology has led to the development of recombinant vaccines where only the immunogenic regions of a pathogen are expressed in heterologous host and are used as vaccine [95]. Recombinant proteins as vaccine antigens have been demonstrated to give useful protection against various kinds of human and animal pathogens [80]. The recent developments in vaccine technology are:

1. DNA vaccines
2. Recombinant vaccines
3. Vector technology
4. Genetically attenuated pathogens
5. Synthetic peptide vaccine
6. Reverse vaccinology

8.1 Deoxyribonucleic acid vaccines

DNA vaccines are recent types of vaccines which are the result of the advancement of molecular biology. As traditional oil-based vaccines show similar efficacies as the bacterial DNA vaccines, and no suitable oil-based vaccines are available against viral diseases, efforts are now on to develop DNA vaccines against fish viral pathogens. Despite an enormous amount of effort invested in the development of DNA vaccines to protect veterinary animal species and humans against viruses, only a few have reached the market. To develop a DNA vaccine, there is no need to use the antigen as a vaccine, rather the gene that code for the antigen is taken by molecular techniques and can be given as a vaccine. DNA vaccines are composed of a particular portion of genetic material that can, after being incorporated into the animal, produce a particular immune-stimulating portion of a pathogen (i.e., antigen) continuously, thus providing an “internal” source of vaccine material [87]. Technically, the principal of DNA vaccine is to deliver to the host fish a DNA or RNA sequence coding for an antigen. The DNA is taken up by host cells and transcribed to mRNA, from which the vaccine proteins are then translated. These proteins will be recognized by the fish immune system as a foreigner or antigen and will induce an immune response. Once induced, the fish immune system response involves both cell-mediated and humoral components [96].

Similarly, more than 20 different virus DNA vaccines have been developed experimentally for prophylactic use in fish targeting viruses such as rhabdoviridae, orthomyxoviridae, togaviridae, and nodaviridae. The rhabdoviridae DNA vaccines (e.g., VHSV and IHNV) have shown high levels of efficacies, whereas others have in most instances possessed moderate to low efficacies [97]. Apex-IHN (Aqua Health Ltd., an affiliate of Novartis Animal Health Inc.) is the recombinant vaccine recently developed and commercialized for the protection of salmonids against IHNV [98]. Apex-IHN DNA vaccine has proved to be very successful, while other DNA vaccines against other piscine viruses are in the advanced pipeline. In 2016, the European Medicine Agency (EMA) recommended granting a marketing authorization in the EU for “Clynav,” a DNA vaccine against salmon pancreas disease (salmonid alphavirus-3) [97]. Advances in genome sequencing of pathogens can accelerate the opening of opportunities to investigate new-generation vaccines. To meet the challenge to develop efficacious vaccines, systems vaccinology approach using both transcriptomics, epigenetic, proteomics, and metabolomics platforms together with bioinformatics may be necessary [97]. Some of the vaccines commercially available against fish bacterial and viral diseases have been presented in [Table 10.5](#).

An optimal vaccine must be able to induce innate mechanisms, a sufficient antibody response, induce T-cell response(s), and generate specific immune memory in the host fish species. In this respect, Apex-IHN DNA vaccine has proved to be very successful, while other DNA vaccines against other piscine viruses are in the advanced pipeline [97]. Intramuscular (IM) injection of these vaccines confers immediate and a durable protection from diseases in farmed salmonids against economically important diseases such as IHNV and VHSV which were controlled by DNA vaccines. It has been demonstrated that DNA vaccination induces a strong and protective immunity to some viral infections in fish, particularly the Rhabdoviruses infecting rainbow trout and Atlantic salmon, and also for channel catfish herpesvirus infection [102].

TABLE 10.5 Fish vaccines available or field trialled against major infectious bacterial and viral diseases of fish.

Name of vaccine	Used in fish species	Against diseases	Mode of application	References
<i>Aeromonas</i> spp. (alphaject Panga2)	Striped catfish	Motile <i>Aeromonas</i> septicemia (MAS)	Ip injection	[64]
Polyvalent vaccines (different <i>vibrio</i> species and <i>Aeromonas salmonicida</i> as antigens)	Salmonids	Vibriosis and furunculosis	Ip injection, immersion	[87]
<i>Edwardsiella ictaluri</i> (AquaVac-ESC)	Catfish	Enteric septicemia of catfish	Ip injection, immersion, oral	[99]
AQUAVAC-ESCO: a modified live <i>E. ictaluri</i> vaccine (intervet inc.)	Catfish	Enteric septicemia of catfish	Ip injection, immersion	[88,89]
<i>Flavobacterium columnare</i>	Channel catfishSalmonid	Columnaris disease	Immersion	[99]
<i>Yersinia ruckeri</i> (Ermogen; AquaVac, ERM; AquaVac ERM oral)	Salmonids/Rainbow Trout, eel, tilapia	Yersiniosis/enteric red mouth	Immersion, oral	[99]
Polyvalent vaccines of <i>Vibrio anguillarum</i> , <i>V. ordalii</i> , <i>Vibrio salmonicida</i> , <i>A. salmonicida</i> , <i>Moritella viscosa</i> , and infectious pancreatic necrosis virus	Salmonids	Bacterial diseases and IPNV	Ip injection	[88]
Formalin-inactivated whole-cell cultures of <i>Y. Ruckeri</i> serovar I, biotype I (hagerman strain)	Salmonids/Rainbow Trout, eel, tilapia	Enteric red mouth disease (<i>Yersiniosis</i>)	Ip injection, immersion	[88]
Renogen: An aqueous live vaccine against bacterial kidney disease (BKD) (novartis)	Salmonids/Rainbow Trout, eel	BKD caused by <i>Renibacterium salmoninarum</i>	Ip injection, immersion	[88]
Polyvalent <i>Pasteurella/Vibriosis</i> vaccine (ICTHIOVAC VR/PD) containing four antigens (<i>Photobacterium damsela</i> subsp. <i>piscicida</i> , <i>Listonella anguillarum</i> serotype O1, <i>L. anguillarum</i>)	Salmonids/Rainbow Trout, eel, tilapia	Vibriosis	Oral or immersion	[79]
AQUAVAC VIBRIO (<i>V. anguillarum</i> biotype I and biotype II strains)	Salmonids/Rainbow Trout, eel, tilapia	Vibriosis	Oral or immersion	[79]
MULTIVAC: Polyvalent <i>A. salmonicida</i> and <i>V. anguillarum</i>	Salmon	Furunculosis and vibriosis	Ip injection	[80]
Renogen: Live-attenuated <i>V. salmonicida</i>	Salmon	Vibriosis	Immersion	[94]
AQUAVAC-ESC: Live-attenuated <i>R. salmoninarum</i>	Catfish	Enteric septicemia of catfish	Immersion	[94]
Brivax II: A genetically attenuated <i>A. salmonicida</i> vaccine	Rainbow trout	Furunculosis	Ip injection	[100]
Viral vaccines				
Inactivated IPNV (alpha Ject 1000)	Salmon	IPNV	Ip injection	[19][80]
Formalin-inactivated iridoviral vaccine	Red sea bream	Red sea bream iridoviral disease	Ip injection, immersion	[82]
Inactivated SVCV (bioveta)	Carp	SVC disease	Ip injection	[19][80]
IHHNV DNA vaccine	Salmon	IHHNV	IM injection	[95]
IPNV DNA vaccine	Rainbow trout	IPNV	IM injection	[95]
Apex-IHN DNA vaccine (novartis Animal Health inc.)	Salmonids	Infectious hematopoietic necrosis virus (IHN)	Ip injection, immersion	[98]

Continued

TABLE 10.5 Fish vaccines available or field trialled against major infectious bacterial and viral diseases of fish.—cont'd

Name of vaccine	Used in fish species	Against diseases	Mode of application	References
IPNV VP2—based recombinant vaccine (Microtek International Canada)	Rainbow trout, salmon	Infectious pancreatic necrosis virus disease	Ip injection	[101]
ISAV hemagglutinin-esterase gene-based recombinant vaccine (Centrovet, Chile)	Rainbow trout, salmon	Infectious salmon anemia viral disease	Ip injection	[101]
IPNV recombinant subunit vaccines utilizing VP2 and VP3 capsid proteins (AquaVac)	Rainbow trout, salmon	IPNV	Ip injection	[80]
IPNV oral recombinant subunit vaccines by Merck Animal Health, New Jersey, USA	Rainbow trout, salmon	IPNV	Oral	[80]
IPNV recombinant subunit vaccines with VP2 capsid protein (norvax and Minova-6 from Intervet International BV, Netherlands)	Rainbow trout, salmon	IPNV	Ip injection	[82]
Synthetic peptide vaccine against IHNV, nodavirus, VHS	Rainbow trout	IHNV disease, nodavirus disease, VHS disease	Ip injection	[80]

DNA vaccine is designed first by identifying and cloning a protective antigen from the pathogen. For example, for some pathogenic viruses of fish like VHSV and IHNV, protective antibodies are known to be against surface glycoprotein of the viruses. Therefore, the glycoprotein gene and the regulatory sequences that allow expression in eukaryotic cells were an option for the development of DNA vaccines. To administer as a vaccine the plasmid is produced in bacterial culture, purified, and quality-assured. After this process, a DNA vaccine will be administered and taken by cells of the host to produce the glycoprotein. This situation leads to detection of the antigen by the immune system of the fish. DNA vaccines have theoretical advantages over conventional vaccines. The advantage of a DNA vaccine is that it is based on purified plasmid DNA carrying only a single gene from the pathogen, which makes it noninfectious. It is unable to replicate within the host, there is no risk of transferring the actual disease with the vaccine. This vaccine type in fish or other animals is essentially the long-term persistence of immunogenicity, the safety, and the stability of the vaccine for storage and shipping [79]. That is why DNA vaccines are considered safer than conventional vaccines. These vaccines do not use adjuvants to administer like that of conventional vaccines which make them free of post-vaccination side effects. The main disadvantages of DNA vaccines are the possibility to induce antibodies against DNA and immunologic tolerance by antigens expressed inside the host body. As the DNA sequence encodes only a single microbial gene, there should be no possibility of reversion to virulence, which is a critical factor in relation to environmental safety in aquaculture [88].

8.2 Recombinant vector vaccines

Recombinant vector vaccines are the result of biotechnological advancement prepared by taking only the immunogenic regions of a pathogen and expressing it in a heterologous host. When culturing of the organism is difficult, these vaccines become useful by taking immunogenic part and using it as a vaccine. The immunogenic part of the organism is taken and expressed to carriers. The proteins are then produced in large quantities in vitro and then purified for use as a vaccine. Recognition of the gene sequence of pathogen's protective antigen is important for designing a recombinant protein. This antigen can be inserted into a production host and can be cultured on a large scale, from which the protective antigen is purified and used in vaccine formulation [80,95]. Various kinds of expression systems such as bacteria, cell culture, yeast, insect cells, microalgae, and transgenic plants are being employed for vaccine production [80,87]. Being easy to produce in large quantities of proteins and effectively expressing the antigenic protein are the main factors considered during vector selection. These vaccines have vital and potent effects in the industry of aquaculture since they yield an alternative pathway to conventional formalin-killed vaccines. These are also safe and economical. The first subunit vaccine for rainbow trout (*O. mykiss*) was developed by including IHNV glycoprotein (G) expressed in *E. coli* [103]. Two subunit vaccines have been developed against IPNV: one utilizing VP2 and VP3 capsid proteins, marketed as AquaVac and IPN Oral manufactured from Merck Animal Health, New Jersey, USA, which can be used in Canada by oral route administration, while the second type of subunit vaccine which is readily injectable via intraperitoneal (IP) route uses VP2 capsid protein and is

commercially available as Norvax and Minova-6 from Intervet International BV, Netherlands [80,82]. In infectious salmon anemia and infectious hematopoietic necrosis disease viruses have been expressed in vectors as a vaccine to protect salmon. Recombinant vaccine comprising of IPNV VP2—based vaccine from Microtek International (Canada) and ISAV hemagglutinin-esterase gene—based vaccine from Centrovet (Chile) have been successfully developed and used in the market [101]. Subunit vaccines are safe for use but their immunogenic nature is very poor compared to inactivated, whole organism vaccines. Adjuvants are therefore needed to improve immunogenicity. The vector technology vaccine method is very similar to recombinant technology but utilizes mainly viral production hosts which express the protein of another pathogen as a vaccine antigen [95].

8.3 Genetically modified vaccines

Production of live-attenuated vaccine by utilizing genetic engineering is typically done by deletion, disruption, or insertion of metabolic pathway or virulence gene that causes an attenuation in pathogen [80]. In vitro passaging of organisms results in a build-up of genome mutations that make the organism weaken. Genetically, microorganisms may be attenuated by molecular approaches that involve removal of genes responsible for its pathogenicity. The resulting mutant pathogen then serves as an avirulent pathogen inducing a protective immune response, yet it does not cause disease [95]. Live-attenuated vaccine will replicate to a lower titer and can stimulate humoral and cellular immunity. *A. salmonicida* in salmon can be prevented by these preparations. Related safety concerns to the vaccinated animals as well as for the environmental aspects are likely the major reason for these vaccines not being accepted and/or gaining any more attention in aquaculture [80].

8.4 Synthetic peptide vaccines

These types of vaccines are produced from short sequences of amino acids prepared synthetically to act as antigens. These can be used as a suitable antigenic site to serve as a subunit vaccine. Studies showed that vaccinating fish with peptides is less practicable because of lack of understanding the fish immune response to different antigens and being not potent enough and thus requires a carrier molecule. These vaccines have been in use as prevention of infectious disease like nodavirus, viral hemorrhagic septicemia, rhabdovirus, and birnavirus [104]. The results of these studies showed that although vaccinating fish with peptides is possible, this approach is limited because of the need for greater fundamental knowledge about fish immune mechanisms to different antigens [80].

9. Methods of vaccine delivery

Vaccine can be administered to fish through three different methods, viz.: 1. injection method, which is the most effective method but need to anesthetize and handle the fish, 2. immersion method is practical for mass vaccination of small fish only, does not work for all vaccines, and 3. oral method, which is most suitable for mass vaccination but dosage uncertain and sometimes, poor potency. The route of vaccine administration may determine the outcome of induced immunological responses and level of protection against pathogens of interest [95]. The most effective vaccine delivery method will depend upon the pathogen, route of infection, vaccine production techniques, status of immunological memory, life stage of host/fish, water temperature during the immunization process, workers' understanding of vaccination principles, and labor costs [80].

9.1 Injection vaccination

The vaccination by injection is the most common method of vaccine delivery in fish with high efficiency for the stimulation of humoral and cellular response. Two methods are used: (i) IP is injection of vaccine in the abdominal cavity of fish, or (ii) intramuscular. IP is the most common method used for most vaccines. IP method is the most efficient injectable method of fish immunization, and hence many of the current vaccines are predominantly delivered by this route. On the other hand, IM route is preferred for DNA vaccination into fish [80]. Vaccine (usually 0.1–0.2 mL) is injected in the abdominal area of each anesthetized fish (>50g). Now also microvaccines (0.05 mL) can be applied using automatic injection guns. Fish held ventral side up and the head away from the operator's body. The needle is inserted into the peritoneal cavity at a 45 degrees angle to a depth of approximately 0.5 cm. Advantages of injection vaccination is that there is long duration of protection, i.e., for over a year. Again, multiple antigens can be combined in a single vaccine and, therefore, in a single administration. In this method every fish in the population get the vaccine and at the correct dose. Injection method is in general superior to any other vaccine application method; however, from a practical point of view, they can only be applied to fish of 10g or

more (usually larger). Bacterin is generally administered via the intraperitoneal route while the relatively recently developed DNA vaccines against IHN and VHS viruses are most effective when administered by IM injection [105]. Sometimes adjuvants can be used in the vaccine for better immunity. Adjuvants stimulate nonspecific defense mechanisms: Short-term protection may be due to nonspecific defense factors, enhance specific immune response, and result in release of interleukins which in turn result in nonspecific activation of macrophages [78].

Advantage of injection vaccination method is that multiple antigens from different pathogens can be delivered simultaneously in the form of a multivalent vaccine. However, injection method of vaccination can cause stress to fish, resulting from the handling and injection of the fish. The major problem with injected vaccines is that they cannot be economically administered multiple times in the fish production cycle, and these vaccines cannot be administered in early life stages due to underdeveloped immune system [106]. If taken due care, no mortality is associated with this vaccination process per se, although some weak fish may die due to the handling process.

9.2 Immersion vaccination

Immersion vaccines consist of suspension of live-attenuated bacteria or live bacterial or vector vaccines. Vaccination by immersion of fish in a vaccine solution is done for seconds to minutes. Generally, two application methods are used: (i) dip and (ii) bath vaccination. The dip vaccination method is widely used. In this case the small-sized fish (fish fingerlings 1–15g size) are immersed for very short duration (30 s) in a highly concentrated vaccine solution (1 part vaccine to 9 parts water). Dip vaccination method is rapid and can vaccinate large numbers of fish (up to 100 kg of fish per liter of vaccine). In bath vaccination, usually larger fish are exposed for a longer period, usually 1 to several hours, in a lower concentration of vaccine [78]. In bath vaccination large groups of fish cut off from the rest in a cage are used for vaccination. A low dose of diluted anesthetic is added to reduce stress to animals. Air or oxygen is continuously pumped in to avoid anoxia during the vaccination process.

Suspension of formalin-inactivated bacterial as well as live bacterial vaccines primarily comprises the commercial immersion vaccines [80]. By immersion method, fish get in contact directly with the antigen through the skin, the gill, and/or ingestion. Immersion vaccination is particularly recommended for smaller fishes weighing between 1 and 4 g. Immersion is suitable for mass vaccination of all fish sizes, less stressful, with low labor costs and less risk for the vaccination person. Immersion method imparts relatively good protection. However, this method has some limitations like the duration of immunity is not very long and a booster vaccination is required when the disease prevails over longer periods. This method is impractical for larger fish due to cost-effectiveness and the stress that could be induced by vaccination [80]. In smaller fish, usually, in fish smaller than 1 g, the immune system might still be immature, and therefore, the vaccine efficacy may be reduced [78]. Immersion delivery method does not induce as strong humoral immunity as injection vaccination [106].

To increase immersion vaccine efficacy, some researchers have tried hyperosmotic environment, coating of positively charged chitosan to enhance adhesion and uptake of antigens by and use of nanoliposomes for recombinant live viruses expressing protective antigens [79]. Application of ultrasound for delivery of vaccine is a newer concept in aquaculture industry. Sound intensity of 20 kHz can improve the cellular permeability of vaccine. *V. alginolyticus* bacterin delivered by ultrasound method to *Epinephelus awoara* showed that this method produced equal immunity similar to IP injection [80].

9.3 Oral vaccination

This type of vaccination is most suitable for mass vaccination but dosage uncertain and sometimes poor potency. In this method, the vaccine is either mixed with the feed, coated on top of the feed (top dressed), or bioencapsulated [78]. In oral administration method, the vaccine powder could be spread on the fish feed using an adhesive agent like edible oils or gelatine, or else the vaccine could be incorporated into the feed during the feed preparation process [79]. When antigens are to be incorporated in feed, the heat sensitivity of the antigen has to be considered. When vaccines are used as top dressing in feed, a coating agent is usually applied, either to prevent leaching of the antigen from the pellets or to prevent breakdown of the antigen in the acidic environment of the stomach. Hence, the stability of this type of vaccine is a big issue. Oral administration of antigens does elicit local immunity but has been reported to vary in the efficacy of protection. Primary vaccination when administered orally does not induce a robust immune response, rather booster oral dose does induce a robust secondary immune response [80].

The oral route presents the ease pathway of administration of antigens; it is less stressful and safe labor than other routes; it applies to small- and large-sized fish; it also provides a procedure for oral boosting during grow-out periods in cages or ponds. Several factors like nature of the antigen, dosage regimen, and formulation of the vaccine influence the

efficacy of oral vaccines [107]. Hence the vaccine need to be heat and acid resistant and protects the antigenic epitopes when administered orally. One commercialized AquaVac Oral Vaccine is incorporated feed pellets. In the acid environment of the fish stomach, the feed pellets are digested. The antigens themselves are protected from acid action and pass through foregut intact. Antigens are delivered to the area of the hind gut where they are absorbed and activate an effective immune response in the fish. Antigens reach intraepithelial macrophages that show antigen-presenting function. GALT contains lymphoid cells such as macrophages, granulocytes, and plasma cells [78]. In some instances, recombinant subunit and attenuated viruses are delivered orally. To deliver recombinant subunit vaccine, fishes were fed *Artemia* nauplii encapsulated with recombinant bacteria containing the antigen of interest [108]. Fishes consumed *Artemia*, thereby encapsulated vaccine agent is released inside the body. *Artemia* sp. containing particular bacteria are usually fed to juvenile fish immersed in water and when these *Artemia* are fed by fish these antigens can elicit immunity [80].

10. Nanovaccines—nanoparticle-based vaccine delivery

Nanoparticles are gaining attention of researchers in the field of developing prophylactics and for aquaculture vaccines. Nanovaccines constitute an innovative approach in vaccinology, enabled by the nanotechnology advances on the synthesis, functionalization, and application of nanoparticles for immunization. Currently, several organic materials such as chitosan, poly(lactic-co-glycolic acid) (PLGA), nanopolyplexes, and viruslike particles (VLPs) have been used for nanovaccine development to fight against fish diseases [109]. These comprise of dispersion of nanosized materials with specific/defined physical characteristics in which immunostimulants and antigens are incorporated to improve the vaccine delivery in a controlled and targeted manner and increase the intensity of desired immune responses [80,110]. Angulo et al. [109] reported that the oral route is the most practical and economical for fish vaccination; thus, several oral vaccine candidates have been developed by using NPs in which the antigen is encapsulated, surface exposed, or recombinantly ensembled (VLPs), leading to promising results in terms of protection capacity against infectious diseases.

Three different compounds have been mainly used to develop oral nanovaccines against infectious diseases that affect fish: chitosan, PLGA, and nanopolyplexes. Additionally, recent works using VLPs as carriers for oral nanovaccines have shown promising results [109]. Chitosan consists of a linear chain of polysaccharides, obtained by partial deacetylation of chitin, a component of the crustacean exoskeleton. Chitosan is nontoxic, biocompatible, and biodegradable. The biodegradability consists in the transformation of chitosan into small polysaccharides that are excreted or assimilated by the organism. Currently, chitosan has many applications including its use in delivery vehicles of pharmaceuticals, such as antimicrobial agents and vaccines against infectious diseases affecting animals and humans [111].

PLGA is one of the most promising biodegradable polymers for nanovaccine development, and it is approved by the FDA and European Medicine Agency (EMA) for use in drug delivery systems for humans [112]. Several oral nanovaccine prototypes using chitosan NPs have been developed in the fight against a variety of pathogens with very promising results [113]. The use of chitosan has gained interest as the delivery vehicle of oral nanovaccines for aquaculture industry, and several efforts have been made in this field to generate alternatives to prevent and control bacterial and viral diseases [109].

In their study on oral DNA vaccine in Asian sea bass (*Lates calcarifer*) using chitosan and chitosan/tripolyphosphate nanoparticles, Vimal et al. [114] noted good level of induced immunity against *V. anguillarum*. However, further studies are required to utilize the tremendous advances being carried out in the field of nanotechnology regarding various applications of nanoparticles to design newer vaccines and improve vaccine delivery systems [80].

Nanopolyplexes are nanoparticles, typically ~100 nm in diameter, formed by electrostatic complexation of cationic polymers (or polypeptides) and DNA. Cationic polymers include natural DNA-binding proteins (e.g., histones), synthetic polypeptides, poly(ethylenimine) (PEI), cationic dendrimers, among others, or carbohydrate-based polymers, such as chitosan [100]. The properties of these complexes have gained interest in the nanotechnology field, including their use for nanovaccine design [115]. Nanopolyplexes have been applied in the development of an oral nanovaccine against *Aeromonas* disease [109].

11. Vaccination strategies

Important considerations for fish vaccination include: (i) fish species, (ii) status of the immune system, (iii) production cycle and life history, (iv) type of diseases needed to control, (v) time or season of diseases occurrence, (vi) farming technology (handling, mechanization), (vii) environment (temperature, salinity), and (viii) stress factors, nutrition, and cost benefit [78]. The effect of temperature can be put to good use during vaccination, e.g., by increasing water temperature an effective antibody response with long-term memory can be achieved which would not occur at low temperatures for salmonids. Fish can be successfully vaccinated in the autumn or onset of winter with the acquired immunity surviving the drop in temperature

over the winter and it is then effective the following spring when water temperatures increase as do the number of disease outbreaks. While vaccination is the most powerful method against disease outbreaks, external factors such as high production costs and antigenic heterogeneity of microbial strains hinder the development of effective vaccine formulations [99][4,5].

12. Conclusion

Disease is inevitable in living organisms belonging to greater complexity of organ systems. Disease outbreaks in aquaculture are the result of a complex network of interactions on aquatic systems between the produced organism, several environmental and zootechnical aspects, and possible pathogenic agents, that present a series of unique challenges in aquatic organism's health. The fish diseases infection caused by bacteria, viruses, and parasites could lead to high fish mortality that affected the aquaculture industry. Improved rapid diagnostics is crucial for the sustainable future for aquaculture. Thus rapid and reliable diagnosis of disease and pathogens constitutes an important aspect of disease management program, so that suitable preventive and control measures could be undertaken to control the mortality and minimize the loss. Traditional diagnostic methods tend to be costly, labor intensive, and slow and might not lead to a definitive diagnosis of disease, and too much time is wasted in arriving conclusion so that decisive action on management could be taken. Rapid diagnosis and prompt removal of infected fish are needed to implement effective control strategies during disease outbreaks. Molecular tools are increasingly relevant to fish diseases. The sequencing of the complete genomes of pathogens is allowing great advances in studying the biology and improving diagnosis and control of pathogens. Using nucleic acid as targets, and new methods of analyzing polymorphism in this nucleic acid, can improve specificity, sensitivity, and speed of diagnosis and offer means of examining the relationships between genotype and phenotype of various pathogens. Systematic strategy using multi-omics platforms has been useful in better understanding of resistance pattern in fish to pathogen infection. These are crucial to disease prevalence monitorization, early detection of emerging exotic and new diseases, and quality management improvement of aquaculture operations. Progress in techniques aids epidemiological studies as well as identifying causes of disease outbreaks or the presence of pathogens. Therefore, molecular biology can be a routine tool in the search for improved methods of diagnosis and control of fish pathogens and the epidemiology of infectious fish diseases.

For environment-friendly aquaculture and human health concerns owing to the rise in incidences of antimicrobial-resistant microbes and food safety hazards, the immunoprophylaxis or vaccination strategies are highly effective and economical in protecting the health of fish and aquaculture animals from various infectious agents. Various vaccines are now commercially available and could be used for enhancing protection in fish against a variety of diseases. This would be of immense use in controlling disease occurrence in aquaculture. The advantages of vaccine formulations based in nanomaterials comprise antigen stability enhancement, possibility to achieve targeted delivery, and finally enhancement of immunogenicity and immunoprotective capacity. Major limitations in fish vaccine developments are less understanding of fish immunology, not cost-effective, and less stressful on administration. It is hoped that next-generation vaccines relied on multiple killed antigens delivered with an adjuvant to enhance vaccine effectiveness.

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Principles of genome editing and its applications in fisheries

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1. Introduction

Aquaculture is one of the fastest-growing food-production sectors and is speedily establishing itself as the chief source of seafood for human and animal nutrition. Selective breeding techniques allow for genetic improvement and production attributes like resistance to disease, but improvement is limited due to the trait heritability of genes and the species' generation gap. Clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9)—mediated genome editing (GE) technology offers one of the most effective ways to speed up genetic gain in aquaculture. GE can make advantageous alterations to the genome at a quicker rate, producing new alleles, integrating alleles from diverse species or strains, or correcting alleles at trait governing loci. Most aquaculture species have great fertility, and external fertilization can facilitate GE for study and application conceivable at a scale not feasible in cultured land mammals.

Food security, along with an increasing need for high-quality animal protein, is a major and growing concern. Crop and animal production will be severely limited due to competition for land utilization [1]. As a result, aquaculture is projected to play a major part in satisfying this expanding nutrition and food requirement. Upscaling and increasing production dependability will necessitate disruptive technologies in engineering, health, nutrition, and genetic enhancement.

Better use of existing genetic resources is required for the development of more effective aquaculture. This additionally includes substantial genetic information in selective breeding methods like marker-assisted breeding. Genome modification like insertion, deletion, or single base alteration is all possible with GE tools [2]. GE technologies opened up several potential possibilities for improving aquaculture stocks, with implications for long-term sustainability and efficiency. Zinc finger nucleases (ZFNs) and transcription activator—like endonucleases (TALENs) were among the earliest GE technologies used. CRISPR-Cas technology, the most recent GE technique, now reigns supreme. This option enables modifications to be made by engineering alleles and trait loci. The CRISPR technology is now recognized as a powerful, most precise, easy-to-use, and cost-effective GE technology [3]. In this chapter, we briefly discuss the CRISPR-Cas—mediated technologies available for performing diverse kinds of GE, review their current status of applications in aquaculture, and highlight the challenges to use those technologies.

2. Genome editing technologies

Researchers are tirelessly working toward gaining the capability to change genomic information since the discovery of DNA structure and function. Restriction endonucleases, ligases, and polymerases are enzymes that allow breaking, ligating, and synthesizing DNA, respectively. Endogenous mechanisms in cells are available to fix DNA strand breaks. The core for GE is the ability to generate breaks at a preferred sequence [4]. Several approaches for changing DNA have evolved in the last 20 years, including nuclease-mediated site-specific mutagenesis and oligonucleotide-directed mutagenesis procedures. The four types of site-directed nucleases are meganucleases, TALEN, ZFN, and CRISPR-Cas [5] (Fig. 11.1). ZFN is constituted of modular DNA recognition and binding proteins combined with FokI nuclease. The complex can be designed to detect certain genomic regions of 9–18 nucleotides, and the FokI enzyme can generate double-strand breaks (DSBs) during dimerization [6]. The use of ZFN was impeded by difficulties in designing,

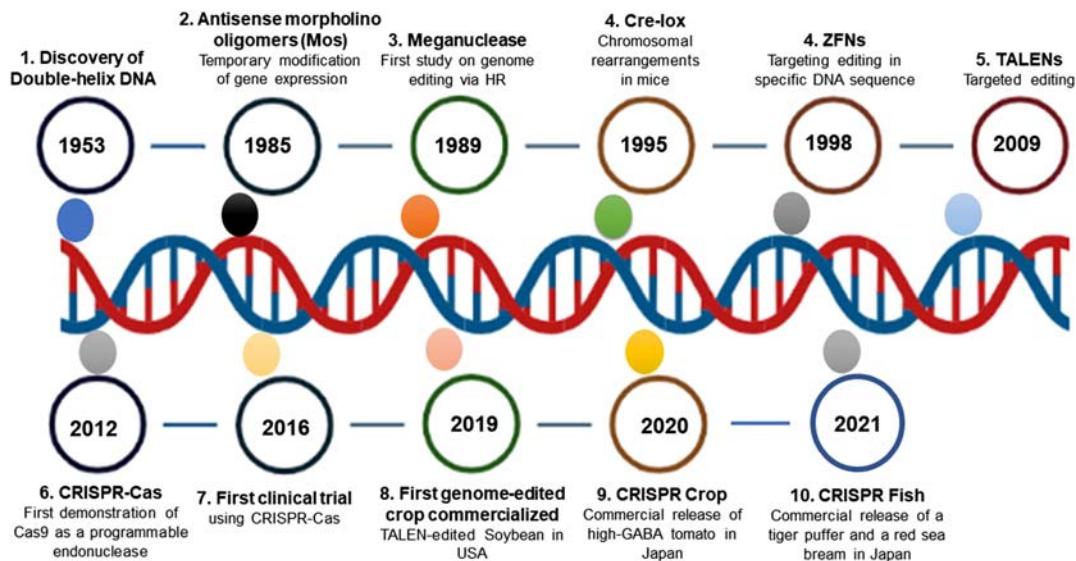


FIGURE 11.1 History and advances of genome editing technologies. CRISPR, clustered regularly interspaced short palindromic repeat; Cas, CRISPR-associated protein; HR, homologous recombination; TALENs, transcription activator–like effector nucleases; ZFNs, zinc finger nucleases.

constructing, and validating. Due to these difficulties, TALEN, a novel tool, was developed in the year 2010–11 [7]. TALEN is though easier to validate and design than ZFN and recognizes fewer nucleotides, making it better than ZFN. However, validation, design, and synthesis of proteins are still cumbersome, limiting the use of this technology.

CRISPR-Cas GE technology, the most recent breakthrough, has been developed by repurposing a prokaryotic immune system [8]. This RNA-mediated mechanism detects and silences alien nucleic acids based on their sequence. Bacterial cells harbor CRISPR arrays consisting of genome-targeting sequences (spacers) interspaced with similar repetitions and Cas enzymes coded in operons. The repurposed CRISPR-Cas9 is a two-component system for GE. The first one is a small guide RNA (gRNA) and the second one is an RNA-directed DNA nuclease, Cas9. The gRNA directs the Cas9 to the specific target DNA sequence to bind and create DSB.

ZFN, TALEN, and CRISPR-Cas systems all can cause targeted DSB at a specific site in DNA. The formation of DSB activates the cell's own repairing systems, viz. nonhomologous end joining (NHEJ) or homology-directed repair (HDR) (Fig. 11.2) [9]. NHEJ is an error-prone repairing process that often leads to alteration of the DNA sequence in the form of deletion, insertion, or substitution of nucleotides [10].

HDR is activated when a donor DNA strand homologous to the flanking sequences of DBS is supplied (Fig. 11.2). The type of insertion, substitution, or restoration of a sequence within DBS is defined by the type of donor. These mutations result in either knock-in (KI) or knockout (KO) of DNA sequence or gene.

To fill the vital gap in precision editing, base editing and prime editing technologies have recently been developed and demonstrated in numerous organisms. These technologies, which are mainly based on Cas9 nickases, can introduce precise changes into the target genome at a single-base resolution [11]. Three types of DNA base editing tools are currently available, viz., cytosine base editor (CBE), adenine base editor (ABE), and C-to-G base editor (CGBE). CBEs were developed to enable C-G to T-A base transition, while ABEs were developed for performing A-T to G-C transition [12–15]. Recently, transversion base editors in the form of CGBE have been developed by several groups to perform C to G base editing [16]. Altogether, these three kinds of base editors present great resources for the precise installation of point mutations in the genome.

For precise insertion and deletion in the genome, a recent GE technology, prime editing, has been developed [17]. Prime editing requires a modified gRNA called prime editing guide RNA and can perform all 12 types of single nucleotide changes and small insertions and small deletions in a precise and targeted manner (Fig. 11.2). However, prime editing needs further improvement to increase the editing efficiency [11,18].

3. Genome editing in aquaculture: an up-to-date status

Currently, GE technology offers plenty of opportunities in aquaculture (Fig. 11.3). Several aquaculture species of Salmonidae, Siluridae, Cyprinidae, as well as gilthead sea bream, Nile tilapia, and Pacific oyster have recently been subjected

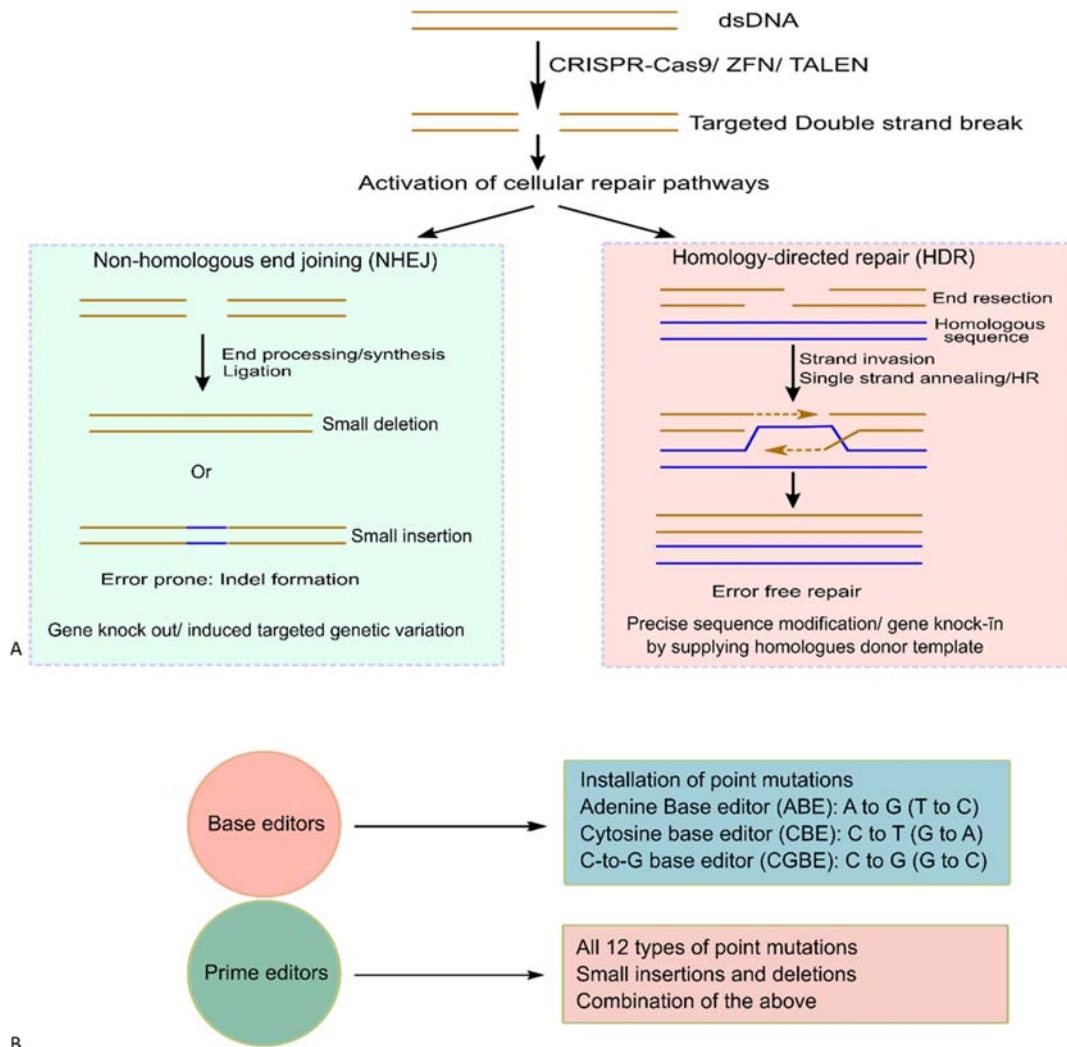


FIGURE 11.2 A, DNA double-strand break (DSB) by CRISPR-Cas or ZFN, or TALEN leads to the activation of cellular repair pathways. NHEJ leads to indel (insertion or deletion) formation causing frameshift mutation and gene knockout. With an additional supply of donor templates, precise modification could be incorporated at or a nearby location of DSB by utilizing the HDR pathway. B, The spectrum of mutations that can be installed with base editors and prime editors. CRISPR, clustered regularly interspaced short palindromic repeat; Cas, CRISPR-associated protein; TALEN, transcription activator–like nuclease; ZFN, zinc finger nuclease.

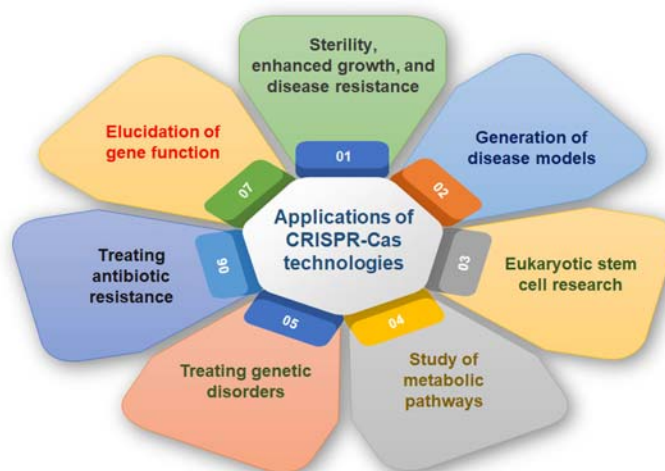


FIGURE 11.3 Application of CRISPR-Cas technologies in basic research and aquaculture. CRISPR, clustered regularly interspaced short palindromic repeat; Cas, CRISPR-associated protein.

to CRISPR-Cas9—based GE in cell lines and/or in vivo (Table 11.1). Successful CRISPR-Cas9—based genome engineering in shrimp (*Penaeus* sp.), one of the primary groups of aquatic organisms, is yet to be documented, which could be due to practical constraints, as addressed briefly below. The majority of the studies were prototypes and applied CRISPR-Cas9 in model organisms like *Danio rerio* (zebrafish) [19]. Most of the studies usually targeted genes with obvious phenotypes to evaluate the effectiveness of editing (e.g., pigmentation). Recently, the CRISPR-Cas9 tool has been used to generate stable and heritable red tilapia phenotype by targeting the *slc45a2* gene [20]. In aquaculture species, the conventional strategy for carrying out in vivo mutagenesis is to inject CRISPR-Cas9 reagents into freshly fertilized eggs at one-cell growth or a near possible stage. In general, mRNA encoding Cas9 is inserted simultaneously along with gRNA, resulting in high editing efficiency that has been confirmed in various species (Table 11.1); using Cas9 protein in place of mRNA has been also effective [21]. Although, in most of the experiments, NHEJ has been used to make mutations, HDR method has been effectively employed in rohu carp by inserting a template DNA [38]. In addition, GE of germline transmission has been described in various studies so far (Table 11.1). Because of widespread mosaicism in edited animals, it has been suggested that Cas9-induced cutting and GE extend beyond the one-cell stage; it is considered as an important issue and needs to be looked after in future research [40].

In aquaculture species, GE has previously been used to target traits like infertility, growth, and resistance to diseases. CRISPR/Cas9-based GE has also been worked out to generate infertility in catfish and Atlantic salmon in order to prevent gene flow with wild stock and to prevent negative production impacts of early maturation [40]. The myostatin gene (known for its main role in double-muscling cattle) has been edited by several groups, resulting in better fish with improved growth-related traits [40]. Immunity responses in Grass carp and Rohu have already been examined via GE, and this area of research is expected to expand as a way of enhancing and comprehending disease resistance. For example, the purposeful mutation of the *TLR22* gene in carp demonstrates how GE might be used [38]. Such simulations can aid in a better understanding of the fish's host reaction to disease, potentially leading to more successful treatment regimens. In a similar manner, GE technologies can be used to improve fish cell lines by wiping off important

TABLE 11.1 Demonstrated cases of genome editing in aquaculture species.

Species	Trait of interest	Target gene	References
Channel catfish, <i>Ictalurus punctatus</i>	Growth	<i>mstn</i>	[21]
	Sterility	<i>LH</i>	[22] and [23]
	Immunity	<i>ticam1/rbl</i>	
Tilapia, <i>Oreochromis niloticus</i>	Reproduction	<i>dmrt1/nanos2-3/foxl2</i>	[24]
	Reproduction	<i>sf-1</i>	[25]
	Reproduction	<i>wt1a/wt1b</i>	[26]
	Reproduction	<i>gsdf</i>	[27]
	Reproduction	<i>aldh1a2/cyp26a1</i>	[28]
Atlantic salmon, <i>Salmo salar</i>	Omega-3 metabolism	<i>elov2</i>	[29]
	Sterility	<i>dnd</i>	[30]
	Pigmentation	<i>tyr/slc45a2</i>	[31]
Red sea bream, <i>Pagrus major</i>	Growth	<i>mstn</i>	[32]
Common carp, <i>Cyprinus carpio</i>	Muscle development	<i>sp7a/sp7b/mstn(ba)</i>	[33]
Pacific oyster, <i>Crassostrea gigas</i>	Growth	<i>mstn</i>	[34]
Rainbow trout, <i>Oncorhynchus mykiss</i>	Growth	<i>2b1/2b2</i>	[35]
Grass carp, <i>Ctenopharyngodon idella</i>	Disease resistance	<i>gcJAM-A</i>	[36]
Southern catfish, <i>Silurus meridionalis</i>	Germ cell development	<i>cyp26a1</i>	[37]
Rohu carp, <i>Labeo rohita</i>	Immunity	<i>TLR22</i>	[38]
Northern Chinese lamprey, <i>Lethenteron morii</i>	Pigmentation/development	<i>slc24a5/kctd10/wee1/soxe2/wnt7b</i>	[39]

constituents of interferon pathway mechanisms, for instance, to enable efficient virus output for subsequent vaccine manufacturing. Two pragmatic reasons why genetic modification has huge potential for research and applications in farmed fish are the large size of their embryos that permits microinjection by hand and the availability of access to many thousands of externally fertilized. The ability to use enormous nuclear families provides for some control over background genetic influences as well as relatively large sizes for assessments of successfully transformed individuals with their full-sibling counterparts later on. It is also typical to be able to perform extensive phenotyping during the early stages of life, such as using well-built illness experiment prototypes so to evaluate defense mechanisms for a number of bacterial and viral infections. Finally, if promising alleles for a specific feature (e.g., resistance against disease) are generated or identified, the enhanced germplasm may be widely disseminated for speedy impact through the above mentioned selective breeding programs. Well-interpreted, high-quality reference genomes are also known for most of the significant species. Given the prevalence of recent whole-genome duplication events in diverse finfish lineages, including salmonids, for successful target gRNA design with discernment and little off-target editing, a superior species-specific reference genome is a prerequisite [41]. Among many constraints, infectious diseases are of the greatest dangers to aquaculture's long-term existence, with an assessed of 40% of total potential productivity loss each year. Due to preliminary stages of taming of many aquaculture species, new option and disease constraints may upsurge the probability that standup genetic variability in populations has to include loci of key effect, which may signify potential 'low-hanging fruit' for GE to surge the rate of the advantageous allele. GE unlocks new exciting possibilities for aquaculture sustainability and productivity. The three major categories in which GE technologies might be used to improve genetics, each one necessitates a unique approach for the study that leads to the finding of operational alleles, are as follows: (1) deleting, detecting, boosting, or solving specified functional sets of genes at multiple or single quantitative trait locus (QTL) segregating within a selective breeding program's current brood stock populations; (2) directed introgression via editing favorable variations from diverse species, strains, or populations to improve or implement novel features in a population; and (3) energy production using de novo useful alleles that have not been seen previously and are used to initiate or improve new features.

4. Harnessing causative variants of quantitative trait locus

The majority of animal breeding and genetics research tries to uncover and apply causal variants for QTL that impact production characteristics; however, there have been few successes. Simulations suggest that employing editing for advantageous causal alleles at many QTLs as part of program can accelerate genetic gain when compared to genomic selection or pedigree alone. The effective identification of functional dissimilarity beneath QTLs, especially those of modest importance, is nevertheless a major stumbling block to the strategy's effective implementation [40]. To do so, researchers can employ a number of functional genomic technologies to limit down a bunch of candidate variants discovered in vast genome-wide association studies. GE could play a major role as functional genomics tools in this regard. Large effect deviations and polygenic deleterious burdens, which are invariably found in populations, could be eliminated using a similar method. Moreover, to have a significant effect with this strategy, many alleles must be edited at once in the same broodstock animal(s), necessitating the improvement and development of multiplex GE strategies.

5. Mimicking beneficial genes/alleles from unlike species or strains by genome editing

One of the most incredibly interesting applications of GE is the ability to acquire genetic variation outside of restricted breeding populations devoid of any need for time-consuming and expensive introgression efforts, or in situations where introgression is inconceivable [40]. CRISPR technology could be used to edit the negative or lethal genes in the target species and/or strain to complement the sequence of the advantageous or nonlethal gene in a related species and/or strain. GE opens up new ways to avoid conventional means of introgression while also preventing the disadvantages of linkage drag (e.g., the deleterious effects of introgression genes from wild strains on growth rate) and permitting utilization of genetic variability in other species and strains that would not be conceivable by means of traditional methods. Parasitic copepod sea lice (*Caligus rogercresseyi* in the Southern Hemisphere and *Lepeophtheirus salmonis* in Northern Hemisphere) cripple Atlantic salmon aquaculture, causing a loss of USD 880 million per year. Aquaculture is distinguished by the close vicinity of farmed grown species to existing wild populations that may have advantageous features. Coho salmon (*Oncorhynchus kisutch*) and pink salmon (*Oncorhynchus gorbuscha*) are tolerant to sea lice attacks and proved to have an efficient immune reaction to the parasite [42]. This tends to raise the exciting prospect of trying to transfer host defenses to

Atlantic salmon, and an extensive effort has been made by scientific community to figure out the reasons governing dissimilarities of resistance mechanisms. It is likely that the major regulatory genes in the pathways that promote species-specific resistance may be altered by GE in Atlantic salmon to mimic the Coho salmon's reaction to sea lice [40]. Targeted GE of coding area and/or alteration of the regulatory sequence may be employed.

6. Creating new variants from scratch based on trait knowledge

Since GE is centered on the known genetic variation and has already made a contribution to possible outcome solutions regarding quality, production, and welfare issues of animals, generating completely novel favorable alleles (distinctive from any naturally existing alleles) is another exciting avenue [40]. Newer interesting alleles can be developed by employing CRISPR/Cas9 system based on pre-existing knowledge of the genetics of character governing genes of interest, or candidate genes can be discovered using genome-wide genetic interruption approaches. Alteration of the *dnd* allele to cause sterility in Atlantic salmon and aiming the *mstn1* gene in various fish species to increase growth have both been utilized in aquaculture (Table 11.1).

CRISPR screens for resistance to disease through genome-wide CRISPR KO strategy is one of the most potent strategies to identify variants responsible for disease-causing or disease-resistant mutations. However, to apply CRISPR screens, well-established cell lines need to be available.

A key barrier in aquaculture research lies in the lack of viable, well-tested and characterized cell lines for several concerned species. Cell lines from chinook salmon, medaka, and carp have been recently transformed with CRISPR/Cas9 reagents with positive outcomes. Virus-related infections are high-priority target features for in vitro research using CRISPR/Cas9. This is because of innate mechanisms of host response that are generally cell intrinsic and therefore feasible to examination. By means of such technologies, it would be simpler to combine large-scale genetic screens to learn more about the genetics of resistance to diseases and generate a pipeline of candidate genes/alleles identification for profitable and commercial aquaculture breeding.

7. Challenges of applying genome editing

To optimize the prospect for using GE systems in aquaculture species, a number of significant technological challenges must be overcome. Firstly, in species where CRISPR/Cas9 has been used, technique enhancement is desired to increase editing efficiency and minimize off-target effects and mosaicism in the F₀ generation. Off-target editing could cause unwanted and nonspecific genomic modifications and could have unanticipated consequences for the organism. Along with targeted sequencing of predicted off-target genomic sites, the cost-effective whole-genome resequencing can allow regular screening for off-target editing. Improved information on sequences of aquaculture species will help to develop unique gRNAs to a precise targeted region. To combat mosaicism, Cas9 proteins with limited half-life have been developed to cause DSBs exclusively during fertilized embryo's one-cell stage of development [43]. It is also plausible to screen for most often generated mutations in F₁ crossings between mosaic F₀ mutants to recognize animals with a homozygous single mutated gene/allele (reasonably than mosaic for more than one edited gene/allele). Base editing, prime editing, and HDR would enable precision editing in F₀ generation, for example, SNP exchange, to generate a premature stop codon or change the amino acid, or install small insertions and deletions of interest. Cleavage efficiency of gRNAs can be evaluated in vitro or in cell culture before in vivo editing to choose the best among in silico-designed gRNAs [44]. Alternative methods of CRISPR/Cas9 delivery such as editing primordial germ cells, microinjection of unfertilized ova, and sperm-mediated transfer could be tested, especially for species where access to newly fertilized embryos is exaggerating, for example, in some shrimp species [40]. Germ cell replacement in animals with a selective edit into numerous sterile surrogates is an alternative way to improve edited alleles of concern [45].

8. Commercialized CRISPR-edited fish

Recently, the sale of two CRISPR-edited fish has been approved in Japan [46]. Genomes of a tiger puffer and a red sea bream were edited by the Kyoto-based company, Regional Fish Institute, in collaboration with Kyoto University and Kindai University. The genome-edited fish grow larger than wild-type counterparts. Deleting the leptin receptor gene, which controls appetite, leads the tiger puffer to consume more and gain weight faster. On the other hand, the *myostatin* gene was disabled in red sea bream to increase muscle growth. The study reported that the tiger puffer grows 1.9 times and red sea bream grows 1.2 times larger than their natural counterpart on the same amount of food.

9. Concluding remarks

Aquaculture species have high fecundity and external fertilization provides excellent possibilities for high-resolution genetic studies for better understanding and improving complex features. CRISPR/Cas9 and other GE technologies have the ability to gear up genetic gain for desired production traits. Infectious disease is one of the important constraints to aquaculture production and productivity, making it a prime goal for GE and selective breeding techniques [40]. Several types of GE applications are possible, which include the following: (1) discovery of contributing variations underlying one or many QTLs affecting attributes of importance, and installing favorable alleles through editing; (2) introgression of beneficial genes from neighboring populations, strains, or species into closed breeding systems through editing; and (3) creation of new alleles with beneficial properties on the trait of interest and subsequent use [40]. Along with the canonical CRISPR-Cas9, advanced tools like base editing and prime editing would greatly facilitate precise genetic modifications for unraveling gene functions and developing the desired trait in fish. Nevertheless, the availability of those tools would unfold new research opportunities that in turn would benefit the sector in long term.

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Ultraviolet-B radiation, a potential threat to the aquatic organisms, and its mitigation

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1. Introduction

Ultraviolet radiation (UVR), the part of the solar emission spectrum, carries more energy per photon than any other wavelength reaching to the surface of the earth. The UVR is divided into three groups based on the wavelength: the UV-A, wavelength 315–400 nm; UV-B, wavelength 280–320 nm; and UV-C, wavelength 200–280 nm. Among these three, UV-C is strongly absorbed by oxygen, ozone, and other gases in the stratosphere and it does not reach the earth surface. Although UV-B is relatively a minor component of the sunlight, it has the highest energy of the daylight spectrum. The intensity of UVR on the earth's surface varies greatly depending on certain factors, viz. latitude, time of day, time of year, and presence of cloud and aerosols. The sun's zenith angle influences the seasonal variation of UVR. This high-energy photon is a potential threat to the terrestrial and aquatic ecosystems. In water bodies, the intensity of UVR depends on two major factors, i.e., the depth and the concentration of particulate and dissolved organic matter of the water. The UV-B can penetrate the significant depths of aquatic ecosystem as water itself absorbs small amount of UV-B [1]. In clean seawater, 10% of surface UVR may penetrate up to 25 m depth, and in lakes with low dissolved organic carbon (DOC), the attenuation depths of UV may be over 10 m [2–4]. UV-B has been considered as one of the major factors for the amphibian declines [5]. The study of impact of UV-B irradiation on the aquatic organisms has ecological as well as aquacultural importance.

The exposure of natural waters to UV-B in the presence of DOC produces hydrogen peroxide (H₂O₂) and other reactive oxygen species (ROS) that likely mediate some of the damage caused by UVR in fish [6,7]. When UV-B radiation induces the generation of ROS in surface water, hydrogen peroxide forms and accumulates as a result of DOC photoactivation [8]. ROS naturally originate from the partial reduction of molecular oxygen during aerobic metabolism. ROS can also be generated from phototoxic chemicals. Uncharged H₂O₂ induces the formation of highly reactive hydroxyl radicals (OH[•]) that induce oxidative damage within cells and tissues. Iron-catalyzed reduction of H₂O by superoxide anions can further generate the highly reactive hydroxyl radical (OH[•]) [9].

The UV-B irradiation may have great impact on the aquatic organisms of the tropical countries like India (latitude: 8°4' and 37°6'N, longitude: 68°7' and 97°25'E). There is a decline of 5%–15% ozone amount per decade over Delhi, Pune, and Trivandrum [10]. The intensity of UV-B has been measured at the water surface of Naini Lake, Delhi, India (latitude: 28°41'26"N, longitude: 77°12'37"E), during June 2013–June 2014 (Fig. 12.1). The minimum UV-B irradiation of 24–27 μW/cm² has been recorded in January–February. It is highest, 180.72–190.45 μW/cm², in the month of June–July [11]. In India, many fishes are breeding and growing in the shallow water bodies/transparent nursery ponds during May–July. The natural water bodies are gradually losing the depth due to the elevation of the basin. There are many surface-dwelling fishes. UV-B–induced behavioral changes are recorded in some fishes (Table 12.1). The exposure may significantly affect the survival rate, physiology, and growth of larvae and adults fishes.

FIGURE 12.1 The intensity of UV-B radiation at the surface water of Naini Lake, North Delhi, and adjacent land monitored during June 2013–June 2014. Four samples were collected at weekly interval for each month.

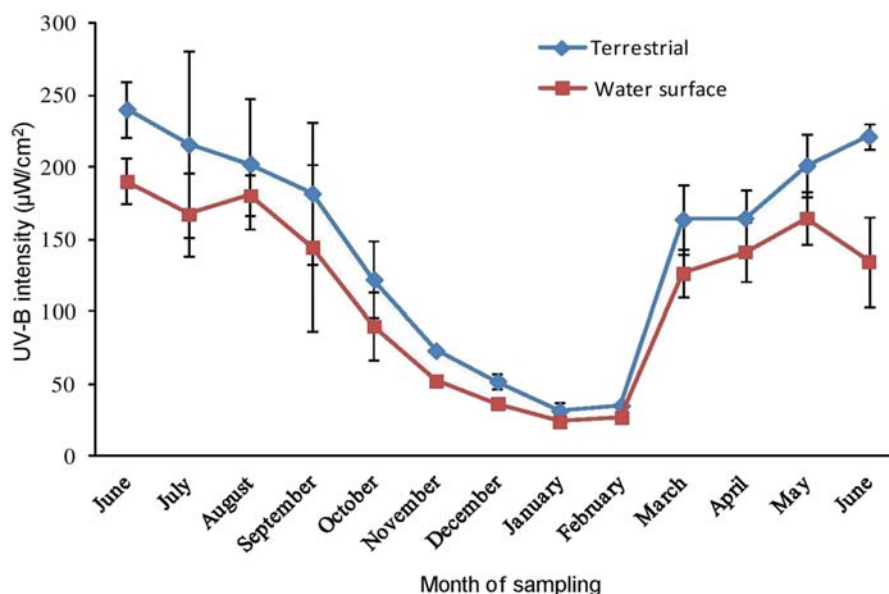


TABLE 12.1 Effect of UV-B radiation on the behavior of fishes.

Name of fish	Specific response	Reference
Pike, <i>Esox lucius</i>	Spiral swimming syndrome	[12]
Red seabream, <i>Pagrus major</i>	Stay at the less illuminated region of the tank	[13]
Rainbow trout, <i>Oncorhynchus mykiss</i>	Increased swimming activity and restless behavior, viz. rapid tail and fin movements and erratic displacement	[14]
Gilt-head seabream, <i>Sparus aurata</i>	Reduced swimming activity, avoiding the exposure, slow movements of the dorsal and caudal fins	[15]

2. Larval survival rate

In natural water bodies (both marine and freshwater ecosystems), eggs and larvae of many fish and shellfish remain near the surface and are exposed to the UVR. Fish larvae are very much sensitive to UV-B as their body is less pigmented and scales are absent in the skin (Fig. 12.2). The pigmented species are more tolerant to UV-B radiation compared to the transparent one. The exposure of early developmental stages may affect the survival rate of the aquatic organisms. The exposure to UV-B radiation in the natural environment/simulated conditions (solar radiation) has seriously affected the survival rate of early developmental stages of many fishes, viz. plaice, *Pleuronectes platessa*; Atlantic cod, *Gadus morhua*; yellow perch, *Perca flavescens*; northern anchovy, *Engraulis mordax*; steelhead trout, *Oncorhynchus mykiss*; coho salmon, *Oncorhynchus kisutch*; and Atlantic salmon, *Salmo salar*. In UVR-exposed yellow perch eggs, 100% mortality has been found [24]. The Kaplan and Meier product-limit model shows an inverse relationship between the survival rate of catla, *Catla catla*, larvae and the exposure duration of UV-B [25]. High mortality at early developmental stage has negative impact on the recruitment and production of aquacrop.

3. Impact of UV-B radiation on the eyes, gills, and skin

The solar UVR avoidance strategy has been recorded in some fishes, like in newly hatched alevins, 2-month-old coho salmon [26], and 25-day-old red seabream, *Pagrus major* [13]. The presence of UV-sensitive photoreceptors, cones, helps the larvae to recognize the intensity of UVR. The UVR reorganization capacity of fish varies during ontogenic



FIGURE 12.2 The early-stage fish larvae with less pigmentation are prone to UV-B irradiation.

development. The exposure to UV-B radiation causes oxidative stress to the lens of the fish eyes. It reduces the hydrogen peroxide—detoxifying capacity of the lens as it reduces the activity of in the epithelial cells of the lens. The UV-B radiation damages the microridges in the eyes of ayu, *Plecoglossus altivelis*, and thereby affects the visual activity of the fish [27]. The occurrence of cataract has been reported in juvenile cod *G. morhua* exposed to UVR (254 nm) used in the recirculating system [28]. In the long term, poor vision affects the feeding behavior of fish and also reduces the escape capacity from the predators.

UV-B radiation affects the primary organ involved in the gaseous and ionic exchange, the gills in fishes. UV-B irradiation damages all three types of cells—the pavement cells (PVCs), mitochondrion-rich cells (MRCs), and accessory cells—present in the gill filaments and lamellae of fish. UV-B destroys the PVCs and exposes the MRCs in catla larvae [29]. PVCs are the most dominant cells in the gill epithelium, constituting more than 90% of the gill surface epithelium. These cells are associated with the gaseous exchange function of the gills.

The skin of fish is most sensitive and prone to UVR damage compared to human [30]. The protective pigment layer is located within the dermis in teleosts. The cases of sunburn have been reported in many fishes like rainbow trout *O. mykiss*, juvenile plaice, and juvenile paddlefish, *Polyodon spathula* [31–33]. The abundance of the microridges (that covers the epidermal cells of the skin) is reduced in UV-B—exposed ayu [27]. The microridges are also fragmented and concentrated in specific areas of the skin. This results in exposure of neuromast and goblet cells. The neuromast cells have specialized functions, viz. help the fish to tolerate osmotic pressure and physical forces of the water and aid cutaneous respiration. Goblet/mucus cells are important components of the fish epidermis. The UV-B—induced damage affects the first line of defense, the skin of fish, and may initiate outbreaks of infectious diseases. The occurrence of flexibacteriosis in Atlantic salmon, *Salmo salar*, has been found [34]. The exposure of fish to UV-B disturbs the epidermal integrity of skin and facilitates the entry of pathogens.

4. Physiological responses of fish

4.1 Impact on metabolic activity

UV-B irradiation shows negative impact on the physiology of fishes in many ways. Proteins, nucleic acids, and lipids are the primary targets of UV-B radiation. Damage induced by UV-B radiation may be at the organ, tissue, and cellular levels, leading to reduced survival and growth of the organism. It affects the metabolic processes in fish, especially during early ontogeny. Significantly reduced amylase, total protease, and trypsin activities have been found in 10–15 min UV-B—exposed catla larvae compared to the unexposed fish [20]. The poor digestive enzyme activities result in improper

digestion of consumed food. The exposure of mosquitofish, *Gambusia holbrooki*, to UV-B suppresses metabolism [35]. This affects the growth and production of fish. Gradually, the fish will become weak and will be prone to the infection.

4.2 Effect on immune system

The effect of UV-B irradiation on the immune system has been studied in many fishes (Table 12.2). In fish, both nonspecific and specific immune systems are downregulated with the UV-B exposure. UV-B irradiation impairs the immunological resistance of fish against pathogens. The immunological parameters like serum lysozyme, myeloperoxidase, hemagglutination titer, and nitric oxide synthase (NOS) indicate the health status of fish. Lysozyme gives protection against microbial invasion to the fish. It splits the cell walls of gram-positive bacteria. Besides an antibacterial function, it promotes phagocytosis by directly activating polymorphonuclear leukocytes and macrophages or indirectly by an opsonin effect. In fish, lysozyme mainly occurs in neutrophils, monocytes, and a small amount in macrophages. Myeloperoxidase is the most abundantly expressed lysosomal protein. It shows phagocytic and bactericidal properties in fish. NOS is a family of enzymes that catalyzes the production of cellular signaling molecule, nitric oxide (NO). Nitric oxide serves as an intracellular messenger that stimulates guanylate cyclase. The reduced levels of these important parameters show the poor immune status of the fishes. The elevated phagocytic activity in the UV-B-exposed fish results in reduction in the abundance of white blood cells.

4.3 Effect on stress parameters

The UVR induces the production of free ROS. The exposure of fish to UV-B increases the activities of enzymes (like superoxide dismutase, SOD) that give protection against oxidative stress. The hematological parameters, like serum protein, albumin, globulin, serum glutamic oxaloacetic transaminase (SGOT), and serum glutamate pyruvate transaminase (SGPT), indicate the physiological status of the fish. The exposure of fish to UV-B shows elevated levels of protein, albumin, and globulin, and it indicates the stress responses of fish (Table 12.3). Higher SGOT and SGPT levels are found in tissue damage. The assay of lipid peroxidation products, malondialdehyde/thiobarbituric acid reactive substance (TBARS), shows the UV-B-induced oxidative damage of cell membranes. In biological samples, the estimation of protein carbonyls (CO) is the most common method to study the end product of ROS-induced protein oxidation. Carbonyl groups (aldehydes and ketones) are produced on protein side chains when they are oxidized. Protein carbonyl derivatives can also be generated through oxidative cleavage of proteins.

Proteins are possibly the most immediate vehicle for inflicting oxidative damage on cells because they are often catalysts rather than stoichiometric mediators. Stress proteins are considered sensitive markers for the evaluation of cell injury as these are associated with the immune response and autoimmune diseases [38]. Heat shock protein 70 (Hsp70) is a widely distributed group of Hsp, and its expression is markedly induced in response to environmental stresses, such as heat shock, UV and γ irradiation, and chemical exposure [39]. It is also known to assist the folding of nascent polypeptide

TABLE 12.2 Effect of UV-B radiation on the immune system of fishes.

Name of fish	Parameters	References
Roach, <i>Rutilus rutilus</i>	Suppressed macrophage migration, respiratory burst activity, and reduced in vitro lymphoproliferative responses	[16,17]
Atlantic cod, <i>Gadus morhua</i>	Reduced levels of hematocrit, plasma protein, and plasma immunoglobulin	[18]
Common carp, <i>Cyprinus carpio</i>	Decreased plasma IgM level and lower total plasma protein and hematocrit concentration	[19]
Rainbow trout, <i>Oncorhynchus mykiss</i>	Reduced levels of plasma IgM, total plasma protein, and hematocrit	[19]
Catla, <i>Catla catla</i>	Reduced lysozyme and nitric oxide synthase levels	[11,20–22]
Rohu, <i>Labeo rohita</i>	Reduced myeloperoxidase and hemagglutination titer levels and decreased number of WBC in exposed fish	[23]
Gilt-head seabream, <i>Sparus aurata</i>	Reduced total antiprotease and peroxidase activities	[15]

TABLE 12.3 The stress responses of UV-B–irradiated fishes.

Name of fish	Study parameters	References
Roach, <i>Rutilus rutilus</i>	High cortisol level	[36]
Catla, <i>Catla catla</i>	Enhanced levels of tissue glutamic oxaloacetic transaminase (GOT), glutamate pyruvate transaminase (GPT), thiobarbituric acid reactive substances (TBARS), and heat shock protein 70 (Hsp70) in exposed fish	[11,20–22]
Rohu, <i>Labeo rohita</i>	Enhanced serum GOT, serum GPT, serum protein, albumin, and globulin; increased tissue GOT, GPT, and TBARS levels in exposed fish	[23,37]

chains, act as a molecular chaperone, and mediate the repair and degradation of altered or denatured protein [40]. The enhanced expression of Hsp70 has been observed in UV-B–irradiated catla (Table 12.3) and that shows UV-B–induced stress in fish.

4.4 UV-B–induced DNA damage

Although organisms possess protective and effective cellular repair mechanisms against UV-B, the excessive exposure may disturb the capability of the organism. The exposure of fish to UV-B for longer period may result in irreversible DNA damage. Then cells undergo complex enzymatic reactions and expend high energy that leads to apoptosis, necrosis, or other forms of cell death [41]. DNA damage induced by UV-B exposure occurs mainly by direct excitation of the nitrogenous bases leading to mutations and chromosome fragmentation. The alterations most frequently induced are cyclobutane pyrimidine dimers and pyrimidine–pyrimidone (6-4) photoproducts [42–44]. UV-B radiation also induces alteration in DNA indirectly through the photosensitization reactions, promoting free radical and formation of ROS. This ROS oxidizes the bases leading to DNA breaks. UV-B–induced DNA damage has been recorded in fishes (Table 12.4).

5. Mitigation of UV-B radiation

The general strategies to cope with the hazardous effect of UV-B are as follows: behavioral avoidance to UVR exposure, acquisition of sunscreens and repair of UVR-induced damage through macromolecules (*viz.* proteins and DNA), and elimination of toxic compounds produced during exposure. Among these, avoidance and photoprotective mechanisms are of great importance for overall protection against UVR. Several photoprotective compounds are found in aquatic organisms, and these are able to absorb UVR directly or indirectly. Sunscreening metabolites and pigments such as mycosporine-like amino acids (MAAs) and carotenoids are widely distributed among aquatic organisms [48,49]. A positive correlation has been found between the concentration of MAAs and the tolerance of UVR exposure in many aquatic organisms. As MAAs can only be synthesized by microorganisms, these are available to the fish through food chain.

TABLE 12.4 The study of UV-B–induced DNA damage in fishes.

Name of fish	Study parameters	References
Rainbow trout, <i>Oncorhynchus mykiss</i>	In larvae, the photoenzymatic repair capacity decreases exponentially with the number of UV-B dose	[45]
Tambaqui, <i>Colossoma macropomum</i> , and pirarucu, <i>Arapaima gigas</i>	Dose-dependent DNA damage, tambaqui is more sensitive to UV-B compared to pirarucu	[43]
Northern pike, <i>Esox lucius</i>	DNA damage in the eyes and brain of fish	[46]
Sea chub, <i>Girella laevis</i>	DNA damage is directly related to exposure duration	[47]
Catla, <i>Catla catla</i>	Enhanced DNA damage, studied indices are tail DNA, tail extent moment, and olive tail moment	[11,22]

Different low-molecular-weight free radical scavengers (e.g., glutathione), including antioxidant enzymes like SOD, CAT, glutathione S-transferase, and glutathione peroxidase, are present in the living organisms to overcome the harmful effects of ROS. Antioxidative compounds like flavonoids, carotenoids, and vitamins C and E help the fish to counteract the harmful effect of UV-B.

Vitamin C is one of the powerful reducing agents available to cells. It serves as a cofactor for the incorporation of molecular oxygen into various substrates. The disease resistance properties of vitamin C have been evaluated in many fishes. Fish and crustacean cannot synthesize vitamin C as they lack the enzyme gulonolactone oxidase, which is necessary for the last step of this biosynthesis [50]. Consequently, they are dependent on constant supplies of adequate quantity of vitamin C through their food. The feeding of catla larvae with vitamin C-supplemented diets (0.5%) gives protection to the fish against UV-B exposure [22]. The antioxidant vitamin C modulates the immune system and provides protection against stress as reduced TBARS, Hsp70, and DNA damages are found in enriched diet-fed catla against the control diet-fed ones.

Supplementation of natural plant ingredients may be useful to mitigate the negative effect of UV-B radiation in fishes. Leaves and seeds of an indigenous medicinal plant, *Achyranthes aspera* L. (family Amaranthaceae), have been incorporated into the diets of fishes at various doses, viz. 0.1%, 0.25%, and 0.5%. It has been found that supplementation of leaves and seeds at 0.5% levels gives maximum protection to UV-B-exposed rohu, *Labeo rohita* [23,37]. The biochemical study shows that seeds and leaves of *A. aspera* are rich sources of protein, lipid, and ash [51]. A total of 18 and 17 essential and nonessential amino acids are found in seeds and leaves, respectively. Seeds and leaves are rich sources of n-6 polyunsaturated fatty acids (n-6 PUFA) and n-3 PUFA. Vitamins A, D₂, E, B₁, B₂, B₆, B₁₂, and C are found in seeds, whereas vitamins A, D₂, E, B₂, and B₁₂ are present in leaves of *A. aspera*. Therefore, the enrichment of diets with these plant ingredients improves the immune system of fish and gives protection against UV-B radiation.

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Applications of biotechnology in aquaculture nutrition and fisheries

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1. Introduction

The human population is growing fastest, and incomes rise most rapidly in Asia. Most additional future demand for fish will come from low- and middle-income consumers in these regions. Fisheries and aquaculture are essential food production sectors, providing nutritional security, contributing to the aquacultural exports, and engaging people in different activities [1]. More than 40% of fish, shellfish, aquatic plants, and algal species are cultivated in a wide variety of freshwater systems globally. The fish production system has grown steadily in the last 50 years, primarily by aquaculture practices, since wild fish capture has been sustained at around 90 million tons over the previous 25 years. In 2018, inland aquaculture produced 51.3 million tonnes of aquatic animals, accounting for 62.5% of the world's farmed food fish production [2]. The total money value is expected at USD 401 billion, of which 82 million tonnes, valued at USD 250 billion, came from aquaculture production. The entire global capture fisheries production reached the highest level ever recorded at 96.4 million tonnes in 2018, an increase of 5.4% from the previous 3 years' average [3].

The use of biotechnology to enhance the production of aquatic species holds great potential to meet demand and improve aquaculture. Biotechnology uses biological systems or living organisms in the production process, has a wide range of practical applications in fisheries and aquaculture, and permits scientists to identify and combine traits in fish and shellfish to increase productivity and enhance quality [4]. Similarly, biotechnology makes it possible to achieve an increased growth rate in farmed species, boost the nutritional value of aquafeeds, improve fish health, help restore and protect the environment, and improve the conservation of wild stocks [5]. Thus, the development and application of biotechnology are possible only when a broad research-based knowledge in biology, physiology, pathology, biochemistry, and genetics of the manipulated organism exists. Hence, this chapter provides ways in which biotechnology is applied to the different sectors of aquaculture nutrition and the future fish farming in the present millennium.

2. Biotechnology in aquaculture production

India is the world's second largest aquaculture producer, with a 10-fold increase in production of 0.37 million tonnes in 1980, which increased to 4.03 million tonnes in 2010, and a compound annual growth rate of above was 6%. The production reached an estimated level of 11.6 million tonnes in 2017 [6]. The global capture fish production in 2018 reached 96.4 million tonnes; this was driven by marine capture and inland fishing. The production of fish in the country reached an all-time high of 14.16 million metric tonnes between 2019 and 2020. The fisheries sector contributes 1.24% to the gross value added (GVA) and 7.28% to the agricultural GVA. The Food and Agriculture Organization in 2020 reported that Asia has the largest share in the global fish production at 89%, since the last 20 years [7].

This speeded growth of aquaculture has significantly benefited both conventional technologies and biotechnologies. It is believed that advanced biotechnologies will further help the sector meet the global demand for aquatic food in the coming decades. Aquaculture is a novel production system in many developing and developed countries than livestock or crop production. Its techniques offer powerful tools for the sustainable development of aquaculture, fisheries, and the food industry in general [8]. While biotechnologies are being applied in fisheries management, their use is minimal compared to

aquaculture. From the past several decades, advances in biotechnology have provided the tools necessary for the artificial manipulation of genes and chromosomes in living organisms [9]. As biotechnology emerged, aquaculture's knowledge is more widely adopted to improve its success rate and sustainability. The aquaculture industry needs fast-growing, disease-resistant varieties, the development of cheap and effective vaccines, disease diagnostic methods, cell lines, and probiotics. Biotechnology can improve aquaculture production through superior breeds, fish health management, population genetics biodiversity, and molecular markers. The demand for aquaculture is growing, and biotechnology can help meet that demand [10].

2.1 Biotechnology in nutritional requirements

Globally, the consumption of food fishes is projected to reach 165 million tonnes by 2030 [11]. As wild-catch fisheries are now approaching full exploitation worldwide, a large part of this increase will come from aquaculture. Thus, the main goal of fish nutrition is to produce feeds that support reasonable growth rates while maintaining fish quality and fish health, resulting in a safe and healthy product for the consumer at a low cost. Identifying alternative fish species and suitable feed ingredients for their diet formulations has become essential. Many experts are investigating the safe use of nutrients and their interactions when alternative plant-based feed ingredients and marine substances are substituted for traditional (and costly) fish meals and oil [12].

Biotechnology helps to answer some of the technical and environmental concerns of fish farming, including aquaculture nutrition and many of these centers around what the fish eat [13]. It is further anticipated that aquaculture production will continue to increase, and since nutrition and feeding play a pivotal role in sustainable aquaculture, feed constitutes about 40%–50% of the total cost of aquaculture production. The nutritionally balanced and completely formulated meal may continue to play a dominant role in finfish production [14]. Hence, biotechnologically improved feed ingredients should be sought, along with pond management improvements and pond productivity manipulation.

2.2 Biotechnology in fish feed production

Fish meal is a high-quality by-product of fish processing with high protein content, and during aquaculture, it is the most common protein source for fish [15]. At the same time, the use of fish meals as a protein source for fish has several drawbacks. First, it is costly, so there is a need for a cheap alternative for fish meals. Second, the supply of fish meal sometimes becomes irregular, and third, the application of fish meal is not environmentally friendly for aquaculture purposes because it may cause several environmental problems [16]. The main reason for that is that it contains a high level of phosphorus. This excess phosphorus mixed with water causes algal growth, otherwise known as eutrophication [17]. So, replacing fish meals with any alternative plant-based protein source can be the best option to solve this problem. At present, about 20 million tons of manufactured aquafeed are being used in the aquaculture, of which the majority is consumed in aquaculture [18]. If the fast growth of aquaculture persists, the feed requirement may increase many folds. Hence, more scientific understanding and interventions are required for sustainable aquaculture of the country. Biotechnology is maintained to answer some technical and environmental concerns with fish meals. Scientists are using biotechnology to produce alternative plant-based protein sources and insect larva sources [19]. Insect (black soldier fly larvae [BSFL]) oil acts as a potential substitute for fish, or soy oil [20] explained that in the fish meal–based diet of juvenile rainbow trout (*Oncorhynchus mykiss*). Similarly, Hossain et al. [18] explained that insect (BSFL) meal inclusion as a novel feed ingredient in soy-based diets improves the performance of rainbow trout (*O. mykiss*).

3. Biotechnology in fish health management

In the field of fish health management, biotechnology has a wide range of applications. Monoclonal antibodies (mAbs) can be used to quickly identify pathogens; antibodies to immunoglobulins from different fish species can be used to monitor the host response after vaccination, and mAbs can also be used to screen brood stock for previous pathogen exposure [21]. Molecular techniques including the polymerase chain reaction (PCR), real-time PCR, and nucleic acid sequence–based amplification have made it possible to detect, identify, and quantify low quantities of aquatic diseases [22]. Microarray technologies add a new level to pathogen and host response multiplex screening [23]. Recombinant DNA technology [24] allows for large-scale, low-cost vaccine manufacture; additionally, DNA vaccination, proteomics, adjuvant design, and oral vaccine delivery will surely contribute to the future development of efficient fish vaccines.

Biotechnology can directly impact many of the main elements of fish health management, with knock-on effects on other vital issues. Rapid detection and identification of pathogens, for example, is crucial for effective disease management,

considering the interplaying genetic, nutritional, and environmental factors, and this, in turn, leads to a reduction in the application of antibiotics and chemicals in the environment; prevention of disease by vaccination significantly reduces antibiotic use [25]. Disease outbreaks are a severe constraint to the development of intensive aquaculture systems and can have a high impact on production due to mortality and decreased growth [26]. Fishes in culture are susceptible to a wide range of bacterial, viral, parasitic, and fungal infections. The losses through disease currently significantly impact the quality and quantum of global fish production. Molecular diagnostic methods and vaccines with immunostimulants are gaining popularity worldwide for improving the resistance to viral diseases in fish and shellfish species [27].

3.1 Biotechnology in fish vaccines and vaccination

One of the most significant breakthroughs of biotechnological application is vaccination. Vaccination is a multidisciplinary approach whereby each vaccine is formulated against a specific disease, with the delivery method, delivery timing, and revaccination means [28]. The knowledge of vaccination has been adopted and applied to the aquafarming industry. The incorporation of vaccination has played a vital role in aquafarming's health and disease management, augmenting its global yield of production to satisfy the food demand of the burgeoning human population [29]. In aquaculture, several vaccines against bacteria have been developed, some of which have been conventional vaccines consisting of killed microorganisms, but new-generation vaccines consisting of protein subunit vaccines, genetically engineered organisms, and DNA vaccines are currently under development [30]. Recent studies have shown that the nonspecific defense system can be stimulated by using microbial products such as lipopolysaccharides, peptidoglycans, or glucans. Among the immunostimulants effective in fish, glucan and levamisole enhance phagocytic activities and specific antibody responses [31]. The latest technology is also of great value in vaccines and immunostimulants for aquaculture species. These allow preventive measures to fight against disease through vaccination. Fish vaccines have become an established, proven, and cost-effective strategy for controlling certain infectious illnesses in cultured animals around the world in the last 2 to 3 decades [32].

The vaccines can be administered via additives in feeds, immersion, or injection. Genetically engineered vaccines are also being developed to protect fish against pathogens. Disease problem area is the major constraint for the development of aquaculture. Biotechnological tools such as gene probes and PCR show great potential in protecting fish from infections [33]. Gene probes and PCR-based diagnostic methods have been developed for several pathogens affecting fish and shrimp. In the case of finfish aquaculture, several vaccines against bacteria and viruses have been created [34]. Still, a new generation of a vaccine composed of protein subunit vaccines, genetically engineered organisms, and DNA vaccine is currently under development.

3.2 Biotechnology in biosecurity and disease control

Disease outbreaks are a severe constraint to intensive aquaculture systems and can significantly impact production due to mortality and decreased growth. It has been recognized that disease is the most significant factor affecting the intensive production of shrimp, salmon, carp, and tilapia, with losses of 10–90% of the total output [35]. Although many aquatic animal pathogens are well studied, unlike in terrestrial animals, pathogens are spread easily through water, and control is difficult due to the high-density culture in a fluid environment. The disease occurs in all systems, from extensive to intensive, although heavy losses are always possible in intensive production systems [35].

Intensive and semi-intensive aquaculture can significantly affect the quality of the aquatic environment where the animals are reared. Poor water quality resulting from increased waste products, inadequate farm management, increased stocking densities within farms, and increased viscosity of aquaculture units per sector can increase the likelihood of disease outbreaks and other environmental problems such as eutrophication, episodic oxygen shortages, and algal blooms, all of them potentially resulting in high mortalities [36]. Therefore, a more “systems-oriented approach” is needed to provide suitable husbandry for adequate growth and effectively control disease outbreaks. There is a greater need for management intervention in intensive systems. Here biotechnological tools can be a valuable part of management approaches. Their scope of application is broad—they can be used as sensors in the production environment, for waste management, and for disease detection and control [37]. Traditionally, disease control is often carried out only after observed mortality. In the past, the diagnosis of fish diseases has been achieved primarily using histopathological methods supported by parasitological, bacteriological, and viral studies based on autopsy and *in vitro* cell culture [38]. These are well-proven techniques. However, we require a high level of expertise, which is often quite time-consuming, not amenable to automation. For these reasons, although expert training is required, PCR technology (described later) has become an essential tool for pathogen assessment in developing countries, for example, in the shrimp industries of Asia and Latin America [39].

3.3 Biotechnology in gene transfer and transgenesis

In the 1980s, due to the fast development of molecular biology and the establishment of DNA recombination techniques, it has become possible for molecular biologists to isolate single gene sequences, assemble them into plasmid vectors, amplify them *in vitro*, and introduce them into microorganisms for investigating their expression activity and regulation mechanisms [40]. By using this new technology, molecular biologists also speculated that when a foreign gene was introduced into an animal cell or fertilized egg, it would be integrated into the cell or egg genome and express its activity during cell differentiation or developmental process. It was also thought that, in some cases, the integrated genes could be kept in the germline of the developed animal individuals and finally be transmitted into further generations. They also believe that since the unique function of genes transferred into the fertilized animal eggs is well known, the transferred genes must be a sort of “target genes.” Therefore, changes in animals’ phenotypes caused by the expression of those integrated foreign genes in recipient eggs or embryos can be predicted to perform their function in unique ways in developing animals. The animal thus obtained is the so-called ‘transgenic animal.’ Theoretically, this method seems an attractive and ideal method for cultivating new animal breeds [41].

Transgenesis or transgenics may be defined as introducing exogenous gene/DNA into the host genome, resulting in its stable maintenance, transmission, and expression [42]. The technique offers an excellent opportunity for modifying or improving the genetic traits of commercially important fishers, mollusks, and crustaceans for aquaculture. Gene transfer is an essential tool to enhance fish stocks for aquaculture and a powerful technique to study gene regulation in living cells or organisms [43]. Palmiter et al. [44] first reported that they obtained “super mouse” (transgenic mouse) by introducing a growth hormone gene into mouse eggs. Later, other authors [45] said that some transgenic chickens, cows, pigs, rabbits, and sheep were also obtained. Some authors are interested in using gene transfer in fish eggs to study insertional mutagenesis and gene expression control during development [46]. In these cases, to use fish species, fish with well-known genetic background, short life cycle, and suitable eggs for operation and cultivation in experimental conditions have been selected for this purpose.

3.4 Biotechnology in environmental management and bioremediation

Aquaculture has been accused of being unsustainable and not environmentally friendly. Although in some cases, where aquaculture development has failed to live up to the global expectations of sustainable development, these allegations are not entirely unfounded; most aquaculture is practiced sustainably and with a high degree of environmental conscientiousness. Reducing the impact of effluent discharge, improving water quality, and responsible water use are vital areas to consider during aquaculture development. Many biotechnologies are being used to address these areas: bioremediation process used for the degradation of hazardous wastes; the vaccination and probiotics to reduce antimicrobial use; and the use of DNA-based methodologies for the early detection of toxin-producing algae [47]. The methods of disease in fish production are strongly connected to environmental factors. Further, a biotechnology that has developed in aquaculture is bioremediation because of the nature of this relationship. This applies to the use of probiotics to treat feeds and discourages the development of “unfriendly” bacteria that potentially would cause disease.

Immunization against disease is a common practice. However, the immune system of fish is rather poorly developed; biotechnological tools are helpful for the development of molecules, which can stimulate this immune system of shrimp. Recent studies have shown that the nonspecific defense system can be produced using microbial products such as lipopolysaccharides, peptidoglycans, or glucans [48]. Among the immunostimulants effective in fish, glucans and levamisole enhance phagocytic activities and specific antibody responses [49]. Aquatic animals are more sensitive to their immediate environment than land animals. The water they depend on for oxygen and a range of other essential chemicals takes up their waste products and may carry pollution from the nearby environment. The effective control and treatment of disease in aquatic animals necessitates rapid, dependable, and susceptible testing facilities [50]. DNA-based technologies are increasingly being employed to characterize various disease species and strains. These are utilized to improve fish health through traditional disease resistance selection and pathogen characterization and diagnosis using molecular methods. Pathogens are also cultured directly in many cases. These approaches, however, are time-consuming and expensive, and there has not been a viable virus culture method accessible. As a result of these issues, immunoassay and DNA-based diagnostic approaches and PCR amplification techniques have emerged [51].

4. Biotechnology in fish breeding

Hypophysation is a technique where pituitary extract used to generate the breeding faced more failures than success besides the limitation of the source. The fish breeding technology suffered for a long time due to the lack of suitable commercial

products to spawn economically important fish [52]. The situation has changed dramatically since discovering gonadotropin-releasing hormone (GnRH), which binds to the membrane receptor of pituitary gonadotroph cells and releases gonadotropic hormone (GtH). An abrupt surge of GtH, roughly 10 times greater than the typical circulating level of GtH, needs the final maturation and release of germ cells, spermatozoa, and oocytes to the water for fertilization [53]. GnRH hormone is a decapeptide, but its gene encodes 92 amino acids containing large molecules; therefore, the injection of GnRH causes this acute GtH surge [54]. Numbers of GnRH structure have been elucidated, and depending on salmon GnRH peptide sequence, a chemical analogue has now been marketed under the name “Ovaprim.” Robust research background is needed to know GnRH mechanism and signal transduction pathway involved in GnRH-mediated GtH function on germ cell maturation and release. These scientific investigations have contributed significantly to designing the superactive GnRH analogues. Biotechnology input in fish breeding is undoubtedly highly appreciable as it provides the cultivators with a riskless method [55]. However, research on biotechnology application in different laboratories is still in progress [56]. Although India has several indigenous culturable bony fish varieties, practically nothing is known about their GnRH. It is a miserable situation. We still must depend on the supply from other countries to investigate the reproductive biology of our economically crucial culturable fish as GnRH is the key regulator of the reproductive control mechanism [57]. For the isolation of this peptide, an assay system to identify the desired molecule at each step of purification is needed.

5. Biotechnology in water quality management

Water quality management is one of the utmost priorities in aquaculture, which requires close monitoring around the clock [58]. Water quality of culture medium is the crucial success or failure factor of the entire industry, determining the survival of culture species. In many countries, the issue of poor water quality is critical due to toxic discharge from industrial waste, agriculture runoff, and domestic sewage. The contaminated water source may carry hazardous heavy metals, high levels of poisonous chemicals from pesticides and herbicides, and toxic pathogens, which eventually reflect the water quality parameters such as deficient dissolved oxygen (DO) and extreme pH levels [59]. Under such severe conditions, certain specific species may manage to adapt and be fortunate enough to survive. However, adverse effects are inevitable. Some classic examples would be reproductive dysfunction with males displaying feminization and food infection on human beings due to biomagnification and bioaccumulation in the food chain. The 24-hour monitoring of water quality and health of the aquaculture species is never easy due to the intensive manpower involved [60]. Different portable digital measuring devices are available in the market, providing accurate and straightforward water quality parameters. However, the result of the subsample collected might be inaccurate due to different factors. Therefore, to improve animal and environmental monitoring using conventional measuring digital devices, biosensors are adopted in aquafarming to obtain real-time valuable and accurate insight into the well-being of cultured animals under water and their living conditions. Biosensors portrayed outstanding performance capabilities compared to the conventional measuring techniques, as it only involves simple technology, yet high specificity and sensitivity [61]. The response time is short and rapid, thus providing 24/7 real-time analysis.

Additionally, what adds to its merits is the low cost involved and relatively compacted size. It can be integrated into a 4G cloud-based technology that can be monitored remotely [62]. All these advantages ultimately contribute to a better aquafarm management system with capabilities of increasing product yield and productivity. Venugopal [63] reported that the first biosensors for fish quality measurement were developed in the 1980s by Watanabe's group by using nucleotide concentration to assess the fish freshness. More biosensors systems are being designed as technology improves, such as using xanthine oxidase electrode, amperometric electrode, and ammonia ion-selective electrode to achieve different measurements. As per research findings of Andrewartha et al. [64], biosensors have been introduced to an oyster farm in Australia to collect variables associated with oyster health, namely DO, salinity, chlorophyll a concentration, heart rate, and the stress level for a monitoring period of 6 months. All this valuable information collected using biosensors can assist aquafarm operators in providing an optimum environmental condition for the cultured species and making critical management decisions.

6. Conclusion

Biotechnology is a technique that uses biological systems and living beings to generate or create various goods, and it has grown rapidly as a result of the new ability to alter the genetic material of organisms (DNA). Biotechnology now influences every part of our lives, including the foods we eat, the safety of the water we drink, the clothing we wear and how we wash it, the prescriptions we take, and the gasoline we put in our automobiles.

The use of synthetic hormones in induced breeding; the production of monosex, uniparental, and polyploidy populations; molecular biology; transgenic fish; gene banking; improved feeds and health management; and the development of natural products from aquatic organisms are all potential areas of biotechnology in aquaculture. All recent biotechnology breakthroughs in the field of aquaculture nutrition are promising and have a beneficial impact on overall system diversification. Aquaculture biotechnology is advancing, allowing for the production of healthy, fast-growing, and high-quality aquatic creatures in an environmentally benign manner. The application of biotechnology in aquaculture is making a substantial impact. However, it should be utilized in conjunction with traditional technologies rather than as a replacement. Although it is a promising area for increasing fish production, its use should be driven by demand rather than technology.

In summary, biotechnology applications provide significant benefits and are the most significant contributor to the development of today's aquaculture industry. Developing countries may adopt demand-driven approaches instead of technology-driven approaches when adopting biotechnology application in the aquaculture industry to maximize their economic effect and achieve self-sufficiency. Additionally, in the perspective of social responsibility, DNA-vaccinated or hybridized products sold in the market should be clearly identified to keep consumers well informed.

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Fish DNA barcoding: advances and challenges

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1. Introduction

1.1 Overview on DNA barcoding

Increased anthropogenic activities on planet earth have created pernicious pressure on biodiversity in both mainland and aquatic ecosystems. Destruction of natural habitats coupled with unsustainable exploitation of natural resources due to rapid increase in human population has led to many unregistered species becoming extinct at an unprecedented rate [1,2]. As of today, many species in the wild, some of which are at the verge of extinction, are yet to be taxonomically identified [3]. The traditional taxonomic tools for taxa identification based on morphology, physiology, internal anatomy, behavior, proteins, and biogeography have been widely used over the past two and half centuries [4]. Despite of being useful, these techniques are expensive, time consuming, and subtle in delineation of morphologically similar but genetically different taxa (cryptic taxa), damaged specimens, and taxa with phenotypic plasticity and immature developmental life stages [5,6]. In addition, these taxonomic tools cannot cater for increased demand for data needed for conservation planning and management amid unprecedented human and climate change—driven biodiversity loss [7]. To overcome challenges associated with morphological-based taxonomy [5], DNA barcoding was introduced in 2003 as an ideal technique for accurate and reliable identification and delimitation of the animal taxa.

The essence of DNA barcoding is to exploit diversity among DNA sequences to identify organisms as universal product codes employ 0–10 alternate numerals to generate unique identifiers to identify retail products [5]. DNA barcoding employs mitochondrial cytochrome oxidase subunit 1 (CO1) gene sequences for discrimination of closely related species. This gene codes for part of respiratory enzyme, cytochrome oxidase, and exhibits high levels of conservation among conspecifics and modest levels of genetic variability among congeneric species, allowing for differentiation [5,8,9,10]. The reproducibility and simple barcoding experimental procedures have made it easier for taxonomists and nonexperts to identify specimens based on a short stretch of nucleotides sequence in standardized gene regions [11].

2. An overview on barcoding project

The Consortium for the Barcode of Life (CBOL) was established in 2004 after the Hebert proposition of CO1 as standardized fragment for global identification and delineation of animal species. The aim was to develop the barcode library for all animal species around the globe [3]. The shortcomings in CBOL including limitation of barcoding research to certain taxonomic groups and geographical regions due to discrepancy in resource capacity among researchers led to the launch of an International Barcode of Life (iBOL) project in 2010 [3,12]. The iBOL comprising 39 member and 7 associate member nations has a mandate to extend barcode reference library coverage across geographical range with a focus on endangered species [3]. It was also mandated to develop protocols and bioinformatics avenues for processing huge quantity of data as much as 500,000 species by the year 2019 [7,12]. Like CBOL, iBOL uses a web-based platform, the Barcode of Life Data System (BOLD), for assembling, cataloging, and providing public access to stored barcode information of all animal species based on CO1 sequences [13]. The online platform was established in 2005 to provide avenue for

collection, submission, organization, validation, specimen management, and publication of barcode data. It also provides platform for alignment of specimen barcodes under investigation to those in reference barcode library for various purposes such as identification, quality authenticity, and generation of genetic distance graphs for conspecific and congeneric species as well as construction of phylogenetic trees [12].

The iBOL has a number of working groups, each assigned to oversee certain taxa. These include land plants, fungi, animal parasites, pathogens and vectors, agricultural and forestry pests and their parasitoids, pollinators, freshwater bio-surveillance, marine biosurveillance, terrestrial biosurveillance, polar life, and vertebrate barcoding group [14]. iBOL successfully achieved its USD 125 million worth project that targeted to deliver DNA barcodes representing 500,000 species via BOLD database in the year 2015. Following successful completion of the project, iBOL launched a new BIOSCAN project in June 2019. The project worth USD 180 million is a consortium project involving researchers from 30 countries, and its broad aim is to accelerate reviewing and describing species lacking scientific names through the Barcode Index Number system [14]. The achievement of this project, which is due to expire in the year 2027, will enhance the ability of scientists to explore and track the patterns of communities and ecosystems through time and space for better understanding of hyperdiverse groups and megadiverse regions while expanding the barcode library [14]. iBOL works in tandem with a number of other organizations including the Global Biodiversity Information Facility, National Centre for Biotechnology Information (NCBI), and many other taxonomic communities and web-based projects [2]. Currently, the BOLD database contains 13,320,188 specimen records with 9,952,713 barcoded specimens from 327,232 species across all biological taxa [13].

3. The fish barcode of life campaign

The Fish Barcode of Life (FISH-BOL) campaign, which is part of BOLD, was established in 2005 to catalog DNA barcodes for all fish groups, to monitor DNA barcoding project progress at regional level and make fish CO1 sequence library available to the public [4,15]. The campaign was further mandated to flag previously overlooked species, expand the barcoding range for known species, facilitate ichthyofaunal identification, and enable identification where morphological-based taxonomic tools fail [4,16]. FISH-BOL consists of 10 regional working groups representing Australia; Africa; North, South, and Central Americas; Oceania; Europe; India; Northeast Asia; and Southeast Asia [7], each mandated to oversee collections, identification, and barcoding of ichthyofaunal species in their respective regions [17]. The collective fundamental goal of FISH-BOL is to gather short, standardized 5' CO1 sequence barcodes for about 35,000 existing marine and terrestrial inland freshwater fish species [4]. The initiative further intended to facilitate rapid, accurate, and cost-effective DNA-based identification of all fishes, regardless of life stage, sex, or specimen condition [17]. The initiative collaborates with other platforms such as FishBase, Catalog of Fishes, and Integrated Taxonomic Information System [17].

Under FISH-BOL, DNA is extracted from a fish specimen's tissue or fin clip, amplified using CO1 designed or adopted primers, and sequenced to obtain barcode sequences, which are compared with the sequences from BOLD or NCBI identification engines [15]. The database engine compares the specimen sequence with similar/closely related sequence repositories and gives the output of resemblance percentage [12]. Archiving of fish barcode data in BOLD public domain requires the name of species, voucher data (catalog number and repository institution), information on data collection (collector, collection date, GPS coordinates, photographs), specimen identifier, size of amplified CO1 gene of at least 500 bp, and trace file of raw electropherograms from sequence analyses [12].

4. Mitochondrial DNA cytochrome oxidase 1 marker

Mitochondrial DNA (Fig. 14.1) is a maternally inherited autonomous portion of the genome (1% of the organism's genome) confined in the energy-generating organelle, mitochondrion, in eukaryotic cells. The genome is a double-helical circular molecule whose size slightly differs among vertebrates [2]. It is about 16,000 bp in size among cichlids with 13 coding genes in vertebrates (ATPase8, ATPase6, ND4L, ND5, ND6, ND1, ND2, CO1, COII, COIII, ND3, ND4, and Cytb), 2 ribosomal genes, 22 tRNAs, and a noncoding control region also known as D-loop (Fig. 14.1) [2,18]. The mtDNA is characterized by the absence of recombination since it is maternally inherited as a single haploid molecule [7,11].

Most of coding and noncoding regions of this organelle have wide applicability in taxonomic studies. The applicability is attributed by lack of introns, absence of recombination, high copy number of the organelle, and maternal inheritance nature, which makes it easier to trace maternal lineage [5,19]. The genome is highly prone to mutation due to the absence

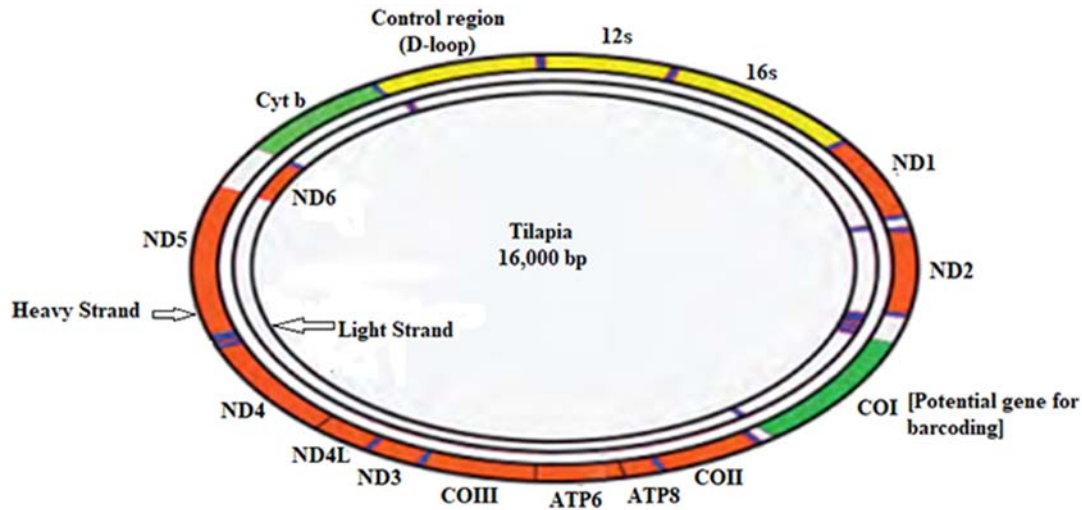


FIGURE 14.1 Diagrammatic representation of mtDNA genome with 13 coding genes, 2 ribosomal RNA, and a noncoding region (D-loop).

of postreplication proofreading and repair machinery, making it highly variable, hence ideal, for conspecific and congeneric taxonomic studies [20].

The COI is one of the three mitochondrial cytochrome genes that collectively encode for three subunits of a transmembrane enzyme, cytochrome oxidase, which helps in cellular respiration [2,12]. The three subunit proteins incorporate with other subunits encoded by nuclear genes to form an enzyme complex involved in the catalysis of oxygen into water (reduction) in the electron transport chain during cellular respiration. The COI gene whose size slightly varies among fish taxa [18] encodes for a catalytic functional subunit of the enzyme [2]. The gene is characterized by higher rate of molecular evolution with unique information in terms of single nucleotide polymorphism and indels (insertions/deletions), making it more variable compared to other protein-coding genes in the genome [5,11]. The variability within the gene has made COI ideal for discrimination of closely related species [2,5,9]. The gene has been found robust in discrimination of congeneric than conspecific species, and based on this, it is not sufficient enough to delineate specimens below species level [19,20,21]. The variability property of the gene, coupled with the presence of universal robust primers [22,23] for recovery of the 5' of the gene, prompted Hebert et al. [5] to make COI gene as an ideal global standard marker for barcoding of animal species.

Delimitation of species through barcoding is attributed by the fact that DNA sequences among conspecifics are more similar than those among congeneric species [5,24]. Species delimitation through barcoding is made possible by subjecting COI specimen sequences to Kimura two-parameter model (K2P) algorithm [25]. K2P mean values within same species have been observed to be lower than those among different species by values greater than fivefold, making COI barcoding robust in delimiting species [9,26]. Hebert et al. [5] proposed that species can be delimited by COI sequences with a threshold genetic divergence of 2%. However, different taxa have been found to have different threshold values for species delimitation. The lack of universality in genetic divergence threshold has been criticized, thereby reducing the credibility of COI barcoding in delineating taxa [27,28]. Despite this, the barcoding of a 658-bp COI fragment is a pillar in identification and delimitation of species and potential cornerstone in solving various issues pertaining to forensic investigation, characterization, and drawing of evolutionary relationship across biological taxa [4,5,15]. The effectiveness of this gene in barcoding across biological taxa is exemplified by the barcoding success reported in different organisms including birds [29], insects [30], fish [4], mammals [31,32], reptiles [33,34], and amphibians [33]. Currently, the BOLD database contains about 10 million barcodes, 234,000 of which are for animal species [13]. Generally, barcoding initiatives have led to the discovery of many species, some of which were erroneously identified through morphological-based techniques.

5. DNA barcoding as a fish identification tool

Fish is a highly diverse vertebrate consisting of groups ranging from agnathans (hagfish and lampreys), cartilaginous fishes (elasmobranchs and holocephalians), to bony fishes (actinopterygians and sarcopterygians) [23]. The group consists of about 35,000 identified extant species worldwide [35,36], representing at least half of all vertebrates on planet earth [23]. The number of species is expected to raise as many species are routinely discovered and more are yet to be discovered [37].

TABLE 14.1 Specimen and species barcodes from Barcode of Life Data System database for all fish classes (Accessed on 15th September 2021).

S.N.	Class	Specimen with barcodes	Species with barcodes
1.	Myxini	387	47
2.	Cephalaspidomorphi	745	46
3.	Elasmobranchii	23,863	1179
4.	Holocephali	577	52
5.	Actinopterygii	375,985	23,361
6.	Sarcopterygii	182	8
	TOTAL	401,739	24,693

Despite of its enormous abundance and distribution across the globe in marine, fresh, and brackish waters, the continued existence of this vertebrate is under threat following anthropogenic interference coupled with climate change [38,39]. Chronic human-driven challenges such as water pollution, illegal fish trade, fish translocation across geographical range, genetic admixture, and overfishing place this vertebrate at jeopardy, with many species at the verge of extinction [37,40]. The ichthyofaunal biodiversity monitoring and conservation initiatives against these challenges primarily require correct, accurate, robust, and reliable systematic identification of fish to species level [8]. Incorrect species identification may lead to errors that may impose significant effect in conservation of species [41].

The morphological identification tools such as meristic, morphometric, and color pattern have been used in fish systematics for the past 250 years [12,42]. These tools have led to identification, cataloging, and assignment of taxonomic status for a number of species. However, majority of species are still unknown and many have erroneously been identified [12]. The slow pace and accuracy in species identification by morphological-based systematics are attributed by the fact that the available tools are inadequate for identification of cryptic species complex, species exhibiting morphological plasticity [19], early fish developmental stages, and processed tissues (in case of identification of illegal fish trade) [37]. Such taxonomic challenges have hampered biodiversity conservation efforts amid increased human and climate change-driven biodiversity extinction. To mitigate such morphological-based taxonomic limitations, various molecular-based taxonomic tools have been developed to aid in accurate and reliable identification of biodiversity of ichthyofaunal species [8].

The use of CO1 gene in barcoding and subsequent launch of a web-based automated fish database system (FISH-BOL) in 2005 has revolutionized the fish identification, delimitation, and automation of sequence data, thereby circumventing taxonomic challenges previously encountered in morphological approach [4]. The global fish CO1 barcode sequence repository database has made it possible to compare specimen sequences for identification at low cost and timely [9,43]. The 16-year success of FISH-BOL is exemplified by its remarkable contribution of CO1 fish barcodes to BOLD database across all fish taxa from all corners of the globe. To date, BOLD database contains about 401,739 barcodes for 24,693 fish species (Table 14.1), representing about 70% of all 35,000 extant species [13].

DNA barcoding and subsequent archiving of ichthyofaunal sequences has received wide application in identification and delimitation of marine and freshwater fish groups to species level [42,44,45,46] and identification of wrongly labeled fish in markets [37,47,48,49]. Others include uncovering cryptic species diversity of marine fishes from different families [24,38,50] and identification and tracking of introduced/invasive species and their source population [6] as well as identification of prey from stomach contents and understanding the ecology and evolution of cryptic species. All these initiatives attributed by accumulation of barcode data in BOLD have contributed remarkably to the conservation of ichthyofaunal biodiversity around the globe in both marine and freshwater ecosystems.

6. Commonly used primers in DNA barcoding of ichthyofauna taxa

The success achieved in DNA barcoding through FISH-BOL avenue is attributed by the presence of readily available set of CO1 primers in the literature [12]. The BOLD portal contains primer database, enabling researchers to access primers for fish barcoding. The presence of these primer sets and their ease to design coupled with the development of standard protocols for the collection, imaging, DNA processing, and archival of barcodes to the database have simplified the species

barcoding process for researchers across the globe [17]. The commonly used primers for fish barcoding are those designed by Ward et al. [23] and Ivanova et al. [32]. Apart from these, primers can easily be designed using web-based user-friendly primer design software such as Primer-BLAST, PRiFi, and BARCRAWL BARTAB [12].

7. Advances in fish DNA barcoding

Since the proposition of Hebert et al. [5] on the use CO1 barcoding as a standard tool for identification of animal species, the technique has received wide acceptance by researchers in all biological taxa. Currently, BOLD database contains about 10 million specimen barcodes from more than 300,000 species from all biological taxa [13]. In fishes, a total of 401,739 specimen barcodes for about 25,000 species representing 70% ($n = 35,000$) [35] of all extant species have been archived in BOLD database from 2003 to date. From the current BOLD records, the ray-finned fishes of the class Actinopterygii have been the most barcoded species, accounting for almost 95% of all CO1 barcoded fish species in the database and 87% of all identified fish in this group (Table 14.1). This could be attributed by the fact that ray-finned fishes are the most diverse, abundant, and widely distributed than other fish taxa. The success in DNA barcoding has made it possible for researchers to resolve various taxonomic issues for different fish groups and eventually contribute to their conservation as explained below.

7.1 Identification and delineation of ichthyofaunal taxa

In the wild, species seldom exist as separate entities. They more often coexist sympatrically with other closely related species or introduced/invasive species; thus, a correct description and delineation is a key a priori step to conservation [51]. Normally species receive conservation upper hand based on their evolutionary uniqueness, diversity, and charismatic value in relation to other species [52]. Therefore, accurate identification is key for conservation prioritization and management of species [53]. Furthermore, ex situ conservation or translocation of species requires a clear taxonomic delineation as different species have different conservation requirements. However, unclear delineation of species may result in genetic mixing, which can further exacerbate conservation problems.

Since 2003, when DNA barcoding was launched, a lot of research in marine and freshwater fishes have been documented (Table 14.2). These include barcoding of salmon and trout species from North American markets [9], freshwater fishes of the family Characidae in Brazil [41], sharks in northern Madagascar [54], bill fishes [37], freshwater fishes of Brazil [16,41], native and introduced fishes of the Philippines [26,45], and cichlids of South Africa [55], just to mention a few. In most of these studies, samples used were tissues, dried fins, fin clips, and smoked tissues from fresh, processed, or confiscated samples (Table 14.2). Genotyping involved amplification of at least 550 bp in the 5' region of CO1 gene, and similarity in identification queries with archived data in public database domain was at least 97% for surveyed literature (Table 14.2). Meanwhile, the intraspecific genetic divergence K2P values were less than 1%, and interspecific genetic divergence K2P values were between 4.9 and 19.

7.2 Identification and delimitation of cryptic fish taxa

One of the major challenges facing morphological-based taxonomy is identification of cryptic species complex. Cryptic species may be defined as morphologically similar but genetically distinct species inhabiting similar environmental condition [8]. According to Ref. [58], cryptic species may be as a result of four factors, namely recent divergence, parallelism, convergence evolution, and stasis. Under recent divergence dogma, cryptic species are sister species or species complex assumed to have recently diverged from a common ancestor and it was assumed that they did not have had enough time to accumulate substantial morphological differences. In parallelism, cryptic species are assumed to have risen from ancestors that were similar to each other but dissimilar to the extant cryptic species. Convergent concept is similar to parallelism in the sense that cryptic species evolved from the same phenotypes independently of each other but differ with the latter in the sense that ancient and most recent ancestors for cryptic species were dissimilar to each other [58].

Because cryptic species complexes consist of species that are more similar in many phenotypic characteristics, it becomes difficult to discriminate them into separate species by morphologically based taxonomic tools [8]. In fish, cryptic species complexes are common in bony fishes of various families such as cryptic complex of marine fishes of family Carangidae [24], pencilfishes (Lebiasinidae) [50], and goatfishes of family Mullidae [38].

Accurate identification of cryptic species complex is key in uncovering species hidden identity, which is key to conservation and understanding their mode of speciation [38,59]. Through barcoding technology, cryptic lineages with many species can be revealed and discriminated by barcode gap and threshold/cutoff criteria. Through barcode gap,

TABLE 14.2 Efficacy of DNA barcoding in solving taxonomic problems of various fish taxa from selected literature.

Activity	Target fish group (taxa)	Sample	Literature	CO1 sequence length (bp)	K2P within species	K2P between species	Comments on efficacy of barcoding
Species identification	Different fish families in Lake Lanao (cichlidae, anabantidae, cyprinidae, clariidae, channidae, osphronemidae, eleotridae, and gobiidae)	Muscle tissue fragment	[26]	650	0.24	8.31	CO1 gene accurately discriminated 12 species of fish in Lake Lanao
	Salmon and trout species	Muscle tissue fragment	[9]	650	0.26	8.22 (32-fold)	DNA barcoding reliably delimited salmon and trout species in North American markets
	Marine fishes (cchimaerids, rays, sharks, and teleosts)	Muscle tissue fragment	[23]	655	0.39	9.93	CO1 DNA barcoding successfully differentiated 207 Australian marine fish species
	Brazilian freshwater fishes	Tissue fragment, fin clips, and eye tissues	[16]	600	0.5	10.6	DNA barcoding clearly differentiated all species by 100%
	Marine fish species of Taiwan	Muscle tissue fragment	[8]	655	0.21	6.5	CO1 DNA barcoding demonstrated high efficiency in species identification and delimitation
	Tilapia in Indonesia	Muscle tissue fragment	[44]	654	N/A	N/A	Successful identification of species by 100% match through CO1 DNA barcoding
Authentication of fish labeled product in markets	Marine fish	Seafood in restaurants	[37]	652	N/A	N/A	41.2% (n = 236) of fish products erroneously labeled were identified through CO1 barcoding
	Marine fish (tuna, salmon, and butterfly)	Seafood	[49]	N/A	N/A	N/A	DNA barcoding showed 99.84%–100% matches with archived data and no mislabeling was observed
	Brazilian catfish	Tissue fragments from whole stored fish and fillets	[48]	658	N/A	N/A	DNA barcoding revealed 80% (n = 63) of incorrectly labeled fish products identified by 100% match
	Shark (seafood products)	Muscle tissue fragments	[47]	550			DNA barcoding revealed commercial fraud by 80%

Identification of cryptic species	Goatfishes (family mullidae)	Muscle tissue fragment	[38]	655	0.3	6	DNA barcoding successfully uncovered cryptic diversity in goatfishes of family mullidae
	Marine fishes of the family carangidae	Muscle tissue fragment	[24]	652	0.37	10.53	CO1 barcoding effectively revealed cryptic diversity in members of family carangidae
	Freshwater fishes of southern Nigeria	Muscle tissue fragment	[40]	651	0.17	10.29	DNA barcoding was effective in identification and delimitation of cryptic freshwater fishes
	Pencilfishes (lebiasinidae)	Muscle tissue fragment	[50]	650	2	19	DNA barcoding clearly delineated pencilfish cryptic diversity by deeper genetic distance values
Identification of critically endangered species	Shark (products)	Battered, fried, frozen, and fresh tissue	[56]	650	N/A	N/A	CO1 identified critically endangered spiny dogfish products sold in fish markets
Identification of larval fish	Larval fish in the lower Ing River, Thailand	Larval muscle tissue	[57]	640	N/A	N/A	DNA barcoding produced 97%–100% identity with entries in BOLD and gene bank
BOLD, the Barcode of Life Data System; CO1, cytochrome oxidase subunit 1; K2P, Kimura two-parameter model.							

intraspecific genetic divergence should differ from congeneric species genetic divergence by larger margin values greater or equal to 10-fold [50]. Under cutoff point criteria, the genetic distance of at least 2% specimens under study is considered more congeneric than conspecific [4]. The second criterion is controversial and has been under criticism as different taxa have been observed to have different delimitation thresholds.

DNA barcoding has been found to be successful in delineating cryptic species with identification success of more than 98% (Table 14.2). The technique has successfully identified cryptic species of various taxa including marine fishes of family Carangidae [24], pencilfishes of family Lebiasinidae [50], goatfishes of family Mullidae across Egyptian coastal waters [38], and freshwater fishes of North Central Nigeria [59].

7.3 Identification of early developmental stages in fish

Identification and delimitation of fish larvae using morphological-based techniques are highly challenging in terms of accuracy and complexity [57]. Identification of early stages of development in fishes is important in providing an understanding on fish breeding areas, nursery, and hatching grounds [60]. Such information is important in management of fishery resource and prediction of fish population dynamics and estimating the size of fish stocks. Incorrect identification of fish eggs and larvae may thus impede understanding on niche partitioning, speciation, diversity, and other marine/freshwater ecosystem features [7]. Since its endorsement as a standard method for identification and delimitation of species in 2003, substantial literature has been documented on barcoding of larval fish in both freshwater and marine environments with similarity match of 97%–100% in BOLD and NCBI database. The literature includes freshwater fishes of Thailand [57] and marine fish groups in South China Sea [61], eastern Atlantic Ocean [62], and many more.

7.4 Authentication of fish products in markets

The application of DNA barcoding in fisheries has made it possible to authenticate the labeling and origin of fish or fish products sold in markets across the globe. The commercial price of fish in markets is determined by supply–demand chain as well as customer preference determined by the quality of fish products. These fish price–determining factors have prompted some dishonest fish processors to substitute more economically valuable customer-preferred species to low-value species by giving them counterfeit labels to lure customers [63]. These incorrect labeling and market substitutions have been found to lower market value for highly commercialized species, especially those facing shortage in the wild [48]. Thus, mislabeling of fish products in markets may lead to illegal trade of protected species by concealing illegal catches. It may also lead to economic fraud especially when a less valuable fish is being replaced with a more valuable customer-preferred fish species [64]. It may also pose adverse health consequences by consuming potentially harmful unlabeled products, especially those derived from highly polluted waters [48,63].

It should be noted that most of the fish products sold in market are processed or in fillet form, thus lack morphological attributes for species identification, making it difficult for customers to prove the authenticity of the fish products they purchase [47,63,64]. Therefore, if left unchecked, fraudulent and mislabeling of fish products may have detrimental effect not only to the economy but also to ichthyofaunal biodiversity through harvesting and illegal marketing of critically endangered species. In this case, DNA barcoding has been exploited in food industry and food chain not only to uncover food safety but also to combat fish piracy through identification of incorrectly labeled fish markets across the world [11]. DNA barcoding has been found to identify fish product mislabeling and fraud with a success rate of 40 [37] to 80% [47,48] at 99%–100% match with archived barcodes. Several studies have shown successful application of DNA barcoding in uncovering of fraudulent substitutions and incorrect product labeling in various fish groups, some of which are critically endangered [37,47,49,56].

7.5 Application of fish DNA barcoding in fish aquaculture

Aquaculture subsector plays a vital role in economic empowerment through direct employment, consequent contribution to the GDP, and conservation of wild fish resource through indirect reduction in overexploitation [44]. The practice also plays an important role in food and nutritional security by acting as a major cheap source of protein and minerals to human communities in both developed and developing countries [65]. The subsector is also vital in poverty alleviation, empowerment of rural microeconomics, and major source of animal feeds. According to the US Food and Agriculture Organization report on the State of World Fisheries and Aquaculture, 2020, the global aquaculture fish production was 82 million tons worth of USD 250 billion, contributing 47% of the total fish produced. The leading aquaculture fish species were grass carp, *Ctenopharyngodon idellus* (10.5%); silver carp, *Hypophthalmichthys molitrix* (8.5%); and Nile tilapia, *Oreochromis*

niloticus (8.3%). Asian countries excluding China contributed 34%, while China alone contributed 35% of the total global fish production. The United States, Europe, Africa, and Oceania contributed 14%, 10%, 7%, and 1%, respectively [21].

Despite of the contribution to economic and food security sectors, the aquaculture subsector especially in developing countries is hampered by several factors, which include inadequate seed quality, lack of selective breeding programs for brood stock development, hybridization of related stocks, and inbreeding in hatcheries [65]. The sector is further setback by inadequate genetic screening programs to identify the nature and origin of brood stocks, which is essential to avoid use of strains from unknown source or from stocks with founder effect [65]. Such strains could lead to poorly performing fingerlings in aquaculture as such suffer high degrees of inbreeding, which consequently results in inbreeding depression and loss of genetic variability resulting in fish with low-performing phenotypic traits of economic significance such as reduced fecundity rates, lower survival, and growth rates [66].

Global fish aquaculture especially in developing countries relies on fingerlings derived from wild fish genetic resource [20,65,67]. Fish brood stock germplasms utilized in hatcheries, which are main source of fingerlings widely utilized by small- and large-scale aquaculturists across the globe, have been developed through selective breeding of wild fish genetic resource [67]. For instance, most of tilapia fingerlings utilized in aquaculture across East Africa are derived from tilapia genetic resource available in natural catchments across East Africa [65]. Even the famous genetically improved farmed tilapia strains developed in Asia in the late 1980s and globally widely used for aquaculture were obtained by crossing and subsequent selective breeding of wild tilapia strains from Africa (Egypt, Ghana, Kenya, and Senegal) and farmed tilapia from the Philippines [67]. Additionally, postselective breeding genetic tools such as hybridization, crossbreeding, transgenesis, and chromosomal manipulation require accurate and reliable identification of wild fish genetic resource [68]. Hence, proper identification of fish wild genetic variability is an a priori and most important step not only in management of fish biodiversity resource and conservation but also in utilization of potential fish genetic resource for aquaculture [44,69,70].

Selection of potential fish wild genetic resource for aquaculture is hampered by incorrect identification of species especially at early stages of development or hybrids. Fishes of aquaculture importance, particularly cichlids, have overlapping morphometric and meristic features that pose a great challenge in proper identification to species level based on morphological features even in adult fishes [71]. The situation is even more cumbersome in identification of eggs, fries, and fingerling by ordinary morphological tools. This in turn impedes authenticity of fingerlings sold to farmers as they are not perfectly sure on the exact species of fish they culture. In this case, genetic screening through DNA barcoding could be an essential tool for correct identification of fish at any life stage development both in the wild and in hatcheries [57].

Despite of the wide applicability of CO1 DNA barcoding in the management and conservation of fishes, there is paucity in literature on direct application of this valuable technique in aquaculture practices. In our view, if given emphasis in aquaculture practices, DNA barcoding could play the following roles.

7.5.1 Identification of wild fish genetic resource potential for aquaculture

The diversity and genetic status of native wild fish resource potential for aquaculture in developing countries with Africa in particular is anecdotal [72]. The anonymity in genetic status has hampered the aquaculture sector as farmers and hatcheries are utilizing wild stocks of mixed species. The increased demand for better-performing stocks has prompted stakeholders to illegally import fingerlings from abroad, which poses pernicious conservation concerns to native species in case of spillage [73]. Thus, the potential of DNA barcoding to identify and delimit species cannot only be exploited in fishery management but can also be applied in getting wild fish genetic resource, which can potentially be used in aquaculture.

DNA barcoding of fish wild genetic resource is an essential initial step in selection and development of strains with phenotypic attributes that can be exploited for aquaculture [74]. The initiative can also help in uncovering genetic diversity among fish species that can be harnessed in breeding programs [69], which consequently can be exploited in aquaculture to maximize profit. Although the barcoding power does not identify subpopulations (stocks) below species level [21], it may be crucial in laying down foundation for other nuclear-based molecular markers such as microsatellites in identification of stocks that can be applied in aquaculture.

7.5.2 Cross-checking the authenticity of germplasm and fingerlings in hatcheries

A survey made across Tanzania by Kajungiro et al. [72] found that most of the hatcheries used wild-derived brood stock and germplasm of mixed tilapia species, leading to low yield. In this case, the potential power of CO1 barcoding to identify cryptic and immature stages of life can be deployed to screen the authenticity of germplasm and brood stock before stocking in hatcheries and prior to distribution to farmers. This will uncover mislabeling and thus enable farmers to rear

actual/intended species as each species has its own culturing requirements. However, this can be challenging in case the specimens are genetically compromised through hybridization or introgression as barcoding is not ideal for identification of hybrids.

7.5.3 Identifying the origin/source of fingerlings utilized in aquaculture

DNA barcoding may help in identifying the source of fingerlings utilized by farmers. Globally, there are many commercial hatcheries supplying fingerlings to small- and large-scale farmers across the globe. However, the origin of fingerlings used mostly by small-scale aquaculturists in developing countries is not known as it involves sourcing from unaccredited suppliers who sometimes illegally import brood stocks from unsubstantiated sources. The anonymity of fingerling origin poses severe concern in aquaculture in case the reared fish maladapt the new aquaculture environment. In addition, it may pose severe conservation concern to the ichthyofaunal biodiversity in case of fingerlings spillage/escape to the natural ecosystems. Thus, the application of DNA barcoding will make it possible to integrate molecular DNA, fish images, geographic location, taxonomy, and nomenclature for domestic and imported brood stocks.

8. Challenges facing fish DNA barcoding

Since its establishment in 2003 by Hebert et al., DNA barcoding has received an upper hand as a potential tool in identification and delimitation of species. However, the molecular-based taxonomic tool is facing a number of challenges and critics from members of scientific community as highlighted below.

8.1 DNA barcoding is inadequate in population genetic studies

The identification and delimitation potential of CO1 barcoding are limited to species level due to limited genetic variability, which cannot discriminate individuals to strain level [20,73]. In this case, population and subpopulation genetic diversity, which are of interest to conservation, cannot be resolved through CO1 barcoding. It should be kept in mind that species seldom existing as single evolutionary entities instead may exist as intraspecific subunits that may evolve independently in response to environmental change [75]. In this case, populations of the same species tend to exist as discrete units, each with a unique genetic pattern in time and space, provided that there is no or partial gene flow among them [53]. These separate units (demes) within the species itself are fundamental genetic units of evolution, which are given upper hand as their conservation ultimately leads to conservation of the entire species. In fisheries and aquaculture, the key units of conservation are evolutionary significant units, management units, and stocks [76]. Since, DNA barcoding cannot resolve genetic uniqueness below species level, it is ultimately insufficient for studying population genetic structure and gene flow patterns among populations. Therefore, other hypervariable markers such as microsatellites can be applied in place of barcoding.

8.2 Hybridization, introgression, and amplification of nuclear mitochondrial pseudogenes

The success of CO1 DNA barcoding may be impeded by introgression, hybridization, and presence of nuclear mitochondrial pseudogenes [12,77]. Fishes, particularly cichlids, have a tendency of undergoing hybridization due to their recent evolutionary radiation coupled with weak reproductive barriers [3,78]. Under this circumstance, the CO1 barcode may not be able to identify or delimit hybrids from parental species.

Apart from that, introgression or incomplete lineage sorting may lead to incorrect identification of species as mtDNA genes of one species may be found in a morphologically different species [10]. For instance, Ref. [79] and later Ref. [80] observed that the Nile tilapia (*O. niloticus*) from West Africa possessed mtDNA genes of a sister species blue tilapia (*Oreochromis aureus*) also endemic to West Africa. In a similar way, Ref. [81] revealed the introgression of mtDNA from *Oreochromis leucostictus* from Lake Naivasha to *O. niloticus* of Lake Baringo despite the lakes being apart by a distance of about 150 km. Under these situations, the application of DNA barcoding in identification and delimitation of mtDNA recipient species may be erroneous and misleading.

The presence of nuclear copies of mtDNA sequences (nuclear mitochondrial pseudogenes) may also impede the integrity of CO1 barcoding [82,83]. Various literatures have reported the presence of nuclear copies of mitochondria-derived genes in most taxa of eukaryotic organisms, particularly crustaceans [82,84]. These noncoding, inactive genes can be easily amplified together with mtDNA genes even when universal primers are used, resulting in overestimation of unique species [12]. Although seldom in fishes, this problem may be resolved by using tissue samples as they contain more

mitochondria and amplification of longer mtDNA CO1 fragment of at least 750 bp as pseudogene length is shorter than that [12].

Another problem associated with mtDNA barcoding is lack of consistency (discordance) in genealogical results between mtDNA and nuclear-based markers from the same specimen. The discordance is due to the fact that the two genomes are inherited differently and their genes evolve independently at different rates [3]. The incompatibility in results for the same specimen between the two genomes may have serious repercussions in conservation and management of species. To overcome this, Ref. [3] suggested the use of an integrative taxonomic approach by combining multiple nuclear and mitochondrial markers and morphological, ecological, and geographical data. They further asserted that such combinational approaches will be invaluable in assessing and managing biodiversity.

8.3 Lack of a universal threshold for delimitation of species

One of the great challenges associated with barcoding is lack of a clear boundary for delimitation of taxa based on computation of genetic divergence values. Species delineation through barcoding relies on the use of thresholds set to differentiate intraspecific variation and interspecific divergence [28]. Normally, DNA barcoding is more robust in species delimitation when there is sufficient “barcoding gap” characterized by discrete distribution (no overlap) between conspecific and congeneric genetic variation given that there is reasonable sampled group for evaluation of unknowns [28]. However, when there is more overlap in intraspecific/coalescent variation and interspecific/speciation divergence, delimitation through barcoding is made impossible [10,28].

The problem is exacerbated by lack of universality in K2P genetic distance cutoff points (boundaries/thresholds) for delimiting species. Ref. [28] asserted that setting a threshold or boundary is essential for making delimitation decision on a sample at hand such that if the sequences under study differ from an existing sample by less than a threshold value, it is assumed to belong to that species. However, if it differs from existing sequences by more than the prescribed threshold value, then it represents a new taxon. The problem that exists in barcoding is absence of universal standard threshold for delimitation of taxa [8]. This is exemplified by variation in cutoff points for speciation reported by different authors in different taxa. For instance, Refs. [4,41,83] adopted a 2% cutoff point for delimiting species. However, different delimitation thresholds have been reported in other taxa. The variation in delimitation thresholds is attributed by the fact that in some species, conspecific genetic divergences exceed congeneric genetic distances in other species [3]. Species delimitation thresholds may also be affected by what Meyer and Paulay [28] described as false positives and false negatives. According to them, false positive may arise when intraspecific variation of spurious novel taxa being identified splits into a taxon whose intraspecific variation falls deeper than the threshold value. False negatives may arise due to inaccurate identification, which causes the taxa under identification to lump within a cluster whose interspecific divergence falls below the cutoff point.

Several methods have been devised to create automatic barcode gaps; these include Automatic Barcode Gap Discovery software [85] and localMinima function (in SPIDER package) [86]. These methods automatically detect the first significant gap used to partition sequence data [3]. Barcode gaps can also be examined by plotting a graph of minimum percent interspecific divergence against maximum percent intraspecies divergence. A best line representing a 1:1 ratio between the two values is drawn, where data above the line represent a barcode gap implying that species may be discriminated through DNA barcoding, while those below the line represent absence of barcode gap [9].

8.4 Inappropriate use of neighbor joining trees

The use of neighbor joining (NJ) trees to infer phylogenies in CO1 and inappropriate use of bootstrap resampling have been under criticism [27]. NJ is a distance-based approach that uses genetic matrix of similarity in sequences to construct phylogenetic trees. The method is an important integral part of DNA barcoding and has widely been used to classify and infer relationship among taxa depicted in a phenogram [87]. Its wide applicability compared to other distance matrix—based algorithms is attributed by the ability to generate results more quickly from large quantity of data without producing ambiguous polytomies [5,27]. Despite its applicability, the phylogenetic approach has received criticism from several authors on its credibility in identifying taxa [27]. The criticism is anchored on the fact that it uses topological rather than cladistics identification system [12]. NJ trees use topology and monophyly of mtDNA lineage criteria to identify taxa, contrary to the reciprocal monophyly identification criteria proposed by some species concepts [27]. In this case, NJ identification systems may produce misleading results in case of species level paraphyly and incomplete lineage sorting situations [88]. Alternative distance matrix software—based methods for NJ trees have been proposed by Ref. [27].

9. Conclusions

Fish DNA barcoding through FISH-BOL has played a critical role in identification of unknown specimens even by nontaxonomists. However, DNA barcoding should be integrated with traditional identification methods and other nuclear-based markers for accurate and reliable systematics. Although the CO1 barcoding technology is user friendly, it is costly and time consuming as it employs Sanger sequencing technique contrary to the next-generation sequencing (NGS) methods with rapid capacity of generating large quantity of genomic data. We suggest barcoding to employ NGS technology to mitigate with the fast-growing sequencing to meet conservation needs. From our observation, the barcoding initiatives should extend more to developing countries with limited resources. Our literature survey confirms that most of fish barcoding has been done in developed countries. We suggest barcoding to extend even in remote areas of Africa to capture barcode untapped ichthyofaunal diversity. In this case, the FISH-BOL initiative headquartered in South Africa should expand its wings in all marine and inland water ecosystems to ensure that all unidentified fish get barcodes. The same should apply for FISH-BOL initiatives in Asia and South America. Such initiatives will ensure fostering of conservation measures to save ichthyofaunal diversity at this critical moment of unprecedented extinction.

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Recent advances in marine biotechnology

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1. Introduction and background

Oceans anchor an affluent variety of flora and fauna, which are adapted to exceptionally complex habitats, such as vents in deep-sea and cold environments. As a result of their in-built capacity to adapt, these organisms can be studied as sources of novel genes contributing to their exceptional adaptive fitness and could be used for advanced use in humans and other organisms. The oceanic environment also offers essential economic prospects attributable to enormous biodiversity, sustaining the potential of specialized bioactive chemical entities to develop novel natural products. Advanced biotechnological and genetic tools and interventions propound enormous potential to utilize rich resources of marine ecosystem. These also help to characterize and document the rich biodiversity of the oceans, recognizing the structure of intraspecific diversity of marine resources to identify stock boundaries, and furthering traceability of captured fish and aquacultured species. Improving the mariculture productivity can be accomplished by selective breeding by means of innovative biotechnological tools through controlling economically significant traits and nutrigenomic mediations by considering the relation and dynamics between genes and nutritional parameters. Utilization of genomic tools could be a valuable method for biomonitoring of oceanic pollutants and to rapidly detect the pollutant traces in sentinel species. The use of next-generation sequencing is an emergent research area in marine biotechnology. Gene-transforming technologies encompassing the evolving CRISPR/Cas9 technique are acquiring greater attention. Genetic and genomic tools support the documentation and characterization of biodiversity of the great oceans, recognizing the structure of subpopulation and intraspecific miscellany of marine resources to identify stock boundaries and furthering traceability. Notwithstanding the availability of advanced genomic tools and technologies, the excessive cost and dearth of know-how are major restrictive factors to develop hardcore marine biotechnologies. Development of in vitro factory fish meat is an interesting area of marine biotechnological advances. The enormous potential of marine bioprospecting along with the development of marine bioactive compounds and their applications, specifically in the nonfood segment are a cutting-edge area of research. Increasing necessity for the renewable energy is being envisaged by marine algae as a valuable source of biofuels, whereas coproducts from algal biofuel processing industries can be recycled as inputs for pharmaceuticals and functional food products. Marine-originated bioactives and functional food components with prospective health benefits are an emergent research area. Rich miscellany of seaweeds, molluscs, heterotrophic microbiota, marine algae, echinoderms, and sponges in oceanic waters characterize an unexplored reservoir of specialized bioactive molecules with treasured biomedical applications (Fig. 15.1).

Notwithstanding the statistic that oceans comprise two-thirds of the surface of our planet earth, not much progress has been made in the marine biotechnology, a leading-edge area of ocean science. Coastal nations have traditionally banked on the maritime resources, while upkeeping their commercial activities. In various parts of the world, irrational exploitation of a handful of target resources for a prolonged duration puts the oceanic ecosystem in major threat. The mammoth task that today's marine biotechnologists face is to create research and developmental tools and process to conserve and protect marine resources as well as increasing biomass productivity on an enduring basis for judicious usage. With the aim of making biotechnological tools sustainable and cost effective, it is essential to develop pioneering concepts and tools. Currently, the marine biotechnological spheres are enormous with intersecting disciplines, starting from molecular methods of

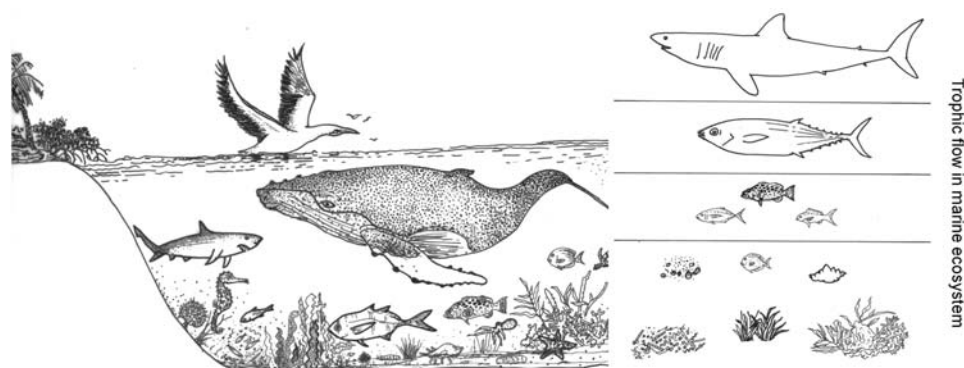


FIGURE 15.1 Marine organisms represent valuable resources for advanced biotechnological applications.

developmental biology to the marine natural product chemistry. This chapter envisages to analyze the current progress in different areas of marine biotechnology, *viz.* molecular biology and transgenic animals, natural products and process, marine bioprospecting, and development of high-value compounds for pharmaceutical, nutraceuticals, and functional food, aquaculture, conservation, developmental biology, cell factories, bioremediation, biodiversity, environmental adaptations, biotechnological process, marine metagenomic approach, in vitro fish flesh production system, and lastly, marine biomaterials.

2. Evolution of marine biotechnology and marine biodiscovery

The concept of marine biotechnology emerged during 1960s upon realizing the prospective of marine organisms and derived natural products for research and commercial utilization. Primarily, the search of marine ecosystems depends on the easily reached organisms, such as seaweed, sponges, and molluscs, besides macroalgae with higher biomass, that were illustrative of oceanic ecosystems [1]. Noticeably, the identified natural products originating from marine environment were primarily isolated from microorganisms. On comprehending the essentially untapped enormous marine microbial biodiversity, application of microbial biotechnology directing to valorize marine resources is a leap forward in the development of the biotechnology sector. Briefly, it has been deliberated that only about 1% of the entire marine microbial population is culturable in laboratory situations [2]. However, recent outcomes proposed that the share of culturable microbial population is higher [3,4]. As various cells in nonhuman milieus are affiliated with novel phyla, new culturing methods by advanced biotechnological interventions would build up the percentage of uncultured microorganisms [4]. Culture-independent techniques with omics methods encompassing proteomics, high-throughput sequencing, transcriptomics, metagenomics, bioinformatics, and metabolomics are nowadays used to identify noncultivable microorganisms. These also include the revelation of metabolic pathways accountable to biosynthesize chemical compounds other than DNA-centered or heterologous systems of expression. However, microbial identification is a preliminary step, and advanced biotechnological interventions are necessary to develop sustainable methods to acquire the required biomass. Genetic engineering approaches are essential to produce high-quality lipids, fatty acids, and proteins, other than high-value bioactive secondary metabolites. Fig. 15.2 provides a diagrammatic illustration of parameters that are to be contemplated for the bioprospecting course, beginning from selecting the marine organisms for cultivation before their use to biosynthesize high-value biocomponents.

Two-thirds of the marine species in the great oceans are yet to be learnt in details. Therefore, oceans are the largest unexploited sources of high-value compounds and biotechnological products, such as enzymes, food supplements, and biomaterials. For example, synthetic bone can be developed from corals, whereas chitin and collagen are obtained from sponges, molluscs, and crustaceans. Oceans anchor a rich miscellany of flora and fauna, which can be investigated as a source of novel genes and high-value products (Fig. 15.3). The marine environment with its unique physicochemical properties harbors an extraordinary source of yet undiscovered organisms and their chemical/biochemical compounds to develop commercially interesting bio-based products.

3. Biotechnological process

Biotechnology encompasses a wide range of procedures and applications. As indicated in the Convention on Biological Diversity Act, the tools comprising bioprocessing/biotechnological processes, and those relating genetic/molecular method with metabolic engineering by genetic manipulation, which could be regarded as molecular biotechnology, are emerging as an area of extraordinary scientific interest as a result of the application of marine genomes.

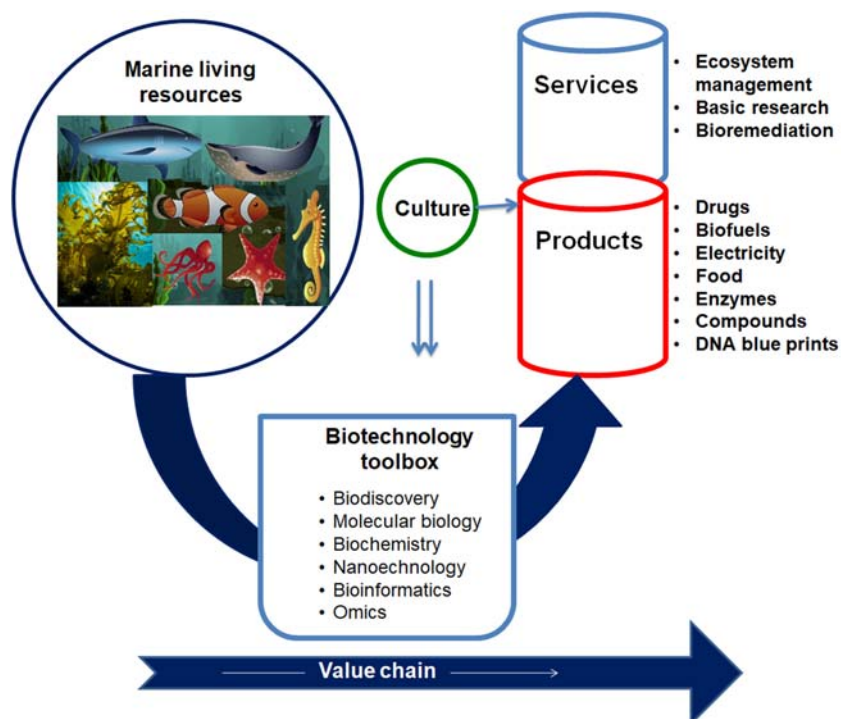


FIGURE 15.2 Marine biotechnology toolbox: products and services.

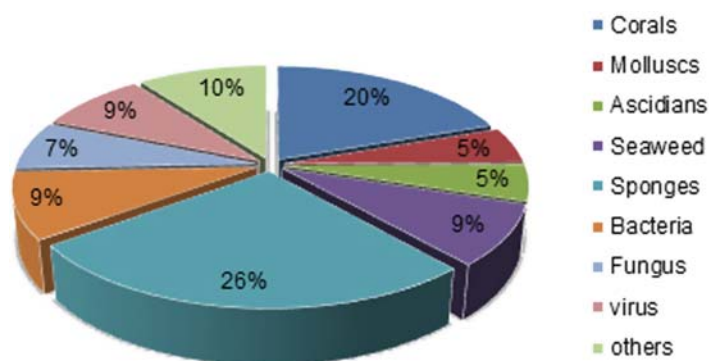


FIGURE 15.3 Percentage share of marine organisms in the marine ecosystem.

3.1 Cell factories

Cultivation of marine organisms, frequently labeled as cell factories, was adopted by human beings for time immemorial. Seaweeds (or marine macroalgae) and microalgae are primarily cultured by (1) open and (2) artificial (closed) culture systems [5–12], each one of those with advantages and limitations (Fig. 15.4, Table 15.1) [13,14]. Noticeably, the production of secondary metabolites in greater yields from algae demands the use of controlled cultivation systems, which led to the development of large-scale bioreactors and artificial culture systems with advanced biotechnological interventions. Bioreactors deliver improved control of growth factors (temperature, nutrients, dissolved CO_2/O_2 , and pH) and preclude contaminants, thereby allowing higher algal cell concentration and greater yields [15].

3.2 Bioprocess technologies

Bioprocess technologies including enzyme-facilitated hydrolysis to yield marine constituents are one of the major methodologies to find marine biological resources, allowing an additional value use of by-products resulting from various fish and shellfish species [16]. Owing to the advanced marine biotechnological tools, it is possible to extract and identify

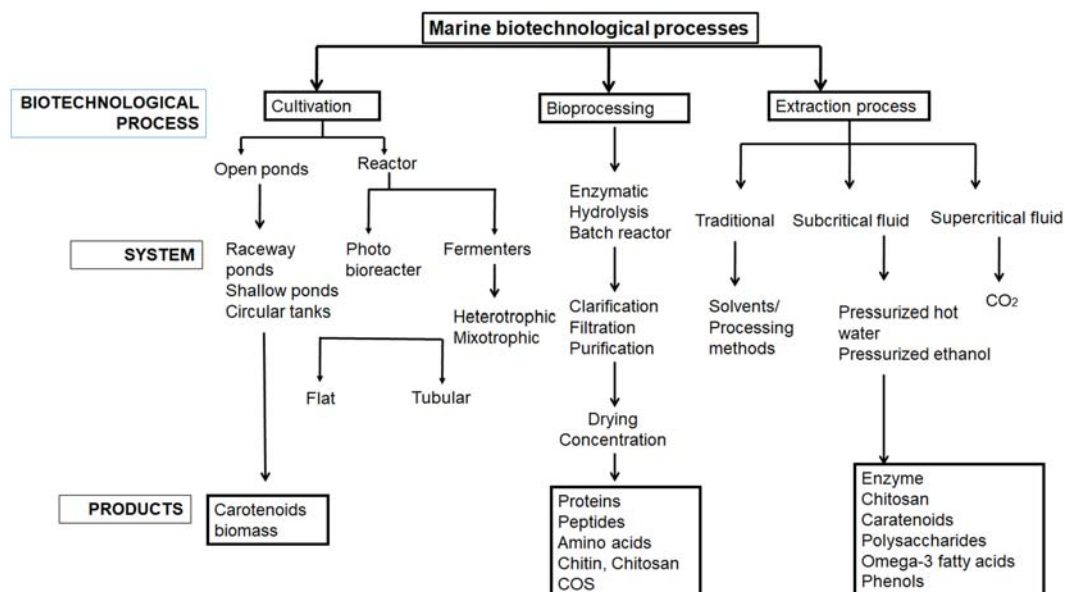


FIGURE 15.4 Marine biotechnological processes.

TABLE 15.1 Marine organism's cultivation in outdoor open and closed system.

Cultivation method	System and characteristics	Species	Production/functional ingredients	References
Open	Open tank in semicontinuous culture	<i>Muriellopsis</i> spp.	Lutein	[5]
	Open tank	<i>Ulva lactuca</i>	Seaweed biomass	[5]
	Open tank	<i>Spirulina platensis</i>	Phycocyanin	[5]
Closed	Bubble column photobioreactor	<i>Scenedesmus almeriensis</i>	Lutein	[6]
	Airlift flat pate photobioreactor	<i>Porphyridium cruentum</i>	Polysaccharide	[7]
	Helix tube photobioreactor	<i>Dunaliella salina</i>	β -Carotene	[8]
	Horizontal tube photobioreactor	<i>Pavlova viridis</i>	Eicosapentaenoic acid	[9]
	Heterotrophic	<i>Nitzschia laevis</i>	Eicosapentaenoic acid	[10]
	Airlift tubular photobioreactor	<i>Spirulina platensis</i>	Biomass	[11]
	Open raceway pond	<i>Spirulina platensis</i>	Algal biomass	[12]

the compounds that stimulate survival in marine environments. Younes and Rinaudo [17] appraised novel methods of chitin extraction from crustacean shell wastes besides microbial/enzymatic hydrolysis as well as substitutes to chemical extraction, for example, fermentation with chitinolytic/proteolytic bacteria. Novel environmentally clean know-hows for extracting fish oil with high-oil content by supercritical fluid extraction and subcritical water extraction as an alternative to chemical extraction were researched in the last few years [18] (Fig. 15.4).

4. Marine metagenomic and transgenic approaches

Even if standard culture practices explained a small fraction of the marine microbial diversity, culture-dependent methods remained to deliver novel chemical entities with promising bioactivities and provide a potential approach to discover new

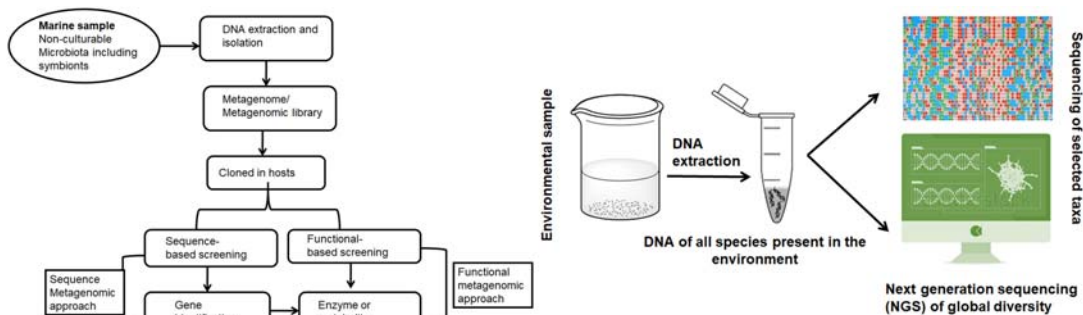


FIGURE 15.5 Marine metagenomic approach: a new tool to derive genetic information of environmental samples.

compounds [19]. Additionally, the understanding of marine genome, particularly of uncultured organisms, would permit to screen for novel genes and obtain new marine microbial-originated compounds. Therefore, it is imperative that greater number of marine microorganisms are considered in advanced genome programs. It is likely to discern whether an organism could produce new compounds by secondary metabolic pathways. This is a prospective biotechnological tool to explore that the genes encrypting biosynthetic enzymes in microorganisms are greater than identified secondary metabolites [20]. Nonetheless, it gives the impression that most biosynthetic gene constellations could express either under cryptic or silent pathways, particularly under laboratory culture conditions [19,20]. Metagenomics is an approach to the study of genetic information of environmental samples encompassing diverse uncultured microbial populations (Fig. 15.5) in that genome fragments (from samples enclosing microbial community), which are cloned and expressed in appropriate hosts. As soon as the genes involved in the biosynthetic pathway of secondary metabolites are known, it becomes probable to understand their expression, and efficiently yield those products and compounds [21–23]. Sequence-built metagenome database along with bioinformatic and functional-based methods of mining of novel gene-encoding enzymes from marine microbial organisms would be potential tools to open new leading-edge marine biotechnological research and development.

Efforts comprising the activity and expression of biosynthetic enzymes in other host are central to the recombinant technology encompassing the recombination of DNAs of different origins in one host. Transformation of photoautotrophs to heterotrophs is of biotechnological importance as light-dependent algal cultivation is less efficient and costly [24]. The potential interest of using transgenic algae is related to improved yield and production of novel metabolites and lipidic substances [25] by expressing specific genes [26]. Metabolic engineering to produce new metabolites or increasing yield of the products could be possible by controlling specificity of biosynthetic enzymes [24].

5. Biotechnological advances to extend the use of ocean bioresources for industrial applications

5.1 Agronomy, food and feed

Marine phytobiomass is presently being investigated for potential applications in agricultural sector (i.e., fertilizers, feed, and biogas plants [27]. Traditionally, seaweeds and seagrasses were utilized as organic soil amendments to improve soil fertility and producing biostimulants [28–30]. Marine protein hydrolysates put forth stimulatory effects on plants [31]. Seaweeds, cyanobacteria, and other algal extracts were acknowledged to contain small molecular bioactives, plant growth regulators, phytohormones, and protein with balanced amino acid profiles [32–35]. Chitosan films are used as coating in leaves and seeds for preventing microbial infections other than their use in slow-release pesticides and fertilizers [36].

Marine organisms are potential sources for novel food components [37], particularly those including mollusc, seaweed or jellyfish. Seaweed has long been part of the traditional diet of coastal communities. It is widely consumed in East Asia, particularly in Japan, China, and Korea. Studies demonstrated that in Japan, the rate of mortality is less, and hence, their life expectancies are very high. It is reasonable to evaluate this result because Japanese are regular consumers of seaweeds [38]. Over and above the whole organisms, bioactive and functional compounds from marine fish, algae, and microorganisms are used in the functional food industries as natural antioxidants, prebiotics, pigments, gelling agents, and nutraceuticals [39]. Marine-originated long-chain *n*-3 polyunsaturated fatty acids, chitin, chitosan, chondroitin sulfate, spirulina, astaxanthin, chito oligosaccharides, and glucosamine from shark cartilage, crustaceans, and cyanobacteria are also used as stabilizing, gelling, flocculent, thickening, and binding agents [40,41]. Food and beverage industries use cold-

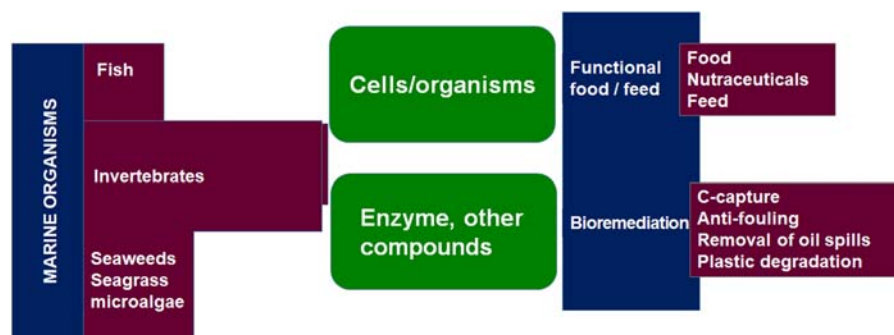


FIGURE 15.6 Potential for using marine organisms in food and feed industries and for bioremediation processes.

active enzymes isolated from marine organisms to process heat-sensitive components, and to deter heat-prompted alterations to organoleptic/nutritional properties of food [42]. An organization of the process of using marine organisms as a source of food or feed is shown in Fig. 15.6.

5.2 Cosmetics and cosmeceuticals

Marine components can be integrated into cosmetic products owing to their drug-like benefits, in which marine bioactive ingredients are supplemented to the oral/topical cosmetics to develop cosmeceuticals and topical ointments with improved properties [43–45]. The prominent examples are (1) photoprotective/antiaging agents, such as mycosporine-like amino acids (potential to absorb UV radiation between 310 and 360 nm), produced by marine cyanobacteria and micro/macroalgae under UV stress [46]; (2) microbial exopolysaccharides to increase the moisture content of the skin [47]; (3) marine algal/crustacean polyphenolic/carotenoid compounds with antioxidant and anti-inflammatory properties [48–50]; and (4) antiaging peptides (protect collagen stores) [51] (Fig. 15.7, Table 15.2).

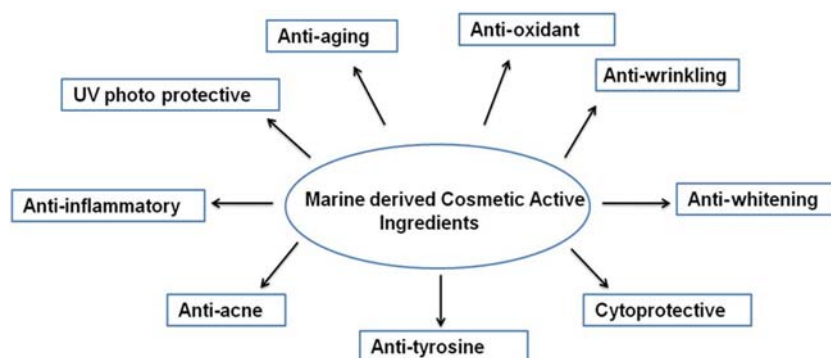


FIGURE 15.7 Marine-derived cosmeceuticals.

TABLE 15.2 Marine-derived cosmeceuticals and their applications.

Cosmeceutical	Marine organisms	Purpose	References
Mycosporine-like amino acids	Cyanobacteria, algae	Antiaging	[52]
Exopolysaccharides	Microorganisms	Moisturizing	[47]
Carotenoid	Microalgae	Antioxidants	[48]
Seaweed-based hydrogel	Macroalgae-associated heterotroph	Anti-inflammatory and wound healing	[53]
Enzymes	Marine invertebrates	Antiaging	[51]

TABLE 15.2 Marine-derived cosmeceuticals and their applications.—cont'd

Cosmeceutical	Marine organisms	Purpose	References
Peptides	Microorganisms	Antiaging	[51]
La Prairie	Macroalgae	Antiwrinkle	[49]
XCELL-30	Microalgae	Moisturizing	[49]
Pseudopterosin-type diterpene glycosides	Soft corals	Analgesic	[49]
Hyadisine	Extremophiles	Antiwrinkle	[49]
SeaCode	<i>Pseudoalteromonas</i> sp.	Moisturizing	[49]

5.3 Marine nutraceuticals: a paradigm shift in health care

There has been a growing interest in functional foods (or nutraceuticals) in recent years, and the functional foods, enriched with natural ingredients, are proved to provide beneficial action for human health. Marine-derived bioactive components and the functional food ingredients demonstrated to possess potential health benefits. Recent trends in functional foods and supplements have demonstrated that bioactive molecules play major therapeutic roles in human disease. Nutritionists and biomedical scientists are working together to discover new bioactive molecules for therapeutic benefits. Marine flora and fauna remain largely unexplored. Approximately 71% of the molecular entities listed in the *Dictionary of Marine Natural Products* have novel molecular structures compared to ~40% of those listed in the *Dictionary of Natural Products*. Noticeably, marine life has evolved over a long period of time in severe and hostile marine environmental conditions. In preclinical cytotoxicity screen, marine organisms show higher incidence of pharmaceutical potential: 1% versus 0.1% for terrestrial organisms. Number of new marine compounds reported in each year is increasing >1000 compounds. The rich diversity of marine organisms represents an untapped reservoir of bioactive compounds with valuable pharmaceutical and biomedical use. Pioneering research work at ICAR-Central Marine Fisheries Research Institute of India developed marine nutraceuticals for hypercholesterolemia, hypertension, diabetes, inflammation, hypothyroidism, and hypertension; immunoboost agent; and a library of molecules with bioactive potential (Fig. 15.8, Table 15.3).

**FIGURE 15.8** Marine-derived nutraceuticals from molluscs and seaweeds.

TABLE 15.3 Marine nutraceuticals.

Categories	Nutraceuticals	Applications	Marine sources	Health benefits	References
Proteins and peptides	Collagen	Edible coating in meat industry (e.g., sausages)	Fish (albacore tuna, silver-line grunt, catfish, trout, and common horse mackerel)	Antioxidant, antihypertensive, and anti-skin-aging activities	[54]
	Gelatin	Stabilizer, texturizer, or thickener in ice cream, confectionaries, etc.	Fish, especially in cold-water (pollock, cod, haddock, hake, and cusk)	Prevent and treat chronic atrophic gastritis	[55]
	Albumin	Whipping, suspending, or stabilizing agent	Molluscs, crustaceans, low-fat fish	Anticoagulant and antioxidant properties	[56]
Polysaccharides	Carrageenan	Gel formation and coatings in the meat and dairy industry	Macroalgae, e.g., <i>Kappaphycus alvarezii</i> , <i>Eucheuma denticulatum</i> , and <i>Beta-phyucus gelatinum</i>	Anti-HIV and anticoagulant activities	[57]
	Agar-agar	Gel formation and food gums	Red alga is a main source of agar-agar like <i>Gelidium</i> , <i>Gracilaria</i> , <i>Hypnea</i> , and <i>Gigartina</i>	Food gums	[58]
	Chitin, chitosan, and derivatives	Gelling agents, edible protective films, and clarification and deacidification of fruits	Shrimp, crab, lobster, prawn and krill increase dietary fiber and reduce lipid absorption	Antitumor, bactericidal, and fungicidal activities	[59]
Fatty acids	Omega-3 fatty acids	Nutraceuticals (fish oil and capsules), fortification of livestock, and infant formula	Almost all marine sources	Visual and neurodevelopment, reduce cardiovascular risk	[60]
Phenolics/pigments	Chlorophylls	Natural food and beverage colorants	<i>Spirulina platensis</i> and <i>Aphanizomenon flos-aquae</i>	Natural source of pigmentation	[61]
Enzymes	Gastric proteases, chymosins	Cold renneting milk and fish feed digestion aid	Various fish body viscera like Atlantic cod, carp, Harp Seals, and tuna.	Feed digestive aid	[62]
	Serine and cysteine proteases	Preventing unwanted color changes in food products and curing of squid fermentation	Crustaceans, molluscs, and short-finned squid	Prevent color change in food	[59]
	Lipases	Numerous uses in the fats and oils industry	Atlantic cod, seal, salmon, sardine, Indian mackerel, and red sea bream	Lipid metabolism	[63]
	Transglutaminase	Creates protein cross-links to improve rheological properties of gels	Red sea bream, rainbow trout, Atka mackerel, walleye, pollock liver, and scallop	Improve protein structures	[63]
Cadalmin™ GMe	Green mussel extract	Food and nutraceutical industries	Marine bioactive compounds from Asian Green Mussel <i>Perna viridis</i>	Antiarthritic	[64]

TABLE 15.3 Marine nutraceuticals.—cont'd

Categories	Nutraceuticals	Applications	Marine sources	Health benefits	References
Cadalmin™ GAe	Green algal extract	Biopharma and nutraceutical industries	Marine bioactive compounds from seaweeds	Antiarthritic	[65]
Cadalmin™ ADe	Seaweed extract	Food, biopharma, and nutraceutical industries	Marine bioactive compounds from seaweeds	Regulation of blood glucose level	[66]
Cadalmin™ ACe	Seaweed extract	Food, biopharma, and nutraceutical industries	Marine bioactive compounds from seaweeds	Dyslipidemia and obesity	[67]
Cadalmin™ AHe	Seaweed extract	Food, biopharma, and nutraceutical industries	Marine bioactive compounds from seaweeds	Regulation of hypertension	[68]
Cadalmin™ ATe	Seaweed extract	Food, biopharma, and nutraceutical industries	Marine bioactive compounds from seaweeds	Thyroid support	[65]
Cadalmin™ AOe	Seaweed extract	Food, biopharma, and nutraceutical industries	Marine bioactive compounds from seaweeds	Osteoporosis	[69]

The global nutraceutical market comprising functional foods and beverages and dietary supplements was valued at around \$250 billion in 2014. Consumer demand for nutraceuticals is rapidly increasing with the market expected to reach around \$385 billion by 2020. Numerous molecules with biomedical functions are also supplied by marine ecosystems, and are used as pharmaceuticals [41,53].

5.4 Bio-inspired materials

Marine-derived biomaterials have useful characteristics, for example, better salt/pressure tolerance and adaptivity to extremes of heat and cold [70]. Even though there are extensive potentials for use of marine-based compounds to design/develop bioinspired materials in biomedical science (bone implants and replacing heart valves) [71], many challenges must be answered. For instance, isolation of biopolymers could play a crucial role in directed delivery of drug or concentrations for continuous drug release. Polysaccharides, peptides, pigments, lipids, biominerals, and toxins from seaweeds, jellyfish, sponges, crustaceans, mussels, etc., were described as commercially important biomaterial sources, and can be used in biomedical applications [72–74]. Structural arrangement of marine organisms, predominantly sponges, has enthused several technical explanations to fabricate biomaterials, aerodynamics, and architecture [75]. A number of sponge taxa were recognized to produce spicules (an inorganic form of skeletal components) made up of hydrated amorphous siliceous structures [76], which could be used to develop biomaterials for biomedical/optical applications [77–81]. Silicateins are silica-forming enzymes were isolated from hexactinellid/demosponges, and can be used to develop inorganic/organic composite structures with distinct electrical, mechanical, and optical properties [82]. Diatoms were reported to produce highly ordered mesoporous and nano-arranged frustules (biosilicate) possessing wide range of applications [83,84] (Table 15.3).

6. Seaweeds and products: valuable sources of high-value compounds and nutraceutical products

Seaweeds are receiving attention in nutraceutical industries due to their bioactive potential against various chronic diseases. Other than the primary metabolites, the seaweeds possess structurally diverse secondary metabolites, with promising biological activities. Although earlier therapeutic uses of seaweeds were found only in traditional medicines, lately at the end of 1990s, the discovery of bioactive compounds from seaweeds further broadened their utilization in pharmaceutical industries. Noticeably, the long life expectancy and lower rate of cardiovascular diseases of Japanese people might be associated with their regular intake of seaweeds. As a result, most of the Western countries have also started to include seaweeds in their diet. Above all, these have been identified as a new source of ingredients for developing nutraceuticals (Fig. 15.9).

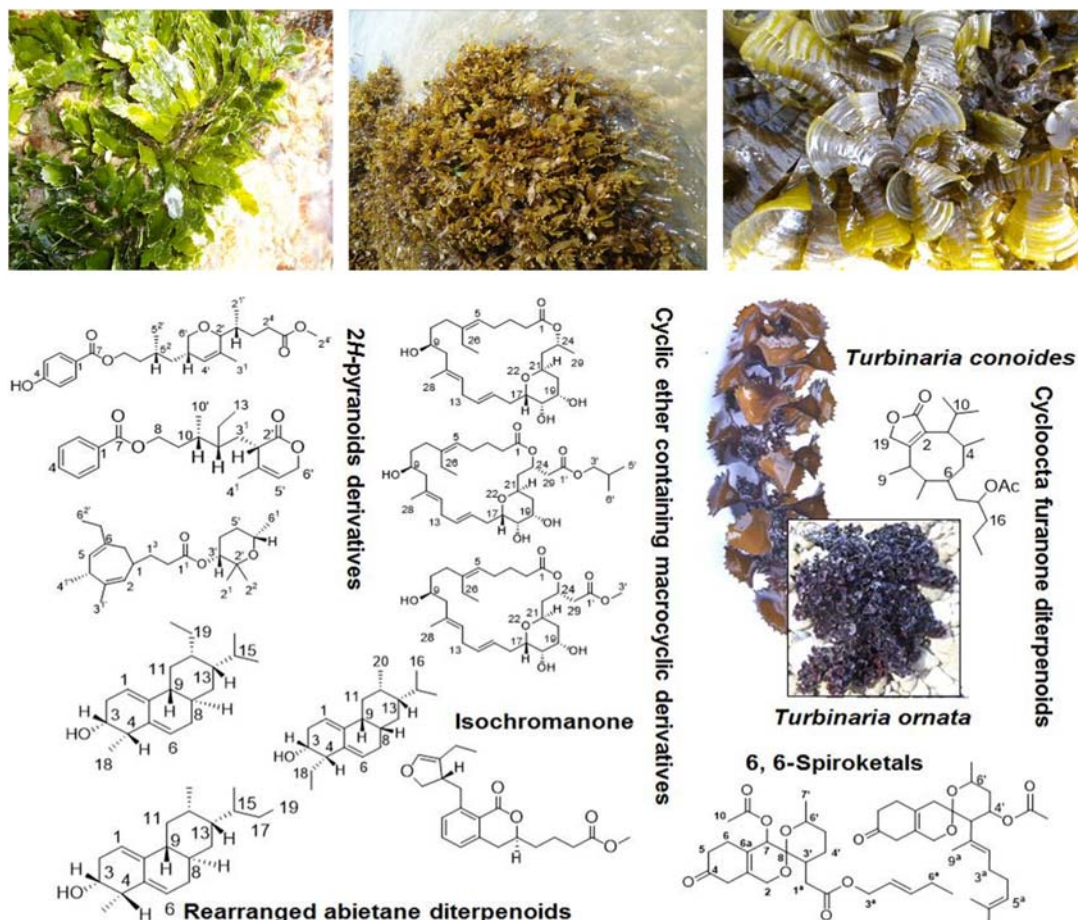


FIGURE 15.9 Seaweeds contain a large assemblage of species that predominate in the coastal shelf areas of Indian subcontinent; various classes of bioactive compounds isolated from brown seaweed *Turbinaria* sp.

Seaweeds were used in the traditional folk medicine for many years in Japan (13,000–300 BCE), Egypt (1550 BCE), China (2700 BCE), and India (300 BCE). The ancient Romans used seaweed to treat burns and wounds. Sargassum has been used in traditional Chinese medicine for more than 1000 years to treat different diseases. Seaweeds are valuable marine macroflora that are proved to have wide-ranging commercial applications. In 2018, global production of seaweeds through mariculture (27.5% of all aquaculture) was estimated at about 32.4 million tons (wet weight), valued at \$13.3 billion (FAO 2020).

During the years 1980–95, seaweeds played a milestone in pharmaceutical industries as well as modern biology. Many of the algae reported to possess anti-inflammatory, antimicrobial, antioxidant, antitumor, anticoagulant, and antihypertensive properties. Certain algae possess powerful cancer-fighting agents that researchers hope would eventually prove effective in the treatment of malignant tumors and leukemia in people. The secondary metabolites obtained from seaweeds demonstrate a broad spectrum of bioactivity varying from neurologically active in humans to algicidal, nematocidal, and insecticidal activities [85]. High content of proteins, polyunsaturated fatty acids, polyphenols, alkaloids, terpenes, pigments, vitamins, minerals, and insoluble and soluble dietary fibers too account for the bioactivities of seaweeds. Apart from these, marine polysaccharides, such as agar, alginate, carrageenan, fucoidan, ulvan, laminarin, and furcellaran from seaweeds, are integral parts of a globally thriving marine phycocolloid industry as it constitutes the most abundant source of polysaccharides. Significant retention capabilities, tunable active units, swelling, and colloidal features make seaweed polysaccharides ideal materials for biomedical applications [86–88].

-
- ✓ Seaweeds are valued commercially for the natural source of phycocolloids like agar-agar, agarose, furcellaran, algin, and carrageenan.
 - ✓ A number of tropical seaweeds including green algae (*Ulva*, *Enteromorpha*, *Monostroma*, and *Caulerpa*) brown seaweeds (*Dictyota*, *Laminaria*, *Cladosiphon*, *Padina*), and red seaweed (*Gracilaria*, *Porphyra*, and *Eucheuma*) are consumed directly (sea vegetables) for their minerals, vitamins, proteins, essential amino acids, and low-fat content.
 - ✓ Although global utilization of marine algae is a multibillion-dollar industry, their bioactive potential is still underexplored. For centuries, the medicinal properties of marine macroalgae were limited to traditional and folk medicines.
 - ✓ However, in recent years, industries have been focusing their attention on the discovery and development of compounds from marine algae.
 - ✓ Seaweeds are the only sources for industrially important phycocolloids like agar, carrageenan, and alginate. They have applications as stabilizer, viscosifier, gelling and emulsifying agents.
-

Studies on natural product chemistry and chemical defense systems of seaweeds recognized the presence of bioactive leads with prominent pharmacological activities. Phlorotannins, sulfated polysaccharides, and polyphenols from the seaweeds have revealed their activities against proliferation of cancer cells and produce some effects on anti-inflammatory and antidiabetic response [89,90,66,91,67,92]. Among various abundantly available seaweed species, brown and red seaweeds (classes Phaeophyceae and Rhodophyceae, respectively) were found to be the potential sources of bioactive substances [93,94]. These bioactive compounds are mainly produced under various stress conditions. The major bioactive compounds isolated from the seaweeds include polysaccharides, phenolics, proteins, peptides, terpenes, terpenoids, carotenoids, sterols, dibutanoids, acetogenins, alkaloids, etc. [94–99]. These molecules are mainly responsible for the healthy properties associated with the seaweeds. Seaweed-derived alginates and laminarin were found to protect the stomach and intestine from potential carcinogens [100–102]. Seaweed protein hydrolysates were used to develop nutraceuticals for controlling hypertension and/or oxidative stress [103]. Seaweed-derived mannitol has been used as a natural sweetener for people with diabetes [104,105]. Exploring the ACE inhibitors from seaweeds displayed a growing interest in pharmaceuticals, nutraceuticals, and functional food industries [92,68,106].

7. Marine-derived pharmaceuticals

Biotechnology researchers have isolated numerous bioactive compounds from marine environment, which held immense potential for treating various human diseases. “Manoalide” from a marine sponge has spawned more than 300 chemical analogues, many of which have gone into clinical trials as anti-inflammatory agents. A study conducted at the University of Hawaii reported “depsipeptide” in the mollusc *Elysia rufescens*. Depsipeptide was reported to be active against tumors, and genetic manipulations of *E. rufescens* could generate adequate amounts of the compound. “Pseudopterosin” is a novel anti-inflammatory diterpene glycoside that underwent clinical trials. A marine bryozoan *Bugula neritina* was reported to produce an antileukemic agent, and further research is underway to isolate the compound [107]. Bioprospecting of previously undescribed natural products from marine organisms has been improved considerably over the previous few decades, resulting into an investigation of newer secondary metabolites [108–110]. It has been stated that the marine organisms are the most prominent sources of natural products, and therefore, they have been favorite choices of natural product chemists [111–113]. Newer natural compounds of marine origin have delivered promising bioactive compounds with previously undescribed structures/skeletons, and these could be used as promising nutraceuticals and therapeutic agents against various ailments [114]. During the past few years, various biopotential components have been isolated, identified, and characterized from different marine organisms, such as molluscs, bryozoans, sponges, tunicates, corals, algae, microorganisms, echinoderms, and cnidarians. Marine natural products have been acknowledged to have highly desirable chemodiversity to support drug discovery and pharmacological applications by their diverse chemical features and the wide range of biological activities [108,109,111,112]. A good number of natural products of interest to the pharmaceutical industry are secondary metabolites, and several such compounds, derived from marine organisms, have been in clinical trials as experimental drugs (Table 15.4).

The distribution of various marine organisms evaluated for chemical investigations and natural product isolations over the period from 1971 to 2015 among different phyla was plotted above (Fig. 15.10). The compounds include alkaloids, peptides, terpenoids, and steroids, which provide natural immunities to the organisms. In National Cancer Institute pre-clinical cytotoxicity screen, marine organisms show higher incidence of antitumor potential: 1% versus 0.1% for terrestrial organisms. The number of new marine compounds reported each year is increasing greater than 1000 compounds.

TABLE 15.4 Bioinspired materials from marine origin.

Biomaterial	Marine organism	Type	Activity	Reference
Collagen	Jellyfish	Collagen V/XI	Healthy food	[115]
	Jellyfish	Collagen I	Enhance cell migration, heal wound, exhibit hemostatic properties, modulate chondrogenic differentiation of human mesenchymal stem cells and cell culture substrate	[116]
	Jellyfish	Collagen I	Trigger similar responses in cell adhesion, proliferation, or migration	[117]
	Sea urchin	Collagen I	Skin regenerative medicine and collagenous extracellular matrix biomaterials for tissue engineering	[118]
	Squid	Collagen II	Degenerative osteoarthritis—relieving effects	[119]
	Persian Gulf squid	Collagen II	Degenerative osteoarthritis—relieving effects	[120,121]
	Squid skin	Collagen I	Anticytotoxic properties	[121]
	Starfish	Collagen I	Production of two-dimensional membranes	[122]
	Catfish	Collagen I	Food, medical, and cosmetic industries; produce high—value added product	[123]
	Catfish	Collagen I	Substitutes for commercial collagen	[124]
	Sponges	Collagen IV	ROS scavenging capability; photoprotective activity as well as wound-healing characteristic	[125]
	Blue shark	Collagen I	Collagen I promotes osteoblast proliferation rates	[126]
	Blue shark	Collagen II	Antioxidant activity and increase apoptotic gene expression	[126]
	Yellowfin tuna	Collagen I	Antioxidant activities	[127]
	Sardine	Collagen I	Mosquito larvae against <i>Aedes aegypti</i>	[128]
	Salmon	Collagen I	Protect DNA against oxidation-induced damage	[129]
Enzymes (L-asparaginase)	Bacterial sources	Asparaginase	Convert L-asparagine into aspartic acid and ammonia	[130]

7.1 Enzymes

Many enzymes were isolated from marine bacteria. Extracellular proteases can be used to clean reverse osmosis membranes and in detergents. *Vibrio alginolyticus* produces collagenase and detergent-resistant alkaline serine exoprotease, which have immense commercial applications in chemical industry. A haloperoxidase isolated from algae could find applications in halogenation process. Advanced biotechnological process was used by the Japanese researchers to develop methods to induce a marine alga to produce greater quantities of superoxidase dismutase with applications in biomedical,

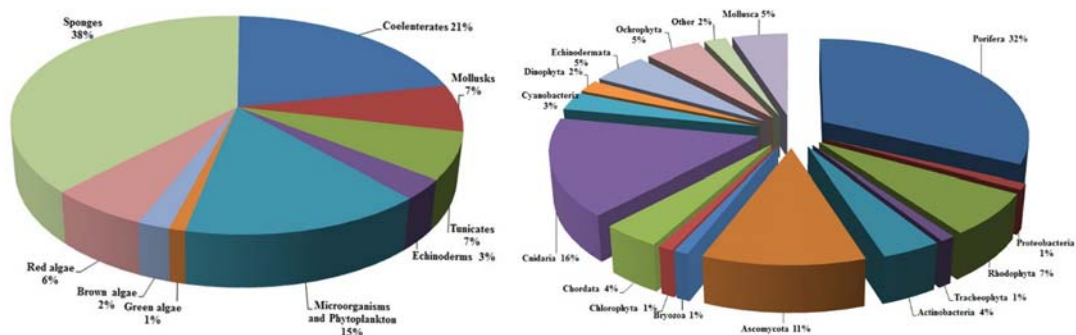


FIGURE 15.10 Distribution of marine natural products by phylum and distribution of marine organisms among phylum collected for natural product isolations over the period from 1971 to 2015.

functional food, and cosmetic industries. Thermostable DNA-modifying polymerases, restriction endonucleases, and ligases have tremendous biotechnological applications. Taq polymerase is a thermostable DNA polymerase I isolated from a deep-sea thermophilic eubacterial microorganism *Thermus aquaticus*. A novel melibiose oligomer hydrolyzing α -galactosidase was isolated by the researches of the Rutgers University from *Thermotoga neapolitana*. These oligomers are most important components of soybean products, and were reported to reduce the amount of soy that can be incorporated into feed for monogastric animals (unable to digest oligomers). The oligomer hydrolyzing α -galactosidase could find its use to remove melibiose/protease inhibitors from soybean products [131].

7.2 Biomolecules and biodegradable polymers

Contemporary studies have revealed that marine biochemical processes could be utilized to produce novel biomaterials. A Chicago-based company has developed a previously undescribed class of biodegradable polymer that forms organic matrices of molluscan shells. Marine coccolithophorids and diatoms produce nanoparticulate mineralized structures (sized with lesser than one billionth meter), which can transform the process of medical implants, protective coating, and other novel products [132]. Oyster shells are used as sources of biodegradable polymers with potential biomedical, agricultural, and industrial applications. An antifreeze compound isolated from winter flounder was used to develop biodegradable antifreeze peptides that were found to control the icing on aircrafts [133].

8. Bioremediation

Bioremediation encompassing the usage of marine microbes or processes to do away with pollutants from marine/coastal environment is attaining greater recognition. Bioremediation holds immense potential to address the problems of mariculture and marine environments. Bioremediation can help stop pollution attributable to oil spills, toxic chemicals/antibiotics/pesticides, managing seafood processing, sewage disposal, and recuperation of minerals. A number of microbial conglomerates were developed to break down petroleum constituents. Introduction of genetically engineered bacterial consortia possessing oil-decomposing properties was tried. Founding work of Anand Chakraborty creating genetically engineered oil-decomposing bacteria with the first ever granted patent on a living organism continues to be a landmark invention in marine biotechnology. Advanced biotechnological methods were developed by the Louisiana State University to metabolize polychlorobiphenyls, polyaromatic hydrocarbons, creosote, and other toxic chemical agents. Gene expression arrays of marine organisms can be deliberated by transcriptome analysis, and genes and their expression profiles can be used to develop biosensors and biomarkers. Plasmid-aided molecular breeding supports to accelerate the oil spill degradation. Recombinant tools were used to transmit plant/animal genes producing metal-binding proteins, metallothioneins, to marine organisms (single-celled alga *Chlamydomonas reinhardtii*) by these scientists to expedite decontamination process in cadmium-polluted waters. Bacteria could play essential roles in mining operations, with special reference of bioleaching of gold, uranium, and copper ores. A single-celled protozoa was found to prompt bacteria to digest oil faster, and scientists are yet to make out how protozoa induced the bacteria to speedily eat up the hydrocarbons. A marine cyanobacterium was used to perceive herbicide concentrations. Noticeably, the cyanobacterium was genetically engineered to carry the *lux* gene expressing a LUX protein triggering light emission in the presence of a reagent (dodecanal). Herbicides act on the photosynthetic mechanism, and in presence of genetically engineered cyanobacterium, emission of light could decrease in such a way that it is possible to compute the herbicide concentration in soil, water, and contaminated sites [134].

9. Role of marine biotechnology in biofouling

Biofouling is the unwanted colonization of surfaces by filamentous algae, microorganisms, and invertebrates, resulting in consequent biodeterioration. This results in the shipping industry worldwide to cost millions of dollars each year. Ever since the ban of tributyltin-based compounds/formulations by reason of high toxicity, novel ecofriendly agents are under development. Several research groups envisage on an encouraging research area enthused by marine biotechnology biomimetics based on surface microtexturing and marine natural antifoulants. Marine biofoulers are recognized to cause severe damages on ship hulls, cages in mariculture systems, and marine infrastructural setup (Fig. 15.11). Among various natural antifouling agents, tribromo-1-methylgramine and 5,6-dichlorogramine showed promising activities against barnacles and mussels [135,136].

10. Applications of marine biotechnology

10.1 Human health

Since time immemorial, extracts of marine organisms were used as medicine. From the 19th century onward, cod liver oil was used as a supplementary nourishment. However, only during the 20th century onward, researchers working in the area of marine biotechnology began to systematically probe ocean for medicines (Table 15.5) [137].

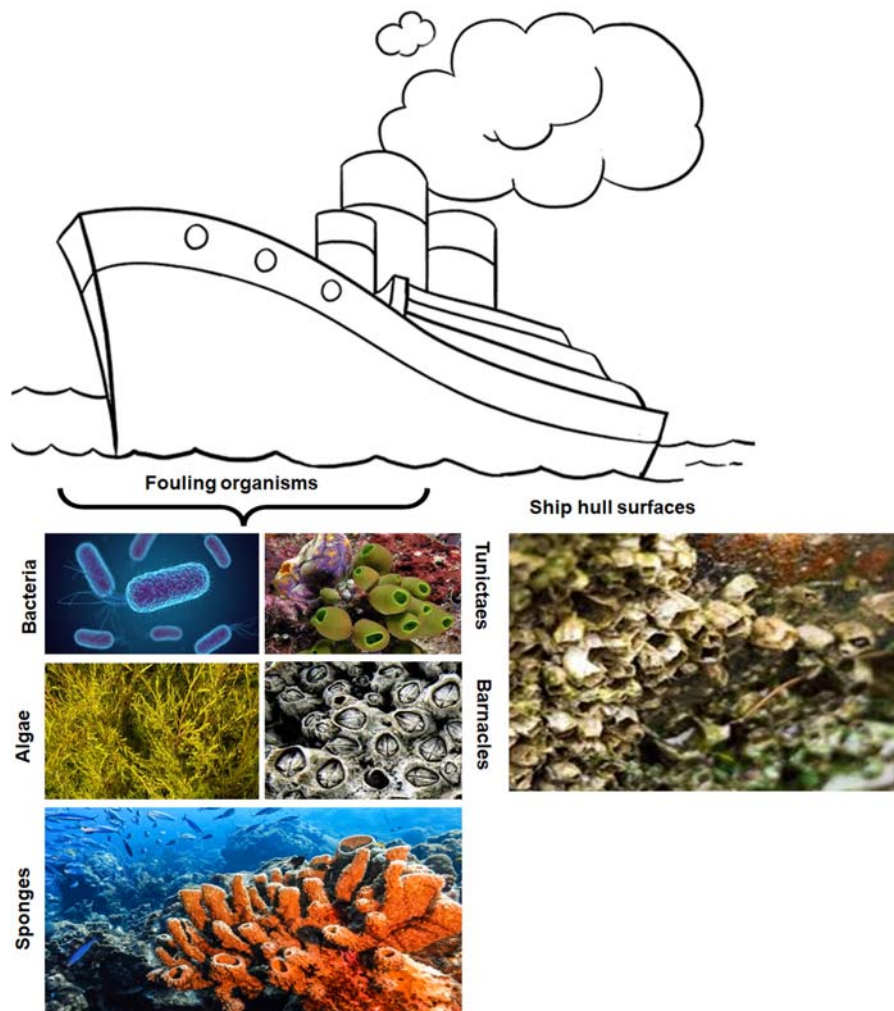


FIGURE 15.11 Marine biofouling.

TABLE 15.5 Marine-derived products.

Subsector	Potential product areas	Specific product areas
Health	Pharmaceuticals	Anticancer/antiviral drugs, novel antibiotics, anti-inflammatory, immunomodulatory agents
	Biomaterials	Bioadhesive, wound dressings, dental biomaterials, alternative disinfectants, medical polymers, artificial bones, medical devices
	Others	Tissue regeneration, 3D tissue culture
Cosmetics	Functional ingredients	UV filter, surfactants, liposomes, carrier systems for active ingredients
	Raw material	Microalgae and macroalgae extracts: colorants, pigments, fragrances, hair-styling raw materials
Food	Functional foods	Prebiotics, omega-3 supplements
	Nutraceuticals	Useful as antioxidants, anti-inflammatory; fat loss; reducing cholesterol; anti-HIV properties; antibiotic and mitogenic properties; antitumor
	Food products and marine ingredients	Stabilizer, suspending agents, bodying agents, foaming agents
	Food packaging and conservation	Films and coating with antimicrobial agents
Energy	Renewable energy processes (micro/macroalgae)	Microalgae produce polysaccharide (sugar) and polyglycerides (fats) that can be used for producing bioethanol and biodiesel Macroalgae: large-scale cultivation of macroalgae for the production of biofuel
	Microbial enhanced oil recovery	Enhanced oil recovery and productive life oil reservoirs
Aquaculture	Seed	Antibur additives for textile printing, binding agent in welding rods, surrogate brood stock technologies, transgenic approaches developing culture species, selective breeding of cultured species for novel and disease-resistant hybrids
	Feed	Fish oil produced from algae; pigments in fish feed
	Disease treatment	Diagnosis treatment of disease and disease-resistant strain
Marine environmental health	Aquaculture system	Treatment of recirculated water

10.2 Transgenic fish in mariculture

Production of transgenic fish is considerably easier than creating other transgenic mammals owing to the fact that fish produce a larger egg volume (a number of dozens to more than a few thousands) that are able to produce greater quantities of genetically even material (Fig. 15.12). A major example as regard to transgenic fish is that the tilapia expressing additional copies of trout growth hormone gene propagate much faster than their nontransgenic equivalents. Transgenic fish were found for their use to study increased growth rate/cold tolerance, developmental regulation of genes, and improved feed utilization. Faster-growing transgenic rainbow trout, salmon, tilapia, and medaka encompassing novel growth hormone genes acquire faster sexual maturity than their nontransgenic counterparts. Competence of feed conversion vis-à-vis growth can be increased in finfish by developing transgenic fish, which integrate a growth hormone—encoding gene construct. Genetically modified fishes were developed for biomedical research comprising organogenesis and embryogenesis other than in-depth study of diseases and producing therapeutic agents [138–140].

10.3 Development of disease resistance in mariculture

Advanced marine molecular biology offers valued evidence regarding mechanism of disease transmission, antibiotic resistance, and pathogenesis, which can improve the comprehension of resistance/host immunity and disease susceptibility. For example, high-density mariculture conditions set forth immense stress upon cultured shrimp/fish, making those

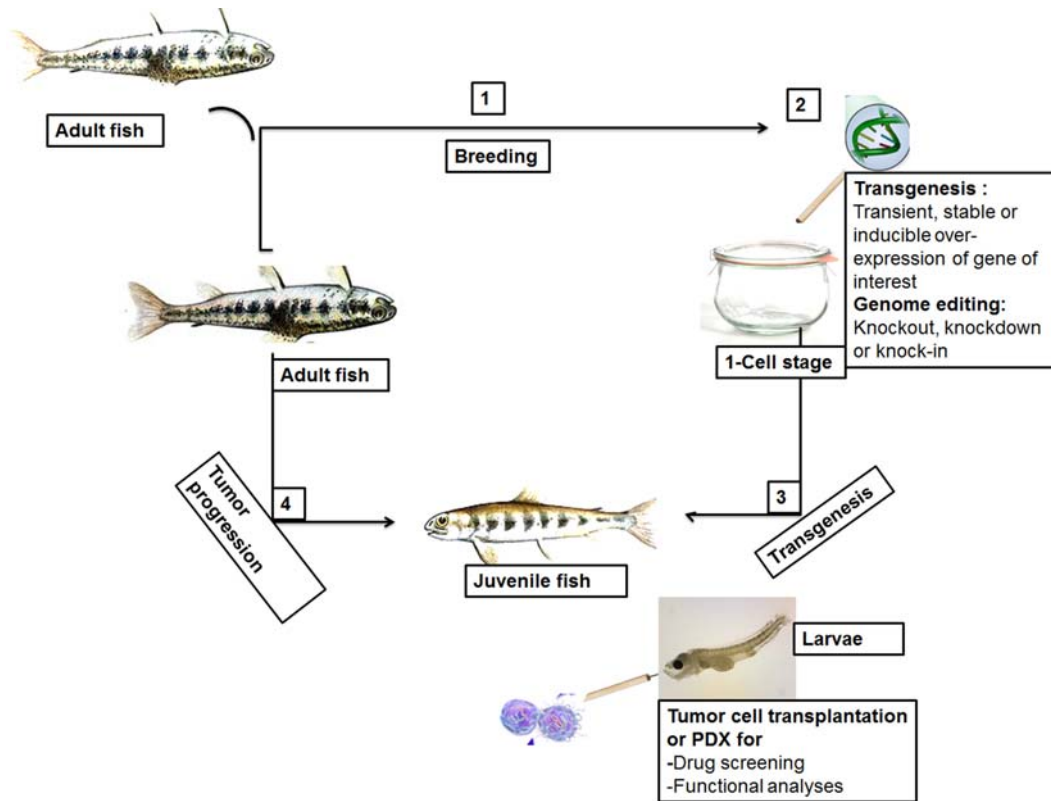


FIGURE 15.12 Transgenic fish.

exceedingly susceptible to pathogenic infection, causing enormous economic losses. This can be circumvented by fostering healthy fish/shrimp strains that can endure the diseases. More than a few transgenic methods were used to improve disease resistance ability in fishes. Viral RNA was destroyed by antisense and ribozyme technologies. Hematopoietic necrosis virus (HNV) causes serious mortalities in salmonids, and destroying this virus could improve their growth [141]. A different method is to express viral coat proteins (e.g., 66 kDa HNV/G-protein) in host membrane prompting the binding to receptor binding positions, and consequently, compete with binding sites of HNV, thereby neutralizing penetration of the virus. Reverse transcriptase polymerase chain reaction (RT-PCR) method could spot aquatic birnavirus, which forms the prevalent group within the family of Birnaviridae (encompassing viruses from several fish and invertebrate species). RT-PCR assay is a rapid and consistent substitute for cell culture methods to detect fish disease agents (e.g., pancreatic necrosis virus). Researchers of the University of California have decoded the cause of an extremely infectious disease afflicting the white sturgeon mariculture and developed protocols for detecting the manifestation of the white sturgeon iridovirus by gene manipulation technique that could help in evolving disease-free breeding stocks [142].

10.4 Freeze-resistant fish

Recombinant methods can be used to transfer an antifreeze protein (AFP) gene to induce freeze resistance on marine fish species. AFPs inhibit the formation of ice crystal in the blood, and are produced by cold-water marine teleosts (e.g., ocean pout, winter flounder, shorthorn, and sculpin sea raven). Noticeably, several commercially significant fishes, such as Atlantic salmon, do not harbor *afp* genes, and in consequence, cannot withstand subzero temperatures. Developing transgenic Atlantic salmon by *afp* gene manipulations can be particularly productive. Transgenic gold fish with *afp* gene also survive in subzero temperatures [143].

10.5 Utility of advanced marine biotechnological tools to increase fish growth rate and reproduction

Genetic manipulations can considerably improve the fish growth rate. Microinjection of growth hormone genes into fertilized salmon eggs could substantially accelerate their growth rates (Fig. 15.13). Placing an additional copy of growth hormone gene in tilapia embryo was found to enhance its growth rate. Nuclei of cells of fertile goldfish kidney were

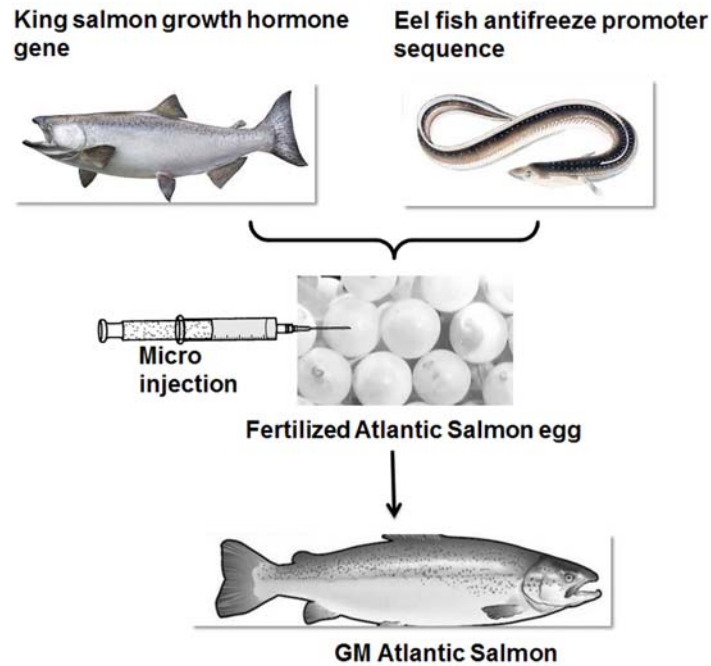


FIGURE 15.13 Improving growth rate using biotechnological tools. *GM*, genetically modified

reprogrammed in enucleated eggs to upkeep its ontogenesis/embryogenesis, which has been the first instance of cloned fish with somatic cells. By microinjection method, recombinant *MThGH* gene was transmitted into fish eggs to develop the first batch of transgenic fish. The *MThGH*-transmitted carp improved growth rate by more than three times in founder juveniles and doubled the body weight in adults. Transgenic carps exhibited superior efficiency to utilize dietary protein than the untreated counterparts. An “all-fish” gene assemblage, *CAGcGH*, was developed by splicing the β -actin gene promoter of common carp on grass carp *gcGH* (growth hormone gene) coding structure. The *CAGcGH*-transmitted Yellow River carps have displayed considerably faster growth attribute. Amalgamation of methods of fish cell culture coupled with gene transformation would produce high-value transgenic fish to realize the human necessity during the 21st century [144,145].

Reproduction is the most important issue for fish aquaculture. Growth rate and quality of the fish flesh depreciates with progressive fish maturity. Advanced biotechnological methods to decelerate the maturity could be usefully exercised to maintain the fish flesh quality. Reproduction of fish can be regulated by producing sterile species, which have immense commercial potential, since sterilized species bring forth no possibility of farm–wild interface. Modified retroviral vectors were developed to modify the gene of marine invertebrate, which constitutes the first application of molecular biology to alter DNA in marine organism. A viral envelope was used to genetically modify the surf clam, thereby permitting the vector to enter in any cell. Other marine biotechnological developments comprise the development of an engineered “reporter gene,” which could prompt the fertilized clam egg to develop a bluish coloration indicative of gene implantation. Electroporation techniques are successful to insert foreign DNA into fish embryos.

Researchers at the University of Minnesota have effectively used genetic insulator systems in fish, and could discover gene regulators, which efficiently function to turn on foreign genes. AquAdvantage salmon with growth hormone gene transferred from Chinook salmon was turned out to be the first instance of genetically engineered fish, which has been permitted for commercial practice. AquAdvantage salmon was demonstrated as safe to consume similar to nongenetically engineered Atlantic salmon, by the USFDA as early as 2015. Subsequently, Canada has also permitted its commercial use in the very next year (2016). Contingent upon the structural variation and bioactivities, various chemical analogues were designed and developed, wherein salmon GnRH analogue (under the name of Ovaprim) is being abundantly used for induced breeding of fish and marketed commercially all over the world [146].

11. Marine biotechnological conservation

Genetic and advanced genomic tools are used to characterize aquatic germplasm comprising various threatened species other than comprehending molecular origin for gene regulation and expression. This also advanced the methods to define

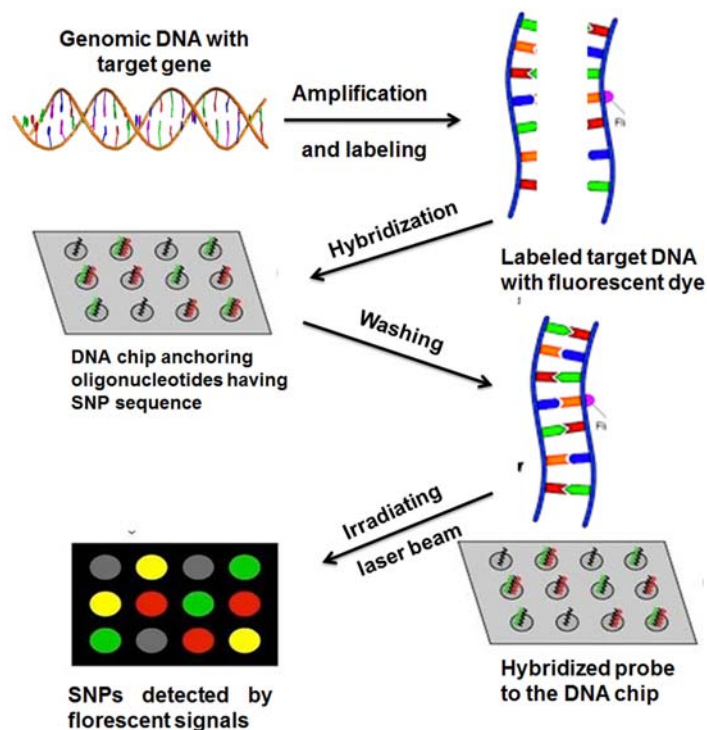


FIGURE 15.14 DNA fingerprinting to know polymorphism in fish stocks. *SNPs*, single nucleotide polymorphisms.

species and stock boundaries, which are necessary for assessing marine fish stocks and traceability and ensuring sustainability [147]. Next-generation sequencing methods are used for transcriptome/complete genome sequencing, characterizing single nucleotide polymorphism and marine fish resources (Fig. 15.14). Marker-aided selection is an indirect selection wherein a trait is selected based on a marker (biochemical/morphological/difference in DNA/RNA) connected to a trait (disease resistance/productivity/quality/abiotic stress tolerance), instead of the trait itself [148]. Development of transgenics delivers methods to quickly bring together new/modified genes/DNA sequences into fish species without hybridizing/cross-breeding. It is a more accurate procedure, but not essentially the same as cross-breeding and genetic selection [149,150]. DNA-level markers find applications in marine fisheries research and mariculture [151].

Cryopreservation is the capability to freeze-thaw while maintaining viability of the material, and gametes/embryos are held in vitro as frozen tissue are stored indefinitely in liquid nitrogen (at -196°C). Biotechnological tools can be used to develop hormonal protocols, which control the spawning of economically important fish (striped bass, Atlantic salmon, flounder, sea bass, sea bream, and other marine tropical species) [152].

12. In vitro fish flesh production system

Science for production of factory fish meat is an extension of the specialized area of biotechnology, termed as tissue engineering. In vitro fish produced has by no means a part of the living animal and could be a safe and ecofriendly substitute to slaughtered fish flesh. This process comprises large-scale culturing of muscle tissue in liquid medium in controlled conditions. Cultured meat forms part of the specialized field of cellular mariculture (Fig. 15.15) [153]. Diverse models to produce cultured meat using methods to produce media and cleaning phases were studied in detail [154]. The companies BlueNalu and Finless Foods produce and market factory fish products from bluefin tuna in a limited manner.

13. Publications and patents in the subject area of marine biotechnology

Research on marine biotechnology-related areas were remarkably high during the period of 1985–2021. Prominent journals of marine biotechnology are *Journal of Ecosystem and Ecography*; *Journal of Biodiversity*; *Chemistry and Biodiversity*, *Marine Natural Products*, *Marine Biotechnology*; international journals focusing on marine genomics; *Molecular Biology and Biotechnology*; *Journal of the Marine Biological Association of the United Kingdom*; *Marine*

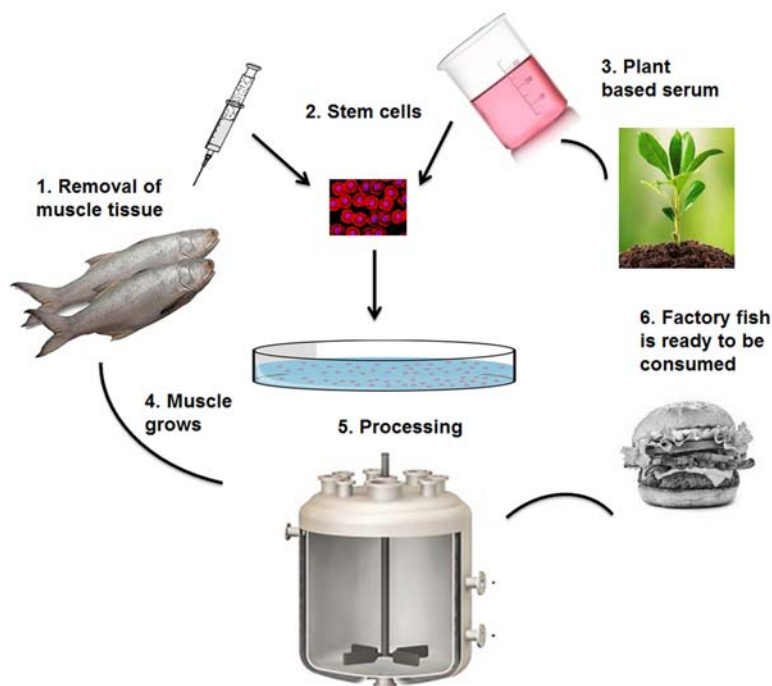


FIGURE 15.15 Illustrative representation of production of factory fish.

Chemistry; Bioorganic Chemistry; Marine Biology; Ocean and Coastal Management Indian Journal of Fisheries; Deep Sea Research; Aquaculture Research; Maritime Studies; Estuarine Coastal and Shelf Science; Marine Ecology Progress Series; ICES Journal of Marine Science, etc. An exponential growth in the publications in the area of marine biotechnology was apparent during 2016–2021 as compared to that in the previous decade.

Investigation into the filed/granted patents on marine biotechnology presented a considerable increase throughout the years. A comparative study from 1985 to 2021 was conducted. The number of patents witnessed a rapid surge after 2015. An increasing interest in marine biotechnological research as evidenced by the scientific publications and patents in the last decade has appropriately demonstrated the possibilities of advanced research and development in this frontier area. Considerable numbers of granted patents during 1995–2019 (retrieved from Google patents, accessed on September 26, 2021) recognized the increasing importance of marine biotechnological research. The number of granted patents was found to be 2% in between the years 1985 and 1990, and was increased to more than 30% during 2010–15, and further increased to more than 50% during the year 2016–21 (Figure 15.16).

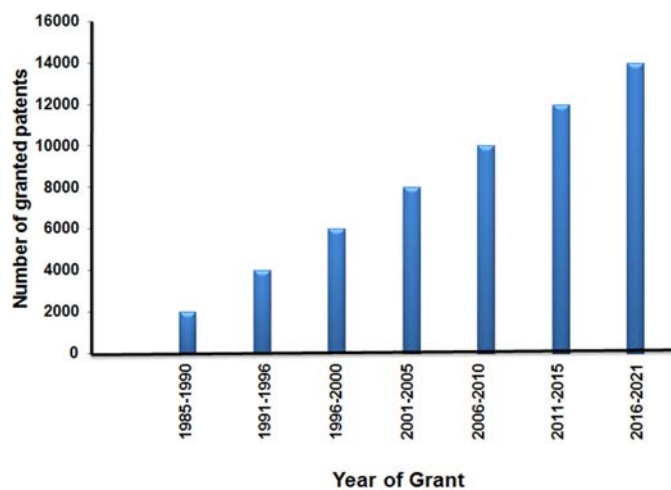


FIGURE 15.16 Graphical representation of the patents granted in the subject area of “marine biotechnology” during 1985–2021.

14. Leading countries undertaking marine biotechnological research

A recent market research report estimates that the global market for products resulting from marine biotechnology might surpass \$15 billion by 2021, and of that marine biomaterials (comprising seaweed hydrocolloids) could contribute more than 40%, wherein marine bioactives for health care would be the most significant and fastest growing sector. The size of this, albeit it is an overestimate, puts forward that the harnessing of marine bioresources through biotechnology and development of products and services should be an important target for any country with significant aquatic biodiversity. The report noted that very few countries have national marine biotechnology R&D programs; and it has also identified the United States as the world leader in the marine biotechnological research and development (Fig. 15.17, Table 15.6). That marine bioresources can give rise to specific molecules of incredible use or potential for human medicine is

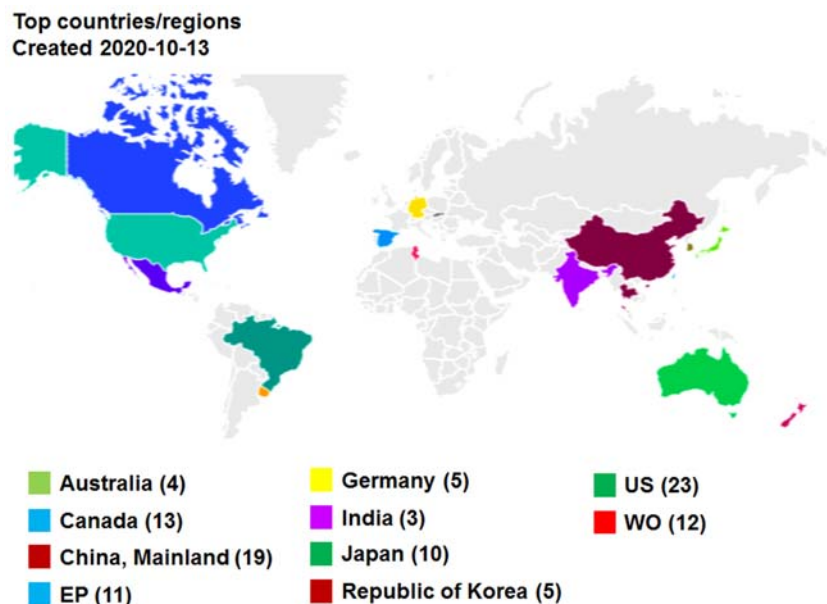


FIGURE 15.17 Geographical distribution of top countries undertaking marine biotechnological research (www.derwent.com).

TABLE 15.6 Examples of marine by-products, which are currently in market or clinical phases.

Product	Source	Application area	Status	References
Ara-A	Marine sponge	Antiviral	Market	[156]
Okadaic acid	Dinoflagellates	Molecular probe	Market	[157]
Manoalide	Marine sponge	Molecular probe	Market	[158]
Vent TM DNA polymerase	Ocean hydrothermal vent bacterium	PCR enzymes	Market	[159]
Aequorin	Bioluminescent jellyfish	Bioluminescent Ca indicator	Market	[160]
Green fluorescent protein	Bioluminescent jellyfish <i>Aequorea victoria</i>	Reporter gene	Market	[161]
Phycocyanin	Red algae	Conjugated antibodies	Market	[162]
Cephalosporins	<i>Cephalosporium</i> sp., marine fungi	Antibiotic	Market	[163]
Yondelis TM	Sea squirt	Cancer	Phase III	[164]
Ziconotide	Cone snail	Chronic pain	Phase III	[165]
Dolastatin	Sea slug	Cancer	Phase II	[166]
Squalamine lactate	Shark	Cancer	Phase II	[167]

TABLE 15.6 Examples of marine by-products, which are currently in market or clinical phases.—cont'd

Product	Source	Application area	Status	References
PL52602 (steroid)	Sponge	Inflammation, asthma	Phase II	[168]
Brentuximab vedotin	Cyanobacteria	Cancer	Market	[169]
Soblidotin (TZT-1027)	Bacterium	Cancer	Phase III	[170]
Tasidotin, synthadotin	Bacterium	Cancer	Phase II	[171]
Polatuzumab vedotin	Cyanobacteria	Non-Hodgkin lymphoma	Phase I	[123]
Salinosporamide A	Bacterium	Cancer	Preclinical	[172]
Chrysopaentin A	<i>Halobacillus salinus</i>	Bacterial infection	Preclinical	[173]
Phenethylamine	Bacterium, lyngbyoic acid	Bacterial infection	Preclinical	[174]
Grassystatins A–C	Bacteria	Immunity	Preclinical	[175]
Pulicatin A	Bacteria	Nervous system	Preclinical	[176]
Omega-3 oil	<i>Schizochytrium</i> sp.	Nutraceuticals	Market	[177]
Omega-3 supplement	<i>Schizochytrium</i> sp.	Nutraceuticals	Market	[177]
Astaxanthin	<i>Spirulina platensis</i>	Carotenoids	Market	[177]
Multivitamin	<i>S. platensis</i>	Nutraceuticals	Market	[177]
β-Carotene	<i>Dunaliella salina</i>	Nutraceuticals	Market	[178]
Phytoplankton powder	Marine phytoplankton	Nutraceuticals	Market	[178]
Assorted algae pastes	<i>Tetraselmis</i> , <i>Nannochloropsis</i> , <i>Isochrysis</i>	Nutraceuticals	Market	[178]
Marine phytoplankton	A blend of marine phytoplankton	Nutraceuticals	Market	[178]
Tasidotin-HCl	Sea hare	Cancer	Phase I	[179]
Spisulosine	Surf clam	Cancer	Phase I	[180]

unquestionable. There are now 4 clinically approved products, 13 in clinical trials, and a large number in preclinical investigation, originating from a wide range of marine organisms from many different parts of the world. Prialit (ziconotide), a painkiller originally isolated from a Pacific (Philippines) cone snail, trabectedin (Yondelis), an anticancer molecule from a Caribbean tunicate *Ecteinascidia turbinata*, and anabaseine (DMXBA) from the ribbon worm *Paranemertes peregrina*, from the Pacific Rim, are few examples [155].

15. Marine microorganism: a potential biofactory to derive high-value compounds

Marine ecosystem encompasses a wide range of habitats and environmental conditions, which host an enormous microbial biodiversity. Marine free-living/heterotrophic bacteria, fungi, and fungus-like protists (thraustochytrids) are among the marine organisms with the maximum potential to produce bioactive compounds that can be used for commercial uses, together with pharmaceutical/nutraceutical and cosmeceutical applications. The details of bioactive compounds and microbial products isolated from microorganisms and their applications are depicted in Table 15.7 [181,182].

TABLE 15.7 Bioactive compounds and microbial products isolated from microorganisms and their applications.

Microbial products	Microbial sources	Applications	References
Antibiotics Polyketides, cephalosporins, quinolones, lincomycins, macrolides, sulfonamides, aminoglycosides, carbapenems	<i>Bacillus</i> spp., <i>Saccharopolyspora</i> sp., <i>Amycolatopsis</i> sp., <i>Micromonospora</i> sp., <i>Fusidium</i> sp., <i>Pseudomonas</i> sp.	Treatment and control of bacterial, fungal, and protozoal infections in humans and animals	[183,184]
Anticancer chemotherapeutics Actinomycin D, bleomycin,	<i>Streptoalloteichus hindustanus</i> , <i>Streptomyces peucetius</i> , <i>Sorangium cellulosum</i>	Treatment of cancers	[137,183]

Continued

TABLE 15.7 Bioactive compounds and microbial products isolated from microorganisms and their applications.—cont'd

Microbial products	Microbial sources	Applications	References
doxorubicin, mithramycin, streptozotocin, epothilones, etc.			
Cholesterol-lowering drugs Lovastatin, simvastatin, mevastatin, pravastatin	<i>Monascus ruber</i> , <i>Aspergillus terreus</i> , <i>Nocardia autotrophica</i> , <i>Penicillium citrinum</i>	In human health, for reducing cholesterol and treating cancer	[185]
Microbial enzymes Amylase, protease, lipase, pullulanase, cellulases, xylanase, glucoamylase, endoglucanase, endoxylanase, β -glucosidase, chitinase, pectinase	<i>Bacillus licheniformis</i> , <i>Bacillus stearothermophilus</i> , <i>Bacillus amyloliquefaciens</i> , <i>Clostridium</i> , <i>Thermotoga</i> , <i>Methanopyrus</i> , <i>Pyrococcus</i> , <i>Thermococcus</i> , <i>Halobacterium</i> , <i>Halobacillus</i> , <i>Penicillium</i>	In food, baking, dairy, biopharmaceuticals, feed, agriculture, paper, pulp, leather, textile, detergents, biofuels, chemical, cosmetics, and bioremediation industries	[186–188]
Biofuels Biodiesel, bioethanol, isoprenoid, butanol, fatty acids, hydrogen	<i>Clostridium acetobutylicum</i> , <i>Chlamydomonas reinhardtii</i> , <i>Escherichia coli</i> , <i>Saccharomyces cerevisiae</i> , <i>Zymomonas mobilis</i> , <i>Synechococcus elongatus</i> , <i>Chlorella protothecoides</i>	An ecofriendly alternative to conventional petroleum-based fuels	[189,190]
Microbial biomass protein, probiotics, and biopharmaceuticals Recombinant human insulin, other hormones, cytokines, growth factors, interleukins, interferons	<i>Spirulina</i> (<i>Arthrospira platensis</i> and <i>Arthrospira maxima</i>), <i>Bacillus amyloliquefaciens</i> , <i>S. cerevisiae</i> , <i>Chlorella</i> , <i>Dunaliella</i> , <i>Paecilomyces</i> , <i>Penicillium</i> , <i>Trichoderma</i> , <i>E. coli</i>	Nutritional supplement for human; feed additive Diabetes, immunological disorders, cancers, growth disorders; as bio-similars and biobetters	[191–193]
Antibacterial agent Cadalm TM antibacterial extract (Cadalm TM ABe)	<i>Bacillus</i> spp.	Cleansing wounds, promotes a moisture balanced environment	[53]
Bacterial polysaccharides and small molecular bioactives Hyaluron	<i>Lactobacillus</i> sp., <i>Geobacillus thermodenitrificans</i> , <i>B. amyloliquefaciens</i> , <i>Brevibacterium otitidis</i> , <i>Xanthomonas</i> sp., <i>Bacillus subtilis</i> , <i>Streptomyces virginia</i> , <i>Shewanella algae</i> , <i>Pseudomonas</i> sp., <i>Geobacillus tepidamans</i> , <i>Pseudoalteromonas</i> sp., <i>Alteromonas</i> sp.	Antiaging affect, cell therapy and tissue engineering, antiviral, immunostimulant, antimicrobial, gelling agent, hydrocolloid anti-inflammatory, antibacterial, face mask, anticancer, etc.	[193–202]

Even though marine microbial flora is known to be an attractive producer of diverse bioactive natural products, they are less characterized in comparison to the terrestrial microbial population. Marine microbial symbionts were found to be the true producers or active participant in the biosynthesis of active natural products [203,204]. Seaweed-associated bacterial flora was reported as potential sources to isolate valuable antimicrobial metabolites to control human food pathogenic strains [205,206]. Therefore, the potential of exploring the bioactive secondary metabolites from symbiotic microbial sources from seaweeds for commercial level exploitation is highly promising (Fig. 15.18).

16. Marine biotechnology and marine population dynamics

Population genetics is a subfield of genetics dealing with genetic differences within and between populations. Its primary founders were Wright, Haldane, and Fisher, who also put forth the fundamentals for the related discipline of quantitative genetics. What sets population genetics separately from phenotypic methods to modeling evolution, for example, adaptive dynamics/evolutionary theory, is its importance on genetic phenomena as epistasis, dominance, mutation, and genetic drift. This makes it appropriate for comparison to population genomics data [207]. Population geneticists typically define “evolution” as any alteration in a population’s genetic configuration over time. These factors that can bring about such a change are natural selection, mutation, random genetic drift, and migration into or out of the population. A fifth factor,

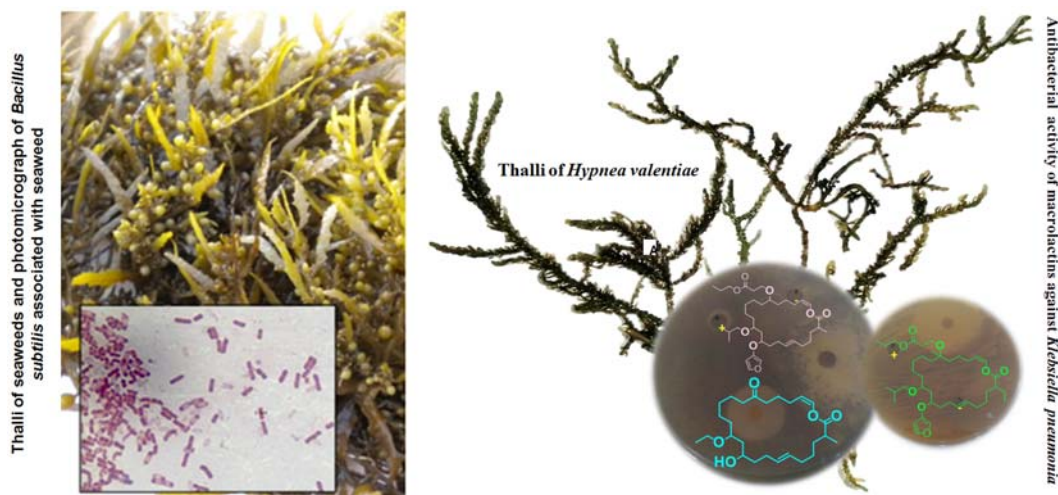


FIGURE 15.18 Seaweed-associated bacteria are novel sources of bioactive molecules.

changes to the mating pattern, can change the *genotype* but not the *allele* frequencies; many theorists would not consider this as an evolutionary change [207].

17. Advances in marine biotechnology in renewable energy sector

Recent marine biotechnological advances can alter natural resources, such as vegetable oils into clean-burning fuels. Biodiesel has manifold advantages owing to its easy biodegradability and high flash point with low volatility, thus causing less air pollution than the conventional diesel. The biodiesel emissions are considerably lesser than those from petroleum, and due to the absence of sulfur in biodiesel, no emission of sulfur dioxide is apparent. Marine microalgae display higher photosynthetic proficiency, production of greater biomass, and faster growth than those of other energy crops. Production of biodiesel from marine algae is developing as a high-value technology as production rates for algae are manifold than those of other energy crops, which are predominantly of terrestrial origin.

18. Marine biomaterials and nanoparticle conjugate from marine source: promising opportunities in clinical applications

These consist of a heterogeneous assembly of ceramic, organic, and polysaccharide-based polymers, which can be used for broad applications. Several microporous materials providing a structure for coral reefs or those forming spine of sea urchins can be used as starting material. By reason of the porous systems and geometric configurations, biomaterials are used for tissue scaffold, constructing vascular graft and orthopedic cares. Biocompatible and implantable biomedical devices built on storage biopolymers are also promising research subjects in marine biotechnological advances. High-value chitin-based biomedical products are promising biomaterials to develop for drug-delivery agents. Chitin and chitosan biopolymers contribute as a base to develop hydrogel-like hosts for various biomedical ingredients and formulations. Storage biopolymers, predominantly those resulting from polyhydroxybutyrates combined with coral residues, can be used to develop biomaterial scaffolds for bone repair.

Nanomedicine and nanodelivery systems are a comparatively new but fast-developing science, where materials in the nanoscale range are used to serve as means of diagnostic tools or to deliver therapeutic agents to specific targeted sites in controlled mode. Nanotechnology offers multiple benefits in treating chronic human diseases by precise target-oriented delivery of medicines. Lately, there are a number of remarkable applications of nanomedicine in the treatment of various diseases. Noticeably, drugs with very low solubility possess various biopharmaceutical delivery issues comprising limited bioaccessibility and lesser membrane diffusion capacity, thus requiring more quantity for intravenous intake and unwanted aftereffects preceding traditional formulated vaccination process. However, these limitations could be overcome by application of nanotechnology approaches in drug delivery mechanism. Seaweed-derived polysaccharide biopolymer-based nanoparticles with their size and drug release potentials have appeared as promising biomaterials for osteogenic differentiation. A metallic nanoparticle (GS-AgNP) prepared from a sulfated polygalactan characterized as $[\rightarrow 3)-2-O-$

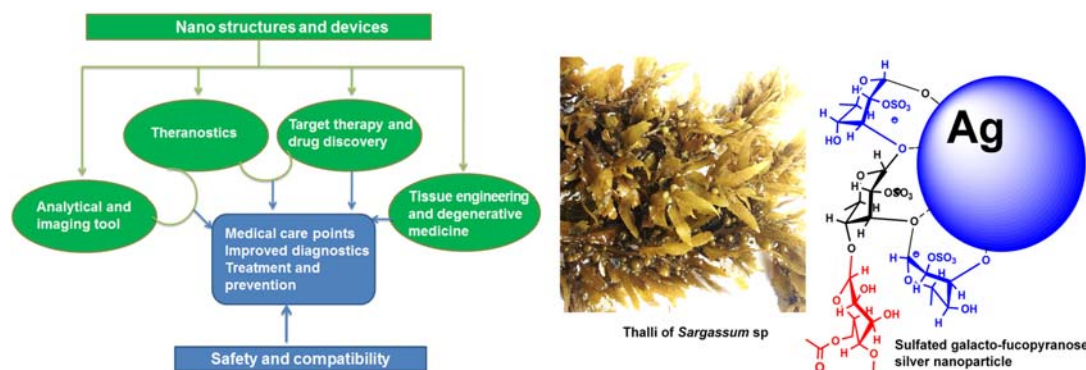


FIGURE 15.19 Application of nanotechnology in human health and well-being; seaweed-based nanoparticle structured of sulfated polygalactofucopyranose demonstrated potential activities associated with osteogenesis.

methyl-*O*-6-sulfonato- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-2-*O*-methyl-3,6-anhydro- α -D-galactopyranose-(1 $\rightarrow$) isolated from a marine macroalga *Gracilaria salicornia* exhibited prospective osteogenic effect [69]. A polygalacto-fucopyranose biopolymer-structured nanoparticle conjugate developed from seaweed *Sargassum* sp. was found to attenuate glucocorticoid-induced osteoporosis (Fig. 15.19) [208]. Recently, there has been enormous developments in the field of delivery systems to provide therapeutic agents or nature-based active compounds to its target location for treatment of various ailments. With the progression of nanomedicine, due to the advancement of drug discovery/design and drug delivery systems, numerous therapeutic procedures have been proposed and traditional clinical diagnostic methods have been studied, to increase the drug specificity and diagnostic accuracy. There are various biopolymeric materials, such as chitosan, alginate, cellulose, liposomes, polymeric micelles, nanocrystals, and quantum dots, which are utilized in the drug delivery systems.

19. Conclusions

Advanced marine biotechnological interventions offer enormous possibilities to harvest the hidden potential of the valuable oceanic resources to meet the sustainable development goals. Modern genomic tools aid to characterize and document the biodiversity of the oceanic ecosystems, identifying stock boundaries, perfecting traceability of capture fish and maricultured products, and recognizing adaptive loci to understand changes related with climatic variations. Bioprospecting for novel bioactives and genes accountable to produce those could be worked out using biotechnological tools. Marine biotechnology in pharmaceuticals and functional food applications is an emerging sector that is globally encouraged by several policies. Augmenting mariculture productivity can be performed by selective breeding techniques with advanced biotechnological tools, considering the interaction between genes, which leads to sustainable mariculture. Marine biotechnological tools are appreciated for pollutant biomonitoring in the deep sea. Marine biotechnology—built omics and pharmacology analysis are fundamental tools to discover novel substances and organisms as candidates for commercial applications. Notwithstanding the advances in marine biotechnology, challenges persist with regard to improved cultivation techniques for some marine organisms. There is further need to carry out advanced marine biotechnological research, even though enormous steps have been taken throughout the last few decades. The genuine challenge is to abridge the marine biotechnological findings and their commercial applications to benefit the mankind.

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Seaweed biotechnology implications to aquaculture

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1. Introduction

Biotechnology industry includes the major sectors such as biopharma, industrial biotechnology, agricultural biotechnology, food biotechnology, environmental biotechnology, and bioinformatics [1]. Among these, biopharma, industrial biotechnology, and food biotechnology contributed major share to biotechnology industries. The worldwide biotechnology sector has been found to grow at 1.3% between 2015 and 2020. It is expected that this domain will achieve a steady increase in the next 5 years and that more investments will be made in research and development (R&D) [2]. In the year 2019, the world biotechnology market size was about USD 295 billion, and the market size is expected to reach USD 2.44 trillion by 2028 [1,3]. Nevertheless, the share of marine biotechnology component has reached USD 3 billion in 2020, and it is expected to reach USD 5.4 billion by 2027, growing at a compound annual growth rate of 5.8% [4].

The global capture fisheries production has registered the production at 96.4 million tonnes (corresponding to 5.4% growth from the previous 3-year average). Furthermore, aquaculture is related to the farming of aquatic species, which includes molluscs, crustaceans, and finfish, along with aquatic plants (commonly algae), in either freshwater, brackish water, or open sea (including inland saline water). The total production encompasses 82.1 million tonnes of aquatic animals, followed by 32.4 million tonnes of aquatic algae and 26,000 tonnes of ornamental seashells and pearls.

The world aquaculture production attained another all-time record high of 114.5 million tonnes in live weight in 2018, with a total farmgate sale value of USD 263.6 billion (the price of the product available at the farm, excluding any separately billed transport or delivery charge). Seaweeds are the only resources for the production of economically important phycocolloids such as agar, carrageenan, and alginic acid. Seaweeds have been utilized for centuries worldwide mainly for food purpose to the coastal communities especially in Asia and the Pacific region [5]. In 2018, 32.4 million tonnes of algae were produced, which included both wild-collected and cultivated biomass, and seaweed farming contributed about 97.1% of total aquatic algal production, [1] which showed of a trade value of USD 11.7 billion in 2016 and recorded 27.3% of the total world marine aquaculture production [6]. The world production of seaweeds has increased threefold in 2018, i.e., from 10.6 million tonnes in 2000 to 32.4 million tonnes in 2018. In the world scenario, about 221 species are having commercial utility and only 10 species are being commercially cultivated, and among these *Eucheuma* spp. (35%), *Laminaria japonica* (27%), *Gracilaria* spp. (13%), *Undaria pinnatifida* (8%), *Kappaphycus alvarezii* (6%), and *Porphyra* spp. (4%) have a major share in global seaweed biomass production [6]. Seaweed cultivation is providing alternative livelihoods for small-scale fishing communities in many tropical countries. Seaweed aquaculture is being extended in tropical waters. The repeated vegetative propagation of the same plants has led to a loss of genetic diversity and resulted in slow growth, reduction in phycocolloid yield with poor quality of phycocolloid, and susceptibility to disease [7]. Periodic replacement of propagules with new stocks (various strains, color, and physical morphotypes) as sources of invigorated plantlets was necessary to prevent the deteriorating agronomic traits of the seedlings used at that time. Production and selection of new seaweed strains could improve the synthesis of natural products with different biological activities, as well as the commercial cultivation of seaweeds via the utilization of higher yield strains. Marine or blue biotechnology techniques such as tissue culture/micropropagation, protoplast isolation and fusion, hybridization, gene

transfer, grafting, utilizing gene marker, parthenocarpy, chimerism, chemical and naturally induced mutants, and haploid spore have drawn a great interest in the last few decades for developing high yield and fast-growing strains with improved qualities and diseases-free seed stock. Marine biotechnology or blue biotechnology for industrial applications has the potential to reach USD 4.8 billion by 2020, rising to USD 6.4 billion by 2025 [8]. India is among the top 12 destinations for biotechnology worldwide. Currently, biotechnology industry makes up just 3% of the overall global biotechnology market (USD 11.1 billion), which is expected to go up to 19% by 2025 (USD 100 billion), about one-fifth of the world's biotechnology sector. The biotechnology industry in India comprises >2700 biotechnology start-ups and is estimated to reach 10,000 by 2024, which aims to provide high-quality drugs, better diagnosis, and MedTec products available at reasonable price (<https://www.blalbiotech.com/blog/top-10-biotechnology-startups-in-india/>) [9]. The marine biotechnology industry also has essential role to play in aquaculture sector. The utilization of biotechnological tools and techniques for enhancing the aquaculture production will be the determining step for boosting global aquaculture. These techniques provide the means to increase the operation intensity as well as capacity. The Southeast Asian Fisheries Development Center proposed tissue culture methodologies for developing fast-growing strain in Philippines [10]. In this chapter, each biotechnological technique for strain improvement for high-quality product and growth rate for aquaculture purpose will be discussed.

2. Protoplast isolation and fusion

Protoplasts are useful tools in genetic manipulation and hybridization. They also have considerable importance in polysaccharide synthesis. In seaweeds, protoplast production is mainly associated with selection and propagation of desired strains as seed material for breeding and cultivation [11]. Protoplast isolation from macrophytic benthic marine algae was reported as early as 1970 using mechanical methods [12–14]. However, the enzymatic method developed by Millner et al. [15] for *Enteromorpha intestinalis* resulted in success in producing a large number of viable protoplasts and became popular only after the plantlet regeneration from the same species was reported by Rusing and Cosson [16]. The details of report on isolation and culture of protoplasts of various seaweeds are listed in Table 16.1.

TABLE 16.1 Protoplast isolation and fusion studies in seaweeds.

S. No	Plant name	Regeneration	References
Green algae			
1.	<i>Enteromorpha intestinalis</i>	Plantlet regeneration	Millner et al. [15], Rusing and Cosson [16]
2.	<i>Enteromorpha</i> spp.		Reddy and Fujita [17]
3.	<i>Enteromorpha</i> spp.		Reddy et al. [18]
4.	<i>Ulva</i> spp.		Reddy and Seth [19]
5.	<i>Ulva reticulata</i>		Vishal et al. [20]
6.	<i>Ulva</i> spp.		Reddy et al. [21]
7.	<i>Monostroma oxyspermum</i>		Kumar et al. [22], Reddy et al. [18]
Red algae			
8.	<i>Caloglossa leprieurii</i>		Zhou and Wang [23]
9.	<i>Gracilaria asiatica</i>	Successful plant regeneration was observed	Bjork et al. [24], Cheney et al. [25], Cheney [26], Yan and Wang [27]
10.	<i>Gracilaria changii</i>	Successful plant regeneration was observed	Yeong et al. [28]

TABLE 16.1 Protoplast isolation and fusion studies in seaweeds.—cont'd

S. No	Plant name	Regeneration	References
11.	<i>Gracilaria chilensis</i>	Successful plant regeneration was observed	Chenny [26]
12.	<i>Gracilaria chorda</i>		Chou and Lu [29]
13.	<i>Gracilaria gigas</i>		Chou and Lu [29]
14.	<i>Gracilaria lemaneiformis</i>		Chou and Lu [29]
15.	<i>Gracilaria salicornia</i>		Chou and Lu [29]
16.	<i>Gracilaria sordida</i>		Chou and Lu [29]
17.	<i>Grateloupia sparsa</i>		Chen and Chiang [30]
18.	<i>Grateloupia filicina</i>		Chen and Chiang [30]
19.	<i>Gracilaria tikvahiae</i>		Chou and Lu [29]
20.	<i>Gracilaria tenuistipitata</i>		Chou and Lu [29]
21.	<i>Gracilaria verrucosa</i>		Araki et al. [31], Bjork et al. [24], Mollet et al. [32]
22.	<i>Kappaphycus alvarezii</i>	Successful plant regeneration was observed	Salvador et al. [33]
23.	<i>Porphyra</i> spp.		Araki et al. [34], Chen [35], Chen and Chiang [30], Chen et al. [36], Chen and Chiang, 1994 [30], Fujita and Migita [37], Polne-Fuller and Gibor [38], Waaland and Dickson [39], Waaland et al. [40], Wang et al. [41]
24.	<i>Porphyra haitanensis</i>	Regeneration of callus/filaments and thallus from protoplast culture	Yan and Wang [42]
25.	<i>Porphyra lanceolata</i>		Polne-Fuller and Gibor [43]
26.	<i>Porphyra leucosticta</i>		Chen [35]
27.	<i>Porphyra linearis</i>		Chen and Chiang [30]
28.	<i>Porphyra nereocystis</i>		Polne-Fuller and Gibo [43]; Waaland and Dickson [39], Waaland et al. [40]
29.	<i>Porphyra okhaenesis</i>		Shikh et al. [44]
30.	<i>Porphyra perforata</i>		Polne-Fuller and Gibor [38], [43]
31.	<i>Porphyra schizophylla</i>		Polne-Fuller and Gibor [43]
32.	<i>P. haitanensis</i>	Only thallus was regenerated	Wang et al. [41]
33.	<i>Porphyra tenera</i>		Araki et al. [34]

Continued

TABLE 16.1 Protoplast isolation and fusion studies in seaweeds.—cont'd

S. No	Plant name	Regeneration	References
34.	<i>Porphyra yezoensis</i>		Araki et al. [34], Fujita and Migita [38]
35.	<i>P. haitanensis</i>	Genetic manipulation by means of heteroplasmic fusion	Dai et al. [45]
36.	<i>Porphyra pseudolinearis</i>		Fujita and Saito [46]
37.	<i>P. yezoensis</i>		Fujita and Migita [47] and Fujita and Saito [46]
Brown algae			
38.	<i>Laminaria japonica</i>	Regeneration of plantlets from protoplasts	Inoue et al. [48], Saga and Sakai [49], Sawabe et al. [50], Sawabe and Ezura [51], Tokuda and Kawashima [52], Matsumura et al. [53]
39.	<i>Laminaria saccharina</i>		Butler et al. [54]
40.	<i>Laminaria digitata</i>		Butler et al. [54]
41.	<i>Macrocystis pyrifera</i>	Regeneration of plantlets from protoplasts	Kloareg et al. [55]
42.	<i>Undaria pinnatifida</i>		Tokuda and Kawashima [52]

Genetic manipulation by means of heteroplasmic fusion was conducted in *Porphyra haitanensis* by Dai et al. [45], *Porphyra pseudolinearis* by Fujita and Saito [46], and *Porphyra yezoensis* by Fujita and Migita [47] and Fujita and Saito [46]. Establishment of suspension culture was reported in *Porphyra linearis* [56], and suspension culture of *Porphyra lanceolata*, *Porphyra nereocystis*, *Porphyra perforate*, and *Porphyra schizophylla* was obtained [43]. Tait et al. [57] have adopted a method involving enzymic treatment and homogenization of fresh tissue to generate successfully cell lines and suspension culture from *Porphyra umbilicalis*. Establishment of suspension cultures would be of advantage for the production of high-cost compounds such as polysaccharides, vitamins, and bioflavors [58,59].

Protoplast isolations from *K. alvarezii* were made with the purpose of improving the genetic characteristics of this species as a source of propagules for commercial cultivation [60]. Digestions with cellulase and *kappa*-carrageenase produced only a few cortical cell protoplasts, while digestions with cellulase and *iota*-carrageenase only produced epidermal cell protoplasts. When both carrageenases were used in the digestion media with cellulase, protoplasts were released from all cell types and yields ranged from 1.0 to 1.2×10^7 cells g^{-1} with sizes from 5 to 200 μm diameter. No regeneration of plantlets was observed while protoplasts were subsequently cultured for cell wall regeneration [60].

Tait et al. [57] isolated a range of cell lines from *P. umbilicalis* tissue protoplasts. Culture of isolated cell lines grown as undifferentiated aggregates formed 4–5 mm small thalli on the surface of aggregates on solid medium. In liquid culture, they reached 0.1–5 mm diameter aggregates and maintained for periods in excess of 1 year without reverting to the foliose growth form. While determining polysaccharide products in the cell lines developed from protoplasts of *P. umbilicalis* tissue, it was found that they were structurally very similar to the native plant's polysaccharides.

3. Chemicals

Chemicals such as ethyl methanesulfonate (EMS) and methyl methanesulfonate esters of methane sulfonic acid and N-methyl-N'-nitro-N-nitrosoguanidine (NNG) are efficient for mutant formation in seaweeds [61,62]. NNG yielded mutants in *Porphyra* thalli, whereas EMS treatments did not yield any mutants. NNG of $25 \mu g mL^{-1}$ for 30 min at $23^\circ C$ treatment on small *Gelidium* sporelings produced approximately 10–15,070 of the surviving sporelings, mutant for color and morphology and low frequency of plant death, and it was found sufficient to provide very high yields of mutant [61,62]. Female sterile mutant of *Gracilaria tikvahiae* when treated with EMS displayed altered branching patterns and

thallus thickness, potentially due to the modification of the size of the medullary and subcortical cells of the algal thallus [63]. Among the generated mutants, i.e., MP-40, MP-44, MP-48, MP-64, MP-42, MP-46, MP-61, and MP-70, MP-40 mutant displayed a partly dominant mutation and other mutants were single locus and recessive. The agar properties also differed among generated mutants in *G. tikvahiae*. Gel strength of agar was higher in the MP-40 mutant, but lower in the MP-61 mutant [63]. The MP-40 mutant showed faster growth rate than the wild type even at high densities and more resistance to epiphytic colonization. It also fixed ammonium rapidly from seawater than other mutants [63].

van der Meer and Zhang [64] reported on induced mutations in germinating tetraspores and small sporelings of *G. tikvahiae*, *Gracilaria sjoestedtii*, and *Gracilaria foliifera* by treating with EMS. Unstable mutants with similar variegated pigmentation were genetically characterized in all three *Gracilaria* species. All three mutants were green plants with spot of red tissue and reverted to wild type due to their genetic behavior, i.e., a single, unstable, nuclear gene. The mutant green phenotypes were all recessive.

Guzmán-Uríostegui et al. [65] stated that when *Gracilaria cornea* was treated with exogenous polyamine spermine, it induced cystocarp maturation, promoted carpospore liberation, and developed cell masses within 4–7 days. Similar observation was also made in *Grateloupia*, where spermine induced the development of cystocarps and promoted liberation, growth, and development of spores [66,67].

4. Chimerism

Chimerism is a typical phenomenon that was formed by fusion of two or more genetically distinct conspecific individuals, and successful chimerism occurred between close relatives species [68]. When two conspecific seaweed germlings were grown closed together, they established secondary pit connections between adjacent cells and a common external cell wall is formed around the contacting discs. Thus, the fusion process between compatible partners has occurred, and the resulted individual was genetically polymorphic chimeric organism [69]. Chimerism has been documented for several algae, as given in Table 16.2.

Chimeric individuals were also obtained from the fusion of genetically different protoplasts. Blades consisting of green and red groups of cells were developed from the fusion products of green (mutant type) and red (wild type) protoplasts of *P. yezoensis* [46,47]. Protoplast fusions between other *Porphyra* species such as *P. yezoensis* and *P. pseudolinearis* resulted in

TABLE 16.2 List of seaweeds reported for chimerism.

S.No	Plant name	Chimerism	References
Red algae			
1.	<i>Chondrus</i> sp.		Santelices [70]
2.	<i>Gracilaria tikvahiae</i>	Bicolor chimeric plants	Cheney [26]
3.	<i>Gracilaria chilensis</i>	Bicolor chimeric plants	Cheney [26], Santelices et al. [69], [68]
4.	<i>Mazzaella laminarioides</i>		Santelices et al. [68]
5.	<i>Porphyra yezoensis</i>	Fusion of genetically different protoplasts	Fujita and Saito [46]
6.	<i>Porphyra pseudolinearis</i>	Protoplast fusion	Fujita and Saito [46]
7.	<i>Sarcothalia</i> sp.		Santelices [70]
Green algae			
8.	<i>Codium</i> sp.		Gonza'lez and Santelices [71]
Brown algae			
9.	<i>Ecklonia radiata</i>		Wernberg [72]
10.	<i>Lessonia berteroa</i>		Gonza'lez et al. [73], Segovia et al. [74]
11.	<i>Lessonia spicata</i>		Gonza'lez et al. [73], Araujo Casares and Faugeron [75]

plants forming mosaic chimeric plants with different color contours [46]. Protoplast fusion between two *Gracilaria* species such as *G. tikvahiae* (green pigmented) and *Gracilaria chilensis* (red pigmented) resulted in bicolor, red and green, chimeric plants [26]. In *Mazzaella laminarioides*, chimeric tissues (mixes of green and red cells) were found in 70%–75% of the young primordial shorter than 5 mm long. Holdfasts, the most common site of somatic fusion, generally exhibited the highest allelic abundance and diversity [68]. The studies of *Gracilaria* and *Mazzaella* have shown that somatic fusion of genetically different conspecific individuals produced a chimeric holdfast [68]. As a result, the resulting axes may be genetically heterogeneous at their basal portions (the chimeric holdfast), but are mostly homogeneous in the more apical portions [68]. In natural populations, this type of holdfast can be considered a priceless in situ reservoir of natural genetic resources and variability. The frequency of chimerism in *Lessonia spicata* was ranged from 33.3% to 86.7% [75], 70%–90% [73], and 60% [74].

Coalescence and chimerism are evolutionary convergent processes observed in all three major groups of seaweeds [73] (González et al. 2014). The process of coalescence was documented in the orders Ahnfeltiales, Corallinales, Gigartinales, Gracilariales, Halymeniales, Palmariales, and Rhodymeniales of the division Rhodophyta [76]. Chimeric-type seaweeds originated by coalescence of genetically different plants, and it is reported in red seaweed genera *Gracilaria*, *Mazzaella*, *Chondrus*, and *Sarcothalia* [70]. Coalescence studies in red algae resulted in higher survival due to the larger size of the coalescent plants during early developmental stages. In later development stages, the coalescing plants form a thick canopy with more branches and greater productivity with greater number of fertile branches than noncoalescing plants [77].

Chimeric plants showed immediate increase in size, higher growth and survival rate, susceptibility to mortality factors, effective reproductive process, and great environment stress tolerance than nonchimeric plants [68,76–80]. In *L. spicata* from protected areas, higher reproductive success and reproductive efficiency were observed in chimeric plants than nonchimeric plants [75]. Field studies of the chimeric plants of *Gracilaria* and *Mazzaella* showed better survival [76,80], growth [76,81], reproduction [77], and environmental stress tolerance [79]. Segovia et al. [74] stated that higher occurrence of chimeric thalli of *Lessonia berteroana* tolerated high adverse conditions. Chimerism theoretically increases the genetic heterogeneity of coalescing organisms, which in turn may increase phenotypic variability in chimeras, allowing them greater tolerance to environmental changes when compared with noncoalescing individuals [79]. Chimeric thalli exhibited higher probability of producing offspring than nonchimeric thalli [75]. Wernberg [72] reported that chimeric plant in *Ecklonia radiata* improved resistance to wave action and increased survival rate in stressful environments.

Coalescing red algae showed unique pattern of postfusion growth, with the capacity to reverse chimerism. Chimeric individuals of coalescing may reverse chimerism in *M. laminarioides* and *G. chilensis*. Patterns of upright axes formation, the fate of the few chimeric branches arising from the chimeric holdfasts, and cell lineage segregation were the three important factors for reversing chimerism [68].

5. Grafting

Grafting is a simple, nonlaborious, and cost-effective protocol for vegetative propagation of seaweeds to improve the biomass yield, improve growth and quality of phycocolloid, withstand the adverse environmental conditions such as grazing, improve high wave actions, improve high water current, improve changes in wind velocity, improve seawater temperature, etc. [82]. Effect of grafting technique on different color strains of *K. alvarezii* from India was tested for its growth and quality of carrageenan [83]. Grafting was done in three color strains, i.e., red (R), brown (B), and green (G), to produce four intragenerically grafted plants, namely RB, RG, GB, and RGB. The physical attachment was observed after 21 and 25 days from the date of initial plantation in the GB and RGB, respectively, due to similar kinds of polysaccharides present in their thalli, which helped in attaching the corresponding mates. Daily growth rate (DGR) and biomass yields were improved considerably in grafted plants than ungrafted plants. Gel strength [82] of the kappa-carrageenan contents obtained from ungrafted plants was ranged from 270 to 350 g cm⁻², while in the grafted plants, it was in the range of 330–690 g cm⁻². Intergeneric grafting was also attempted in *K. alvarezii* (Ka), *Gelidiella acerosa* (Ga), *Gracilaria verrucosa* (Gv), and *Gracilaria dura* (Gd) with the combinations of Ka + Ga, Ka + Gv, Ka + Gd, Ga + Gv, and Gv + Gd. Among intergeneric grafted plants, only Ka + Ga was found attached after 29 days but not survived after 60 days. Intergeneric grafts did not survive due to their different anatomical, physiological, and chemical properties.

6. Haploid cell/spore culture

In seaweeds, obtaining haploid cultures is possible, and haploid cultures depend only on the type of explant (gametophyte) chosen. Thus, somaclonal variation in haploid cells could produce fully homozygous cells, which were readily employed for spontaneous chromosome doubling by colchicine treatment [84] and demonstrated and utilized for strain improvement programs in *Laminaria* [85,86], *Porphyra* [35,37,87], and *Monostroma* [87].

7. Parthenocarpy

Parthenogenetic spore germination was reported in three species of *Ulva* by modification of single cells into reproductive cells that discharge many spores [25]. Balar and Mantri [88] reported that unfertilized male and female gametes parthenocarpically grow into male and female plants, respectively, in *Ulva* spp.

8. Genetic transformation

Genetic transformation occurs at the cellular level and can be used to introduce trait-altering genes into the host genome. Transient transformation of the red alga *K. alvarezii* using particle bombardment was the first report in seaweeds [89]. Microparticle bombardment has been proven to be an efficient technique for transforming algae [90].

Wang et al. [91] developed a method for producing stable transformants by using SV40 promoter—driving lacZ gene into the cells of *K. alvarezii* through particle bombardment of epidermal and medullary cells at 650 psi at a distance of 6 cm to achieve transgenic breeding in *K. alvarezii* and also to eliminate chimeric expression. In another report, a transgenic *K. alvarezii* was successfully produced when a binary vector pMSH1-Lys carrying a chicken lysozyme (Lys) gene was transformed into *Agrobacterium tumefaciens* LBA4404 by triparental mating [92]. The percentage of pMSH1-Lys transformation on *K. alvarezii* was 23.5%, while the efficiency of regeneration was 11.3%. Polymerase chain reaction analysis showed that three of the regenerated thalli contained the lysozyme gene, which has the ability to break down the bacterial cell wall, a significant result in the prevention of “ice-ice” disease in *K. alvarezii*. Successful transformation by using SV40 promoter to express lacZ gene was also reported in *L. japonica*, *U. pinnatifida* [93], and *Gracilaria changii* [94].

Successful transformation of *Gracilaria gracilis* by using three different promoters including the SV40 promoter, the CMV promoter, and the CaMV 35S promoter expressed the lacZ reporter gene in *G. gracilis* transformed by particle bombardment, which helped to produce disease-resistant strain for suspended cultivation [95].

9. Genetic marker

Color-mutated variants, particularly green, are considered to be the most useful genetic markers because they are readily identified and many of them do not seriously affect the viability or growth of the plants [96]. Green strain can be used as the green marker whenever possible in crosses of *Palmaria palmata* and *Gelidium* sp. because it makes it easy to identify the F₁ tetrasporophytes [97,98]. A homozygous green tetrasporophyte is used as the source of green female gametophytes, and thus the only red plants that can develop are tetrasporophytes resulting from fertilization by the normally pigmented male in the cross. Without the green marker gene, small tetrasporophytes would be indistinguishable from small male plants developing in the same flask. All affect the plant pigmentation that happened in the chloroplast DNA. The transmission behavior of such nonnuclear markers confirms what was already suspected from ultrastructural studies on spermatia [99,100]. Unstable mutants with similar variegated pigmentation were genetically characterized in the red algae *G. tikvahiae* (McLachlan), *G. foliifera* (Forsk.) Bmg, and *G. sjostedtii* (Kylin) [64]. All three mutants were green plants with fleck of red tissue where cells had reverted to wild type. The mutant green phenotypes were all recessive, and their genetic behavior in crosses indicated that each was the result of a single, unstable, nuclear gene. Wild-type revertant tissue was stable once it arose. Revertant plants obtained from spores and revertant fronds taken from variegated plants could not be distinguished from the normal wild type, either phenotypically or genetically. Reversion to wild type occurred during all phases of the life cycle. In crosses between the mutants and wild type, most of the F₁ tetrasporophytes were heterozygous wild-type plants, an observation consistent with the recessive nature of the mutations; however, a low frequency of homozygous unstable green F₁ tetrasporophytes was also obtained from these crosses [64]. Few morphological mutants have been characterized, primarily at the phenotypic level. In compensation for their complex life cycles and sometimes challenging culture conditions, these mutants often displayed similar or increased reproductive capabilities in laboratory conditions compared to the wild types. Mutants were obtained either from sampling natural stocks or among offspring of mutagenized populations. In the brown alga *Ectocarpus siliculosus*, the natural immediate upright mutant showed a flaw in the early morphogenesis of the filamentous sporophyte, in which the prostrate basal body is reduced, thereby resembling a gametophyte with mainly upright filaments [101]. This phenotype is due to a single-locus mutation, emerging in the offspring population (gametophytes) from a wild-type sporophyte, probably by spontaneous random mutation.

10. Algal breeding

Among the commercial farmed seaweeds, only a few brown and red species have been subjected for algal breeding through hybridization. The first algal breeding that was successfully hybridized was *Saccharina* (*Laminaria*) done by Chinese scientists during the late 1950s and early 1960s. China pioneered the method of hybridization, considering that *Saccharina japonica* was an introduced species from Hokkaidō, Japan. This hybrid *Saccharina* created a few highly productive strains that were partially responsible for the increasing annual production in China. Researchers developed *Saccharina* (kombu strains) that tolerate high temperature and oligotrophic environments in gametophytic and young sporophytic stages. This was achieved by selection or intra-interspecific crosses using parental plants collected from various areas. The vegetatively propagated gametophytes that exhibited gametogenesis and healthy growth of young sporophytes at a higher temperature and oligotrophic conditions have been preserved. Among them, the hybrid of *Saccharina angustata* with *Saccharina longissima* grew at 17.5°C. The optimal temperature for gametogenesis in *S. longissima* was between 3 and 13°C [102]. In China, three types of hybrids from certified cultivars of *S. japonica* were obtained. Type I is produced by interspecific crossing [103,104]. Type II is produced by first crossing *S. japonica* and *S. longissima* and then by crossing the resultant offspring with *S. japonica* [105]. Type III is produced by crossing geographically isolated individuals of the same species [106]. In all three hybrid types, the sporophytic seedlings need to be produced by crossing vegetatively propagated gametophyte clones [107,108].

Hwang et al. [109] reported that *Undariopsis peterseniana* (male) and *U. pinnatifida* hybrid achieved a maximum blade length much greater than its parents. The hybrid continued growing even after the growth of *U. pinnatifida* ceased in unfavorable season. Thus, the hybrid provided a longer production period and greater biomass production. The successful work of Hwang et al. [110] on the hybridization of female *U. pinnatifida* and male *U. peterseniana* led to the extended period of availability of *Undaria* for abalone feed and cultivation in the Republic of Korea. Using free-living gametophyte seeding and standard on-growing techniques, the second generation (F₂) hybrids were found to have longer pinnate blades and narrower midribs than the first generation (F₁) hybrid and formed only sporophylls. The growth and morphology of F₂ hybrids originating from the sporophyll or sorus of the F₁ hybrids were not morphologically different from each other. Both of the F₂ hybrids exhibited late maturation, with the early stages of sporophylls appearing in April. In order to develop hybrids of *Undaria* for rapidly growing and high water temperature tolerance, reciprocal crosses were made between very early (N₁) or early (N₂) maturing indigenous individuals farmed in Naruto Strait, with an individual (Kn) collected in the sea of Ibusuki city in Kagoshima. The F₁ hybrids of N₁♀Kn♂ and N₂♀Kn♂ showed better blade growth (length and weight) than the parents [111].

An attempt to hybridize *K. alvarezii* and *Eucheuma denticulatum* was successful, as reported by Wang [112], using a somatic cell fusion method to produce non-filamentous hybrids.

Hybridization of *Pyropia* began in Korea in the 1980 [113–118] and genetic analysis started in the early 2000 [119–123]. Hybridization of *Pyropia* was conducted for developing fast-growing, temperature-tolerant cultivars that were rich in highly functional substances (e.g., breeding antioxidants, amino acids, vitamins) based on selection, hybridization, and mutation methods. Hybrid cultivars were derived from crossing *Pyropia dentata* with *Pyropia pseudolinearis* [115] and mutated cultivars were obtained by either mutagen induction [124] or radiation treatment [125,126]. A pure line is created by first isolating a single cell from young gametophytic blades of a selected strain of *Pyropia* [114]. In Japan, *Pyropia tenera* and *P. yezoensis* cultivars were then subjected to hybridization in various locations.

11. Tissue culture/micropropagation

Seaweed tissue culture is an important tool for micropropagation, as well as for the selection of high-yield strains or those with interesting commercial characteristics. Micropropagation or *in vitro* clonal propagation permitted the production of the large number of individuals within a short period and helped in sustainable production of raw materials of commercial interest [126,127]. These individuals can be used as seedlings for seaweed cultivation, rather than collecting them from natural beds. Seaweed tissue culture has been used to select and propagate genotypes that favor specific economic interests [127–129]. In 1978, first successful seaweed tissue culture was reported in *Chondrus crispus* Stackhouse (Rhodophyta) by Chen and Taylor [130], and they described the axenic culture of the medullar region of *C. crispus*. Two types of factors controlled tissue culture: (1) endogenous factors (related to explants characteristics such as origin, physiological state, stage of life history, age, etc.) and (2) exogenous factors (such as temperature, salinity, irradiance, photoperiod, chemical composition and pH of the culture medium, state of medium [liquid or solid], etc.).

Among the three groups of seaweeds, tissue culture/micropropagation studies were reported in 25 green algae, 51 red algae, and 36 brown algae [129]. However, successful plant regeneration was limited. Among 112 attempted seaweeds,

only 23 red algae and 17 brown algae showed complete regeneration (Table 16.3). Various media (artificial media to undefined natural seawater media) available for seaweed tissue culture are listed in Table 16.4.

In Philippines, 4-month-grown seedlings ($n = 5$) developed from tissues cultured from *E. denticulatum* were cultured at 0.50 m of depth and exhibited biomasses that ranged from 354.9 to 1055.9 g wet weight with good growth rate in the range of 5.8%–7.2% day⁻¹ [7].

TABLE 16.3 List of successful report in seaweed tissue culture.

S.No	Plant name	Regeneration	References
Red algae			
1.	<i>Ahnfeltiopsis flabelliformis</i>		Huang and Fujita [131]
2.	<i>Agardhiella subulata</i>	Regeneration was observed	Bradley and Cheney [132], Cheney et al. [133], Huang et al. [134]
3.	<i>Chondrus crispus</i>		Chen and Taylor [130]
4.	<i>Eucheuma alvarezii</i>	Regeneration was observed	Dawes and Koch [135], Dawes et al. [136], Polne-Fuller and Gibor [11]
5.	<i>Eucheuma denticulatum</i>	Regeneration was observed	Dawes and Koch [135], Dawes et al. [136]
6.	<i>Eucheuma uncinatum</i>	Regeneration was observed	Polne- Fuller and Gibor [11]
7.	<i>Gelidiella acerosa</i>	Regeneration was observed	Rajakrishna kumar et al. [137]
8.	<i>Gelidiella robustum</i>	Regeneration was observed	Polne-Fuller and Gibor [11]
9.	<i>Gelidium</i> sp.	Regeneration was observed	Titlyanov and Titlyanova [138]
10.	<i>Gigartina exasperate</i>	Regeneration was observed	Polne-Fuller and Gibor [11]
11.	<i>Gracilaria cornea</i>	Regeneration was observed	Guzman-Uriostegui et al. [65]
12.	<i>Gracilaria corticate</i>	Regeneration was observed	Subbaraju et al. [139]
13.	<i>Grateloupia doryphore</i>	Regeneration was observed	Robina et al. [140]
14.	<i>Gracilaria papenfussii</i>	Regeneration was observed	Polne-Fuller and Gibor [11], Rajakrishna kumar et al. [137]
15.	<i>Gracilaria tenuiformis</i>	Regeneration was observed	Yokoya, [141]
16.	<i>Laurencia</i> sp.	Regeneration was observed	Garcia-Reina et al. [142], Robania et al. [140]
17.	<i>Ochtodes secundiramea</i>	Regeneration was observed	Malaikal et al. [143], Rorrer and Cheney [144]
18.	<i>Porphyra lanceolata</i>	Regeneration was observed	Polne- Fuller and Gibor [11]
19.	<i>Porphyra nereocystis</i>	Regeneration was observed	Polne- Fuller and Gibor [11]
20.	<i>Porphyra perforate</i>	Regeneration was observed	Polne- Fuller and Gibor [11]

Continued

TABLE 16.3 List of successful report in seaweed tissue culture.—cont'd

S.No	Plant name	Regeneration	References
21.	<i>Porphyra yezoensis</i>	Regeneration was observed	Hafting [145], Yamazaki et al. [146]
22.	<i>Smithora naiadum</i>	Regeneration was observed	Polne- Fuller and Gibor [11]
23.	<i>Solieria filiformis</i>	Regeneration was observed	Yokoya and Handro [147]
Brown algae			
24.	<i>Ecklonia radiata</i>	Regeneration was observed	Lawlor et al. [148], Notoya [149]
25.	<i>Ecklonia stolonifera</i>	Regeneration was observed	Notoya [149]
26.	<i>Eisenia bicyclis</i>	Regeneration was observed	Notoya and Aruga [150]
27.	<i>Elachista fucicola</i>	Regeneration was observed	Pedersen [151]
28.	<i>Fucus</i> sp.	Regeneration was observed	Fries [152]
29.	<i>Laminaria angustata</i>	Regeneration was observed	Saga et al. [153], Saga and Sakai [154]
30.	<i>Laminaria digitata</i>	Regeneration was observed	Asensi et al. [155], Fries [156]
31.	<i>Laminaria hyperborea</i>	Regeneration was observed	Fries [156]
32.	<i>Laminaria japonica</i>	Regeneration was observed	Yan [157]
33.	<i>Laminaria saccharina</i>	Regeneration was observed	Lee [158]
34.	<i>Sargassum confusum</i>	Regeneration was observed	Kirihara et al. [159]
35.	<i>Sargassum fluitans</i>	Regeneration was observed	Polne-fuller and Gibor [11]
36.	<i>Sargassum heterophyllum</i>	Regeneration was observed	Mooney and Staden [160]
37.	<i>Sargassum muticum</i>	Regeneration was observed	Polne-Fuller and Gibor [11]
38.	<i>Sargassum polycystum</i>	Regeneration was observed	Rao and Rao [161]
39.	<i>Undaria pinnatifida</i>	Regeneration was observed	Kawashima and Tokuda [162], Notoya and Aruga [163], Zhang [164], Yan [157]
40.	<i>Undaria undarioides</i>	Regeneration was observed	Kimura and Notoya [165]

Cheney et al. [133] obtained a filamentous tissue from the explants of the carrageenan-producing alga, *Agardhiella subulata*. The filaments in the early stage as well as in older clumps bearing callus-like masses and shoot primordia produced a similar *iota*-carrageenan as the whole plants, and this result suggested possible production of carrageenan in the laboratory setting.

TABLE 16.4 Various media used for seaweed tissue culture.

S.No	Type of the medium	Plant name	References
1.	AsPC-1 agar medium	<i>Dictyosiphon foeniculaceus</i>	Saga et al. [166]
2.	ASP12NTAi	<i>Agardhiella subulata</i> <i>Gelidium nudifrons</i> <i>Gelidium robustum</i> <i>Gelidium versicolor</i> <i>Gigartina exasperate</i> <i>Gracilaria papenfussii</i> <i>Grateloupia doryphora</i> <i>Laurencia</i> sp. <i>Meristotheca papulosa</i> <i>Porphyra columbina</i> <i>Porphyra</i> sp. <i>Pterocladia</i> sp. <i>Laminaria sinclairii</i> <i>Sargassum fluitans</i>	Bradley and Cheney [132] Robaina et al. [140], [167], [168], Garcia-Jimenez et al. [64] Huang and Fujita [131] Polne-Fuller and Gibor [11] Xue-Wu and Gordon [169]
3.	Solidified ASP12NTA medium	<i>Gracilaria domingensis</i> <i>Porphyra</i> sp.	Provasoli [170], Goveia et al. [171]
4.	Modified PES medium	<i>Porphyra</i> sp.	Fujita and Saito [46], Goveia et al. [171]
5.	ES enrichment and Erd-Schreiber solution	<i>Gracilaria verrucosa</i>	Kaczyna and Megnet [172]
6.	von Stosch—enriched seawater	<i>G. domingensis</i> <i>Grateloupia filiformis</i> <i>Solieria filiformis</i>	Yokoya et al. [173] Yokoya and Handro [147] Goveia et al. [171]
7.	Liquid or semisolid medium	<i>Agardhiella subulata</i> <i>Eucheuma denticulatum</i> <i>G. filiformis</i> <i>Kappaphycus alvarezii</i> <i>Laurencia</i> sp.	Dawes and Koch [135], Robaina et al. [167], Yokoya et al. [173]
8.	ESS/2 medium	<i>Chondrus crispus</i> <i>Eucheuma</i> sp. <i>Gracilaria</i> sp. <i>Kappaphycus</i> sp. <i>Porphyra</i> sp. <i>Undaria</i> sp.	Chen and Taylor [133] Yang and Wang [27] Polne-Fuller and Gibor [11], Dawes and Koch [135], Dawes et al. [136] [174], Wang [116] Notoya [174] Tokuda and Kawashima [52], Hurtado and Cheny [7]
9.	Seawater enriched with mixed algae fertilizer, natural seaweed extract	<i>K. alvarezii</i>	Yong et al. [175]
10.	Instant ocean + PES media	<i>Acrosiphonia coalita</i>	Rorrer and chenny [144]
11.	ASP12	<i>A. subulata</i>	Rorrer and chenny [144]
12.	Natural seawater + ESS	<i>Portieria hornemannii</i>	Rorrer and chenny [144]
13.	GP2 medium	<i>Laminaria saccharina</i>	Rorrer and chenny [144]
14.	ESS/2 medium	<i>Laminaria</i> sp	Saga and Sakai [154]
15.	PES media	<i>Gelidiella acerosa</i> <i>K. alvarezii</i>	Rajakrishna kumar et al. [137] Reddy et al. [176]
16.	Solidified PES media	<i>K. alvarezii</i>	Reddy et al. [176]
17.	Modified Erd-Schreibers seawater (ESS)	<i>K. alvarezii</i>	Reddy et al. [176]
18.	Modified f/2	<i>K. alvarezii</i>	Reddy et al. [176]

In Indonesia, Kasim et al. [177] compared the growth of tissue culture and clone selection cultivated material of *K. alvarezii* (Rhodophyta) by using horizontal net method. In this study, tissue-cultured seedlings showed slightly higher growth rate than clonal selection material, i.e., 6.7% and 6.3% DGR, respectively. Fadilah et al. [178] employed recurrent selection methods to select high growth rate germplasm from *K. alvarezii*, from a population having a DGR >10%. Incidentally, the selected germplasm also had the favorable characteristics of higher clump weight, average DGR, longer thallus potential, and a greater number of branches with shorter internodes, as compared to the control plants. Tissue culture has a higher growth rate because its branching is greater compared to local seeds. Therefore, thallus absorbs more nutrition from the water. Tissue-cultured *K. alvarezii* thalli have been reported to exhibit higher growth rates and resistance to epiphytes and “ice-ice” disease, compared to the same age, farm-propagated seedlings after outplanting to the open sea [176,179]. Hurtado et al. [180] reported on the production of new and improved *Kappaphycus* thalli grown under optimized growth conditions with the addition of a biostimulant to maintain the high-yielding and rapidly growing seed stock for a commercial nursery. Comparison of the tissue culture versus farm-propagated thalli of *K. alvarezii* revealed economically important quality benefits, for example, high carrageenan yield and quality [177,178], higher nutritional value [180], and less heavy metal contamination [181]. This suggested that the tissue culture strains have a superior quality to serve as planting materials for the future of sustainable seaweed farming and use of the biomass for high-value applications.

In India, the plants of *K. alvarezii* that regenerated through tissue culture were outplanted, and their growth performance was recorded against the wild plants. Higher growth rate over 1.5%–1.8% day⁻¹ by tissue-cultured plants than normal seed in seven subsequent crop cycles helped for commercial farming [176]. Ganesan et al. [182] attempted to culture the tissue-cultured progenies of callus-regenerated *G. acerosa* (73 plantlets each with an average biomass of 70 ± 20 g fresh wt.plant⁻¹) in the lagoon waters, Mandapam coast, Tamil Nadu, by suspended stone method along with wild plants for 90 days, and these exhibited higher biomass yield, growth rate, agar yield, and quality than wild plants. Biomass of the tissue-cultured farm plants ranged from 1.86 to 2.97 kg fresh wt.m⁻² with DGR of 1.87%–2%, whereas wild plants showed a range of 0.44–0.62 kg fresh wt.m⁻² with 1%–1.2% DGR. Higher gel strength of agar was reported in agar extracted from tissue-cultured plants (2400 ± 250 g cm⁻²) than wild plants (1900 ± 135 g cm⁻²). Agar quality of tissue-cultured *G. acerosa* was found to be suitable for microbial culture and DNA electrophoretic applications. Hence, tissue culture progeny of *G. acerosa* may be used as improved seed stock for the large-scale cultivation of *G. acerosa* as it expressed higher biomass yield, better growth rate, and superior agar quality than wild plants [182].

12. Way forward

Several worldwide stakeholders are envisaging enormous interest in investing cutting-edge new biotechnological innovations. Besides product development, seaweed aquaculture provides enormous opportunities like climate change mitigation, biodiversity conservation, and ecosystem servicing. The biotechnological interventions are currently implied in RandD setup or in pilot stage. They are expected to grow and play critical role in commercial success of the industry. Applications of tissue culture techniques have already shown considerable promise in catering to the large number of seedlings of uniform train for commercial exploitation in relatively short time. Furthermore, these technologies have produced clone via somaclonal variation, but reproducibility needs to be ascertained for commercial applications. The grafting has been successful for quality improvement of phycocolloid contents of intrageneric species, but again routine use of this technique has not yet been comically used anywhere in the world. The natural selection and breeding has been the only way today in developing high-yielding and disease-resistant varieties. The numbers of certified varieties of seaweeds for commercial exploitation have increased 308% from only 12 in 2010 to 49 in 2018. These were mostly of foliose Bangiales (*Neoporphyra haitanensis* and *Neopyropia* spp.) and brown taxa *Saccharina* spp. [183]. The species diversification of farmed species also needs to take help of biotechnological tools and techniques especially to gain greater control over reproduction, which is essential in nonclonal species. There are several taxa where life history pattern is heteromorphic and reproductive stages are used for seeding. Additionally developing tool kit, via complementary methodologies including molecular and microscopic techniques, to study and understand disease progression, for example, ice-ice disease of eucheumatoid seaweeds, is necessary. Steps should be taken to standardize high-throughput sequencing–based methodologies such as metagenomics, 16S metabarcoding, and transcriptomic to develop mitigation strategies for controlling and progression of diseases in commercial farming. The developments in cryopreservation techniques to aid commercial seaweed farming are on rise, especially in the area of long-term storage and molecular alteration. The biobanks might not help seaweed farming domain directly in immediate future but have relevance of conserving best germplasm for commercial exploitation, if in case the economically important traits show the decline due to repeated use of the same germplasm generation over generation. In conclusion, the biotechnological

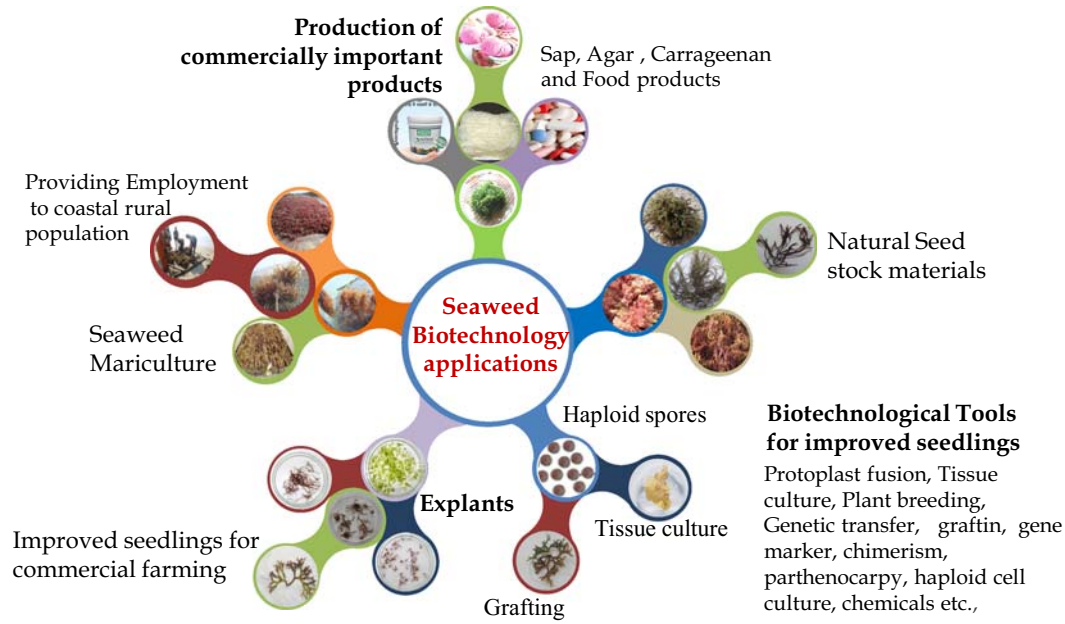


FIGURE 16.1 Thematic diagram of implications of seaweed biotechnology to seaweed mariculture.

interventions are going to be pivotal in coming time to improve the prospects of the seaweed aquaculture domain and to support the sustainable production (Fig. 16.1).

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Transcriptome analysis: implication in characterization of genes involved in biosynthesis of economically valuable products from seaweeds

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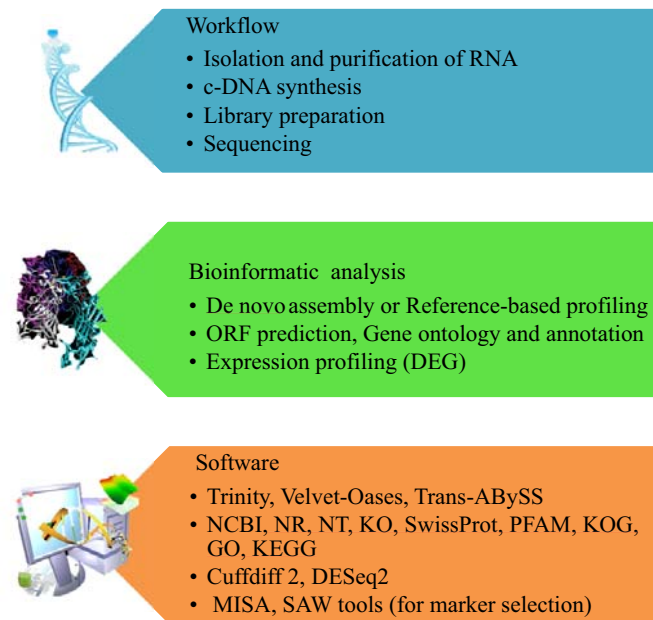
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1. Introduction

Gene as a basic and functional unit of genome governs the cellular processes in a cell. The identification of genes involved in cell metabolism is a key to the understanding of its functional role and biotechnological applications. The functional gene is expressed in the form of RNA or protein. Proteins are the final product of gene expression process, while the RNA can be a directly functional product or play as intermediate product in protein synthesis. The transcriptome is represented by complete set of RNA molecules (both coding and noncoding) present in a cell or organism. Transcriptome analysis is performed to identify differentially expressed genes in different development stages, tissue types, and physiochemical conditions, which lead to understanding the expression, regulation, function, and structure of genes.

The studies of transcripts was started way back in the late 1970s by the synthesis of complementary DNA (cDNA) from mRNA, and further after 2 decades, the technique of construction of libraries of these cDNA as expressed sequence tags (ESTs) was established [1,2]. Reverse-transcription quantitative polymerase chain reaction (qPCR) was also a method of choice to study few targeted genes [3]. The development of different molecular biology techniques such as serial analysis of gene expression [4], cap analysis of gene expression [5], and massively parallel signature sequencing [6] and DNA microarrays [7] had contributed significantly to the transcriptomic studies. The advancement of high-throughput sequencing has revolutionized the genomic studies, which also facilitates the whole-transcriptome study by RNA sequencing (RNA-Seq) (Fig. 17.1). RNA-Seq method utilizes reference genome or transcriptome for alignment to generate transcriptome assembly or de novo assembly approach by using transcript reads [8]. The recent development of single-molecule transcriptome sequencing, a third-generation sequencing technique, has opened up a new approach of studying a full-length transcript sequence without fragmentation and assembly process [9].

“Seaweed” is the colloquial term commonly employed to diverse group of lower plants inhabiting marine ecosystems. The term itself is a bona fide misnomer as none of the species display tendency of weed by harming environment in its natural range of distribution. They form conspicuous flora in both intertidal and subtidal areas of the world oceans. Besides major oxygen producer and sustaining the productivity in the tropical ecosystems, they provide nesting and breeding grounds to diverse marine fauna [10]. Even though seaweeds have simple morphological features, the physiological and biochemical properties are way different to their land-based counterparts due to their complex living habitats. Although seaweeds have been utilized for centuries by humans, the increase in cultivation efforts and phycocolloidal industries in 20th century make them attractive marine resources. In recent times, the global demand for seaweed-derived products is fast increasing, due to biotechnological interventions and new applications those were not conceived before. The list includes functional food, bioactive proteins, specialty chemicals, biostimulant, biofuel, hydrogel scaffolds, drug delivery, pharmaceutical, wellness, etc. [11,12,13,14]. The simple and viable farming techniques need to be credited for the

FIGURE 17.1 Framework of transcriptome analysis in seaweed by RNA sequencing.

development of successful farming industry mostly in Southeast Asian countries. The other advantage of seaweed farming is that they do not need arable land, water for irrigation, fertilizer, and pesticides and thus are looked as futuristic crop. The commercial harvesting of seaweeds has reached the new milestone recording 31.2 million ton year⁻¹ production (95% accounts for farming) worth about US\$ 11.7 billion [15].

The major industrial product from seaweed includes polysaccharides such as agar, alginate, and carrageenan. The industrial production grows at 2%–3% year⁻¹ in the Asia–Pacific region. Indonesia is the largest producer of raw material for agar and carrageen, while China is the largest processor of seaweed hydrocolloids [16]. Similarly, seaweeds are promising source of pigments such as carotenoids, fucoxanthins, phycoerythrin, and phycocyanin. The global market for carotenoid was USD 1577 million in 2017, and it is estimated to be USD 2098 million by 2025 [17]. The quest for new drug molecules from natural resources is the major thrust area in medical research. Seaweeds grow in highly diverse and extreme physiochemical and biological conditions [18]. These conditions elicit different adaptive and defense characteristics in seaweeds in form of active chemical constituents. Therefore, seaweeds can become a major source for bioactive molecules.

Compared to the land flora, seaweeds are underexplored for their capabilities and functions. Considering the increasing popularity of seaweeds, transcriptome analysis becomes a valuable tool that provides greater insights into the role of genes in developmental, physiological, and biochemical processes that govern adaptation, growth, reproduction, and defense. In seaweeds, transcriptome analysis was used to study the response to stress of changing physical conditions [19–21], difference between gametophytic and sporophytic generations [22], interactions between host and its associated microbiome [23], metabolic pathways and networks [24–26], molecular mechanism of developmental process in tissue culture [27], pathways involved in reproduction process [28], etc. This chapter provides an overview of the potential of transcriptome analysis in identifying genes involved in biosynthesis of economically valuable products from seaweeds (Table 17.1).

2. Polysaccharide biosynthesis

Polysaccharides are major constituents of seaweeds that play structural, storage, and functional role in cell metabolism. The content of total carbohydrate may vary with the species. The structural polysaccharide includes agar and carrageenan from red algae and alginate from brown algae. These polysaccharides are termed as phycocolloids for their role in gelling, thickening, clarifying, emulsifying, and stabilizing different food products [29]. Similarly, some minor polysaccharides are also present in cell wall, which include sulfated fucose, xylans, and cellulose and storage polysaccharides such as laminarin and floridean starch [30]. Greater portion of seaweed polysaccharides are not digested by humans and termed as dietary fibers.

TABLE 17.1 Genes identified for biosynthesis of economically valuable products from seaweeds through transcriptome analysis.

Product	Seaweeds species	List of detected genes in transcriptome analysis
Agar	<i>Gracilariopsis lemaneiformis</i> , <i>Gelidium pristoides</i> , <i>Gracilaria gracilis</i> , <i>Gracilaria changii</i> , <i>Gracilaria edulis</i> , and <i>Gracilaria salicornia</i>	Fructose 1,6-bisphosphate aldolase (FBPA), fructose 1,6-bisphosphatase (FBA), galactose-1-phosphate uridylyltransferase (GALT), UDP-galactose 4-epimerase (UGE), GDP-mannose 3',5'-epimerase (GME), GDP-mannose pyrophosphorylase (GMP), glucose-6 phosphate isomerase (GPI), phosphofructokinase (PFK), glycosyltransferases (GTs), sulfo-transferases (STs), methyltransferases (MTs), and pyruvyltransferases (PTs)
Carrageenan	<i>Betaphycus gelatinus</i> , <i>Kappaphycus alvarezii</i> , <i>Eucheuma denticulatum</i> , and <i>Chondrus crispus</i>	Galactosyl transferase, ST, and galactose-2,6-sulfurylases
Alginate	<i>Laminaria digitata</i> , <i>Saccharina japonica</i> , <i>Cladosiphon okamuranus</i> , and <i>Ectocarpus siliculosus</i>	Mannose-6 phosphate isomerase (MPI), phosphomannomutase (PMM), GDP-mannose 6-dehydrogenase (GMD), mannuronan synthase (MS), and mannuronate C5-epimerase (MC5E).
Fucoidan	<i>C. okamuranus</i> , <i>E. siliculosus</i> , and <i>Nemacystus decipiens</i>	GDP-mannose 4,6-dehydratase (GM46D), GDP-L-fucose synthase (GFS), L-fucosidase (FK), GDP-fucose pyrophosphorylase (GFPP), fucosyltransferase, and STs
Allophycocyanin and subunits	<i>Gracilaria chouae</i> , <i>Gracilaria blodgettii</i> , <i>Gracilaria vermiculophylla</i> , and <i>Gp. lemaneiformis</i>	apcA, apcB, apcD, and apcE
Phycocyanin and subunits		cpcA, cpcB, cpcD, and cpcG
Phycoerythrin and subunits		cpeA and cpeB
Carotenoid	<i>Bangia fuscopurpurea</i> , <i>Pyropia umbilicalis</i> , <i>Porphyr a yezoensis</i> , and <i>Undaria pinnatifida</i>	Geranylgeranyl diphosphate synthase (GGPS), phytoene synthase (PSY), phytoene desaturase (PDS), ζ-carotene isomerase (Z-ISO), ζ-carotene desaturase (ZDS), carotenoid isomerase (CRTISO), lycopene β-cyclase (LCYB), lycopene δ-cyclase (LCYE), lycopene β/δ-cyclase (CruP), lycopene cyclase (CruA), carotene hydroxylase (P450-type), and zeaxanthin epoxidase (ZEP)
Terpenoid precursors	<i>Laurencia dendroidea</i>	Acetyl-CoA C-acetyltransferase, mevalonate kinase, 1-deoxy-D-xylulose-5-phosphate synthase, 1-deoxy-D-xylulose-5-phosphate reductoisomerase, 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase, 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate synthase, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase, isopentenyl-diphosphate Δ isomerase, dimethylallyltransferase, GGPS, hexaprenyl diphosphate synthase, geranylgeranyl diphosphate reductase, all-trans-nonaprenyl diphosphate synthase, farnesylcysteine lyase, protein-S-isoprenylcysteine O-methyltransferase, farnesyltransferase, and ditrans,polycis-polyprenyl diphosphate synthase

Continued

TABLE 17.1 Genes identified for biosynthesis of economically valuable products from seaweeds through transcriptome analysis.—cont'd

Product	Seaweeds species	List of detected genes in transcriptome analysis
Monoterpene		(3R)-linalool synthase, (+)-trans-carveol dehydrogenase, (+)-pulegone reductase, (–)-isopiperitenone reductase, and secologanin synthase
Diterpene		Gibberellin 20-oxidase, gibberellin 2-oxidase, and abietadienol/abietadienal oxidase
Triterpene		Farnesyl-diphosphate farnesyltransferase, squalene monooxygenase, squalene-hopene/tetraprenyl-beta-curcumen cyclase, and lupeol synthase 1
Sesquiterpene		Nerolidol synthase, alpha-bisabolene synthase, germacrene-A synthase, germacrene A oxidase, aristolochene synthase, 5-epiaristolochene 1,3-dihydroxylase, pentalenene synthase, (+)-delta-cadinene synthase, and zerumbone synthase

2.1 Galactans

In red algae, the major cell wall polysaccharides are sulfated galactans, which include agar and carrageenan. Galactans are linear polysaccharide of repeating disaccharide units of 3-linked β -D-galactopyranose and 4-linked α -galactopyranose residues [31]. The side chains of backbone may be consisted of sulfate ester, pyruvate ketal, and methyl groups. The hypothetical model of galactan biosynthesis involves three main enzymes, namely galactosyltransferases, sulfotransferase (ST) and sulfohydrolases [32].

The development of ESTs from *Gracilaria gracilis*, which was the first from any algae, reported 200 sequences [33]. Among these, seven ESTs were found to be involved in carbohydrate metabolism, which included galactose-1-phosphate uridylyltransferase (GALT), a key enzyme of D-galactose metabolism. Furthermore, [34] cloned and characterized GALT gene from *G. gracilis* with predicted product length of 399 amino acids. Similarly, [35] characterized GALT gene from *Gracilaria changii* and showed the correlation between expression of GALT and agar content. The expression of GALT was higher in *G. changii* than lower agar-producing species such as *Gracilaria edulis* and *Gracilaria salicornia*. The qPCR analysis of GALT gene in *Gracilariopsis (Gp.) lemaneiformis* reported that the expression of this gene is useful to screen strains of high agar content but cannot be considered as the limiting factor in agar synthesis [36]. An EST analyses in *G. changii* identified several genes that could be the part of putative agar biosynthesis, such as phosphomannomutase (PMM), galactose-1-phosphate uridylyltransferase, GDP-mannose phosphorylase-like enzyme, and NDP-sugar epimerase [37]. One of the important enzymes in Leloir pathway, UDP-galactose 4-epimerase (UGE), was reported in transcriptome analysis of *Gelidium pristoides*, which catalyzes interconversion between UDP-D-glucose and UDP-D-galactose [32]. The increased expression of UGE was found to be correlated with hydrolysis of floridean starch, suggesting the role of UGE in supplying UDP-D-galactose for sulfated galactan synthesis [32]. The sulfate deprivation study in *G. changii* and *G. salicornia* by transcriptome profiling identified a number of differentially expressed genes related to putative agar biosynthesis and sulfur-related metabolic pathways [38]. The study also reported the increase in expression of GALT and GDP-mannose 3',5'-epimerase genes, which supports their putative role in agar biosynthesis. Similarly, they detected the downregulation in some of the genes related to putative agar biosynthesis pathway in *G. changii*, which include galactosyltransferases, STs, methyltransferases, and pyruvyltransferase. [38] also reported the glycosyltransferase (GT) genes, of which two genes got upregulated in sulfate-deprived *G. salicornia*, which also showed increase in agar yield. GT catalyzes polymerization of sugars into polysaccharide chains. About 65 genes of 27 GT families were reported in *Chondrus crispus*, which are speculated to involve in galactan synthesis [39]. Under nutrient limiting conditions of 3 weeks in *Gp. lemaneiformis*, [24] reported positive correlation between expression of GDP-mannose 3,5-epimerase gene and agar content. Similarly, the expression of UDP-glucose pyrophosphorylase gene was found positively correlated with agar yield in culture of *Gp. lemaneiformis* under low salinity (17‰) conditions for 2 weeks [40]. In gene expression analysis of 15 candidate genes associated with agar biosynthesis pathway in *G. changii*, [41] found significant correlation between 4 genes with agar yield and 11 genes with gel strength. Among these, fructose-bisphosphate aldolase and UGE were found as the most potent markers to correlate with agar yield and gel strength.

Along with fructose-6-phosphate, floridean starch and floridoside are also considered as sources of the initial substrates in putative galactan biosynthesis pathway [42]. The transcriptome analysis of *Gracilaria chouae*, *Gracilaria blodgettii*, *Gracilaria vermiculophylla*, and *Gp. lemaneiformis* showed the presence of UDP-glucose 6-dehydrogenase (UGD) of length 305 bp, 329 bp, 320 bp, and 295 bp, respectively, which confirmed that UDP-D-glucose acts as a substrate in biosynthesis of floridean starch [43]. [28] reported the genes involved in uptake, sulfation, transport, and synthesis of polysaccharide, such as floridoside and carrageenan from *Grateloupia imbricata*. They identified that glycerol-3-phosphate dehydrogenase and α -galactosidase were involved in floridoside biosynthesis and galactosyl transferase, ST, and galactose-2,6-sulfurylases were involved in carrageenan biosynthesis. Twelve genes encoding for galactose-2,6-sulfurylases were identified in *C. crispus*, which speculated to be involved in desulfation reaction in carrageenan synthesis [42]. The transcriptome analysis of three red algal species, namely *Betaphycus gelatinus*, *Kappaphycus alvarezii*, and *Eucheuma denticulatum*, showed the presence of 861 differentially expressed genes related to carbohydrate metabolism and 8 for sulfur metabolism, and some of these genes are speculated to be the important part of carrageenan synthesis [44]. However, the complete profile of genes and enzymes involved in carrageenan synthesis is not yet characterized from red algae [28].

2.2 Alginate and fucoidans

Alginate, fucoidans (sulfated polysaccharides), and cellulose are the major components of cell wall polysaccharide in brown algae. Alginate is a polymer composed of two monomeric units, β -D-mannuronic acid (M) and α -L-guluronic acid (G), arranged in MM, GG, and random MG blocks. In the cell wall, M, MG, and G blocks exist and the ratio of them helps in deciding the hardness of cell wall [45]. The putative alginic acid synthesis from fructose 6-phosphate in brown algae was catalyzed by the following enzymes: mannose-6 phosphate isomerase (MPI), PMM, mannose-1-phosphate guanylyl-transferase (MPG), GDP-mannose 6-dehydrogenase (GMD), mannuronan synthase, mannuronate C5-epimerase (MC5E) [46]. The analysis of transcriptomic data and subsequent phylogenetic analysis of MC5E and GMD genes from brown algae revealed the bacterial origin of these genes acquired through horizontal gene transfer [47,48]. However, some of the genes like MPI, PMM, and UGD are likely to be originated from secondary endosymbiotic eukaryotic hosts [47]. Recent genomic studies of brown alga *Ectocarpus siliculosus* showed that most of the known families of genes and enzymes for cell wall biosynthesis are absent in this alga [49,46]. GDP-mannuronic acid is the precursor for the alginate synthesis, and it is a product of oxidation of GDP-mannose catalyzed by GMD. [50] identified and characterized the GMD from *E. siliculosus* genome, which is also a first report of this enzyme from any eukaryote. The final step in alginate biosynthesis of conversion of M into G by MC5E had been characterized at molecular level in brown algae. [51] for the first time isolated and characterized the MC5E genes from *Laminaria digitata*. In case of brown algae, about 28 genes in *E. siliculosus* [49], 45 genes in *L. digitata* [52], and 125 genes in *Saccharina japonica* [53] for MC5E were identified. The expansion of these large multigene families of MC5Es in brown algae is attributed to their capabilities of regulating structural heterogeneity and content of alginate under developmental and different physiological conditions [53,54]. All the potential candidate genes for enzymes in alginate biosynthesis pathway, except the enzyme catalyzing conversion of mannose 1-phosphate to GDP-mannose, were detected in genomic and transcriptomic analysis of *Cladophoron okamuranus* and *E. siliculosus*. However, one of the MPI genes from *Saccharina* was found to encode functional MPG protein [47]. Along with MPG, the enzymatic activity of MPI, PMM, and GMD were also confirmed in this study. The genome sequence and deep transcriptome sequencing of female gametophytes and sporophytes of *S. japonica* revealed the presence of important genes of polysaccharide biosynthesis such as cellulose synthase (GT2 family) for cellulose biosynthesis, MC5Es for alginate biosynthesis, and α -(1,6)-fucosyltransferase (GT23 family) for fucan chain elongation. It was observed that in response to varying physiological conditions, cell wall architecture changes. Alginate degradation is also a part of this cell wall modification process. [55] isolated and characterized the first functional alginate lyase gene from brown algae *S. japonica*, which is an important step toward the understanding of alginate metabolism in brown algae.

Fucoidans are sulfated polysaccharides that occur in brown algae and consist of α -L-fucose residues. Fucoidans are homo- or hetero-polysaccharides that may found be in a variety of composition such as high uronic acid, low sulfur, and homo α -L-fucan chain polymers. GDP-mannose (de novo pathway) and L-fucose (salvage pathway) act as sources for GDP-fucose synthesis, which is further converted to fucan and sulfated fucan [46]. In de novo pathway, GDP-mannose is converted into GDP-fucose in three steps involving enzymes such as GDP-mannose 4,6-dehydratase (GM46D) and GDP-L-fucose synthase (GFS). In salvage pathway, L-fucose is converted into GDP-fucose in two steps involving enzymes such as L-fucosidase (FK) and GDP-fucose pyrophosphorylase (GFPP). The synthesis of fucan and sulfated fucan from GDP-fucose is catalyzed by fucosyltransferase and ST. Genes encoding for these enzymes have been detected in brown algae *C. okamuranus*, *E. siliculosus*, and *Nemacystus decipiens* [46,56,57]. The number of genes encoding GM46D, GFS, FK,

and GFPP was found conserved in the genomes of these algae, but the numbers of genes encoding fucosyltransferase and STs differ among these species. It was observed that FK and GFPP genes are present in fused form and produce a bifunctional enzyme having both the domains [56].

3. Pigments and secondary metabolite synthesis

The current increasing trend in genomic information in the marine realm provides an opportunity to expand our knowledge of the various secondary metabolites from seaweeds. Seaweeds are great source of large number of compounds such as phycobiliproteins, carotenoids, phenolic compounds, alkaloids, and terpenoids, which are receiving attention due to their biological properties and potential benefits for humans [58].

Phycobilisome is the red algal light-harvesting complex composed of phycobiliproteins and linker proteins. The most common phycobiliproteins are phycoerythrin (red pigment), phycocyanin (blue pigment), and allophycocyanin (light blue pigment). Phycobiliproteins have wide range of applications in food industries, pharmaceuticals, cosmetic industry, therapeutics, nutraceuticals, and as fluorescence probes [59]. The analysis of transcriptome sequencing data of *G. chouae*, *G. blodgettii*, *G. vermiculophylla*, and *Gp. lemaneiformis* identified genes encoding phycobiliproteins, including allophycocyanin and subunits (apcA, apcB, apcD, and apcE), phycocyanin and subunits (cpcA, cpcB, and cpcG), and phycoerythrin and subunits (cpeA and cpeB) [43]. Phylogenetic analysis of these genes showed that only rhodophytes and some species of cyanobacteria contained all three types of phycobiliproteins. The study of transcriptome and differential expression of genes in wild type and green mutant of *Gp. lemaneiformis* has shed light on the phycoerythrin synthesis pathway [60]. The transcription of apo-phycoerythrin was found to be downregulated in green mutant strain, but the expression of chlorophyll–protein complex increased. The optical activation of phycoerythrin required the binding of apo-phycoerythrin to phycoerythrobilin. Similarly, genes related to the synthesis of biliverdin (a precursor of phycoerythrin) were highly expressed in green mutants compared to wild strain. However, the increased level of chlorophyll–protein complex possibly involved in the decrease in synthesis of biliverdin and subsequent phycoerythrin synthesis. The transcriptome analysis also helped to identify genes encoding linker proteins. The sequences for CpcD domain, PBS linker polydomains, and γ subunits were identified in transcriptome of *G. chilensis* [61]. The main functions of linker polypeptides are to connect phycobilisome to the phycobiliproteins and thylakoid membrane [62].

The transcriptomic profile of red algae *Laurencia dendroidea* revealed the presence of 34 transcripts involved in mevalonate-independent pathway [23]. This pathway synthesizes isopentenyl diphosphate and dimethylallyl diphosphate, which act as precursor for the synthesis of sterols, terpenoids, carotenoids, and dolichols. Further transcriptome study in *L. dendroidea* detected genes encoding two important enzymes (acetyl-CoA C-acetyltransferase and mevalonate kinase) of mevalonate (MVA) pathway for biosynthesis of isoprenoid precursors indicating simultaneous occurrence of the MVA and the MVA-independent pathway [63]. About 20 genes associated to biosynthesis of terpenoid precursors and 21 related to terpene synthases were identified in *L. dendroidea*. Carotenoids are tetraterpenoids synthesized by all photosynthetic organisms and involved in light harvesting and photoprotection. Carotenoids have many health benefits in humans as antioxidants and pro-vitamin A activity and beneficial effects in prevention of cardiovascular disease, macular eye disease, cataracts, and cancers [64]. The major carotenoids in seaweed include zeaxanthin and lutein in red seaweeds; fucoxanthin in brown seaweeds; and zeaxanthin, violaxanthin, and neoxanthin in green seaweeds [65]. The pathway of β -carotene synthesis from geranylgeranyl pyrophosphate (GGPP) seems to be conserved in algae and terrestrial plants. The transcriptome analysis of *Pyropia umbilicalis* showed the presence of nine EST contigs encoding homologues to the enzymes in carotenoid synthesis pathway [66]. This includes genes for geranylgeranyl diphosphate synthase (GGPS), phytoene synthase (PSY), phytoene desaturase, ζ -carotene isomerase, ζ -carotene desaturase, carotenoid isomerase, lycopene β -cyclase (LCYB), lycopene θ -cyclase (LCYE), lycopene β/θ -cyclase, lycopene cyclase, carotene hydroxylase, and zeaxanthin epoxidase. GGPS catalyzes the synthesis of GGPP, which is the precursor for metabolites such as carotenoids and chlorophylls. The gene encoding GGPP was isolated from *P. umbilicalis*, and the activity of enzyme was characterized by color complementation assay in *Escherichia coli* [67]. The characterization of GGPS from *Bangia fuscopurpurea* showed that it produces only GGPP as its product and also revealed that it interacts with a putative PSY, which helps in channeling GGPS for carotenoid synthesis [68]. Similarly, [66] isolated and functionally characterized the gene encoding carotene hydroxylase, which catalyzes the hydroxylation of β -carotene to zeaxanthin. LCYB and LCYE are the enzymes of important bifurcation step in carotenoid synthesis pathway, which catalyzes the cyclization of open ends of acyclic lycopene for the synthesis of α - and β -carotene. The isolation and characterization of LCYB gene from red alga *B. fuscopurpurea* showed the ability of enzyme in cyclization of lycopene into monocyclic γ -carotene, bicyclic β -carotene,

and α -carotene [69]. The isolation and characterization of LCYs from *P. umbilicalis* and *Porphyra yezoensis* showed that each species has a pair of LCYB and LCYE [70]. It was observed that LCYBs cyclize both ends, whereas LCYEs only cyclize a single end of lycopene. The gene encoding LCYB was also identified and characterized in brown algae *Undaria pinnatifida* by de novo transcriptome analysis, and the activity was determined by using lycopene producing *E. coli* [71].

4. Gap area

The regulatory mechanisms in many metabolic pathways of seaweeds are still not fully understood. For example, the roles of genes like fructose-bisphosphate aldolase (involved in fructose metabolism) and GLE (involved in galactose metabolism) on agar gel strength are still not understood [41]. Similarly, the functional characterization of two sets of fused genes of sulfated fucan biosynthesis pathway (one is FK and GFPP and another is ST-domain-containing gene and an alpha/beta hydrolase gene) reported from *N. decipiens* will provide deeper understanding of this pathway. The sulfated polysaccharide ulvan from Ulvophyceae group of green seaweed has recently got attention due to its beneficial pharmacological effects [72]. Even though [73] proposed the putative pathway for ulvan synthesis, the enzymatic and molecular mechanism underlying the synthesis of this sulfated polysaccharide is still not known. These are only few representative examples showing wide gap in our understanding of metabolic pathways in seaweeds. The lack of functional annotation of large numbers of genes from transcriptome and genomic data also poses difficulty in correlating these genes to the metabolic pathways and their further characterizations.

5. Conclusion and future perspective

In recent years, high-throughput sequencing technology has emerged as a powerful and cost-efficient tool for transcriptome analysis. Transcriptome studies in different seaweeds proved the effectiveness of this technique in identifying genes in nonmodel organisms and provided valuable information for further investigation into metabolic pathways and their cellular responses. Most of the transcriptome studies to date have provided suggestive evidence for putative galactan synthesis pathway. It also helps to correlate many genes to the content, yield, and strength of galactans, which can be used as markers to screen commercially important strains of red algae. The identification of genes and crucial steps in metabolic pathways provide not only new insights into the transcriptional regulatory networks for pharmacologically active compounds but also the solution for reconstruction of complete pathways in heterologous systems for commercial production. Similarly, this knowledge will also be used for the bioengineering of seaweeds using modern tools and techniques such as CRISPR/Cas in near future for the improved production of key secondary metabolites of commercial use. Additionally, transcriptome profiling will be an effective approach to select seaweed strains for commercial farming purpose. Transcriptome analysis of important seaweeds unravels the key information about genes, which will elucidate the molecular mechanism of biosynthesis of industrial important metabolites. Furthermore, transcriptomics with other omics technologies such as proteomics and metabolomics will help to unravel complex mechanism of metabolic pathways and their significance in the evolution of seaweeds. Overall, net outcome of transcriptomics will add a significant impact on the algal industry.

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Proteomic approaches and their applications in salmonid health research

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1. Introduction

The application of omics technologies has transformed the field of molecular biology, generating a huge amount of data and advancing our understanding on human and animal diseases. The early phase of omics era dominated by genomics and transcriptomics gradually paved the way for the development of proteomics. Moreover, the identification of proteins (by Edman sequencing or mass spectrometry [MS]) became possible because of the progress made in nucleotide sequencing, and gene sequences provide resources for correlation of experimentally derived protein sequence with already available sequences in the databases (DisProt, InterPro, MobiDB, neXtProt, Pfam, PRINTS, PROSITE, Protein Information Resource [PIR], SUPERFAMILY, SWISS-PROT, and NCBI) [1]. On the other side, proteins and even the genes cannot be accurately predicted from genomic data alone [2]. In addition, it is now established that the transcription of a gene does not guarantee the formation of a protein due to several factors such as translational regulation, posttranslational modifications, half-lives, and compartmentalization in protein complexes [3]. These advancements have shifted emphasis on proteomics and are increasingly being viewed as a bridge between genomics and cellular phenotype.

The use of the term “proteome” dates back to 1995, when it was first used for the total protein content of the genome. Over time, the proteome became more specifically defined as the protein content of a cell, a tissue, an organism, or a biological fluid at a given time under the specified set of conditions [4]. Changes in the proteome (posttranslational modifications, translocations, synthesis, or degradation of proteins) occur in response to external or internal cues. Thus, the proteome of a sample is dynamic in nature, reflecting the immediate environment in which it is studied [5].

The dynamicity of proteome has given rise to subdisciplines such as expression proteomics, structural proteomics, and interaction proteomics. Expression proteomics (the high-throughput analysis of protein expression) could be important in identifying key proteins in a sample and those that are differentially expressed in samples being compared, for example, diseased and healthy tissue. Two-dimensional polyacrylamide gel electrophoresis and MS are the commonly used tools for expression proteomics. Structural proteomics is the high-throughput analysis of protein structures. Comparing protein structures can aid in identifying the functions of newly discovered genes. Structural analysis can also reveal the binding site of a drug on the target proteins and where the proteins interact with each other. X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy are the tools used to study the structural proteomics. Interaction proteomics (the high-throughput analysis of protein interactions) helps to determine protein functions and reveals how proteins assemble in larger complexes. This approach mainly utilizes the affinity purification, MS, and the yeast two-hybrid system [6].

Proteomics has a diverse and wide range of applications (Fig. 18.1). It can play a key role in elucidating insights into fundamental processes such as the time and place of protein expression, rate of protein production, degradation, steady-state abundance, and protein modification mechanisms. Other important areas where the potential of proteomics is

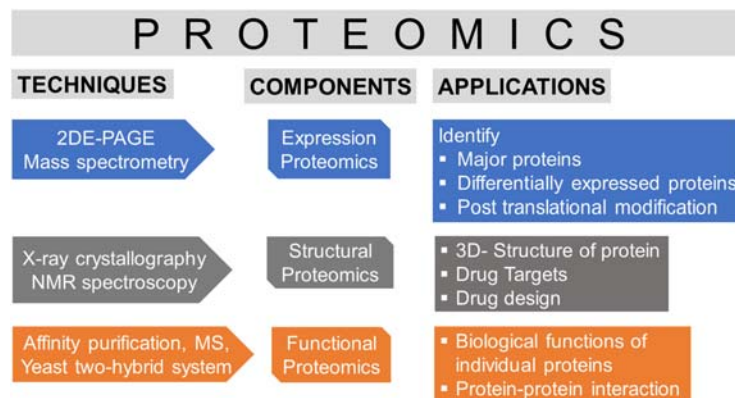


FIGURE 18.1 Techniques, components, and applications of proteomics. Primarily, the components of proteomics include expression, structural, and functional proteomics. Two-dimensional gel electrophoresis coupled with polyacrylamide gel electrophoresis (2-DE-PAGE) and mass spectrometry (MS) are the major techniques employed in expression proteomics. This area of proteomics helps in identification of major proteins expressed, differentially expressed proteins, and posttranslational modifications. Structural proteomics deals with the determination of three-dimensional structure of proteins and drug targets and also aids in drug designing. X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy are the methods of choice for these studies. Functional proteomics includes such investigations, which help in elucidating biological functions of individual proteins and protein–protein interactions. This field of proteomics mainly employs affinity purification, MS, and yeast two-hybrid systems.

evident include biomarker development for disease diagnosis and its early detection, identification of novel therapeutic targets, and evaluation of the drug efficiency and toxicity [7]. The growth and development of proteomics have largely been influenced by the emergence and continued advances of high-throughput proteomic technologies and bioinformatics analyses. The proteomic repertoire includes MS, two-dimensional gel electrophoresis (2-DE), two-hybrid analysis, X-ray crystallography, and NMR spectroscopy.

1.1 Sample preparation, ionization, and mass analysis for protein identification

The major steps involved in a standard proteomic analysis are shown in Fig. 18.2. Sample preparation is the first and major step in any proteomic analysis. Therefore, careful handling and sometimes optimization for each step becomes necessary in order to obtain the maximum amino acid sequence coverage of the protein(s) of interest. Polyacrylamide gel electrophoresis (PAGE) has retained its usefulness as a major method of protein separation until date, although in different

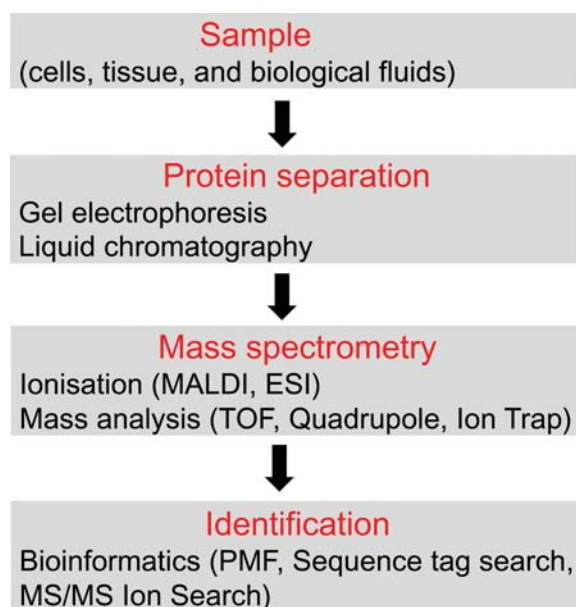


FIGURE 18.2 Major steps in proteomic analysis. Samples for proteome analysis include cells, tissue, and biological fluids. Proteins can be separated from samples using gel-based approach or gel-free approach. Gel-based approach includes one- or two-dimensional gel electrophoresis, whereas gel-free approach includes chromatographic techniques such as liquid chromatography. Once the proteins or peptides are separated, they are ionized mainly by soft ionization techniques such as matrix-assisted laser desorption ionization (MALDI) or electrospray ionization (ESI). Subsequently, the mass analysis is done with the help of techniques like time of flight (ToF) and quadrupole ion trap. Finally, proteins are identified using any of the available bioinformatics tools such as peptide mass fingerprinting (PMF), sequence tag search, ms/ms ion search.

formats. In one-dimensional gel electrophoresis or sodium dodecyl sulfate-polyacrylamide gel electrophoresis, proteins are separated in a single dimension based on their molecular weight [8] or separated as a native complex [9]. In 2-DE, proteins are separated in the first dimension according to their isoelectric point, and in the second dimension by molecular weight [10]. Subsequent to PAGE, proteins can be visualized with an MS-compatible staining procedure such as glutaraldehyde-free silver staining protocols [11], Coomassie blue staining [12], and colloidal Coomassie staining [13]. Importantly, silver staining protocols that use glutaraldehyde to cross-link proteins to the acrylamide gel are incompatible with MS. Before proceeding to MS analysis, some important considerations should be taken into account while preparing the sample. It is more advantageous to extract peptides by in-gel digestion rather than the whole protein itself. Subsequent to recovery from gel, peptides should be purified to remove salts, buffers, and detergents, which may interfere with MS [14]. Most often, peptides have to be concentrated commonly by reverse-phase chromatography.

It is noteworthy to mention that 2-DE suffers from some major limitations. Apart from the limited ability of 2-DE to detect low abundance proteins, very small or large proteins, and basic and membrane/hydrophobic protein [15,16], their automation is quite difficult. This fueled the invention of more robust protein separation technologies such as liquid chromatography coupled with mass spectrometry (LC-MS). Liquid chromatography (LC) exploits the different physical properties of proteins such as size/mass, charge, or hydrophobicity for their separation or fractionation [17]. The different LC techniques include ion exchange, size exclusion, affinity, and reversed phase, as well as electrophoretic separation in liquid-phase techniques (e.g., capillary isoelectrofocusing, capillary zone, and free-flow electrophoresis) [18–20]. In size-exclusion chromatography, the mobile phase contains salt to suppress any specific charge interactions between the column matrix and the proteins. In ion-exchange chromatography, an increasing salt gradient is used for proteins elution, and in chromatographic focusing, proteins are eluted based on a decreasing pH gradient in the presence of urea, which is used as a denaturant. Since high concentrations of salt and urea are incompatible with MS analysis as well as with many enzymatic digestions, the proteins require desalting prior to further analysis. Reversed-phase high-performance liquid chromatography (RP-HPLC) is a widely used method to concentrate and desalt intact proteins, either on its own or downstream from other types of chromatographic separations. The mobile phase is volatile and compatible with MS; therefore, RP-HPLC is the preferred alternative used in various proteomic workflows.

In the protein analysis by MS, protein/peptide ionization and mass analysis succeed sample preparation. The two common approaches for ionizing the peptides are matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). These methods are called as soft ionization methods because they maintain the sample integrity, which is extremely important for accurate mass determination. Samples obtained from in-gel digestion can be used directly without any subsequent purification in MALDI, whereas purification is essential in ESI. Subsequent to ionization, mass analyzers in a mass spectrometer perform the mass analysis of the ions. There are different kinds of mass analyzers such as time of flight (ToF), quadrupole, and ion trap. Among them, ToF is the simplest, and it measures the m/z ratio of an ion by determining the time required for it to traverse the length of a flight tube. Inclusion of an ion mirror at the end of the flight tube has led to increased mass resolution. This is because the ion mirror reflects ions through the flight tube to a detector, thus increasing the length of the flight tube. Additionally, ion mirror corrects the small energy differences among ions.

1.2 MALDI-ToF-MS

MS has remained one of the major proteomic tools for simultaneous analysis of thousands of purified proteins rapidly. This technique is useful for obtaining structural information of proteins such as peptide mass and amino acid sequences. Protein analysis with MS is usually coupled with various techniques as it identifies proteins after vaporization and ionization [21]. Generally, a mass spectrometer comprises four main units, i.e., ionization source, mass analyzer, ion mirror, and detector. The two common methods for ionization include MALDI and ESI. The impact of these two techniques was evident from the fact that Fenn and Koichi Tanaka were awarded the Nobel Prize in 2002 for the discovery of ESI and MALDI, respectively.

1.3 LC-ESI-MS/MS

In LC-ESI-MS/MS approach, LC is used as a protein separation technique, whereas ESI is used for ionization of isolated peptides and tandem mass spectrometry (MS/MS) is used for mass determination. LC-MS is preferred over gas chromatography–mass spectrometry and LC with other detectors, due to the high specificity and the ability to handle complex mixtures. The invention of ESI has provided a simple and robust interface, which makes LC-MS a widely used technology. A wide array of samples can be subjected to LC-MS. Additionally, fast scanning speeds enable a high degree of multiplexing, and many compounds can be measured in a single analytical run. The development of economical and reliable instrument has led LC-MS to be one of the most preferred techniques in several areas of clinical biochemistry.

1.4 Nano LC-MS

Nanoscale liquid chromatography coupled with mass spectrometry (nano LC-MS) is gaining reputation as an indispensable tool in the field of proteomics in sample-limited conditions due to its higher sensitivity compared to conventional LC-MS techniques. Nano LC consists of analytical columns of internal diameter (ID) of 100 μm , 75, and 50 μm , which are called as nanoflow columns and are much smaller than the IDs of traditional columns. Using flow rates in the range of low nanoliter per minute results in high analytical sensitivity of this technique, whereas technical challenges such as lower resolution and extra-column effects are encountered with low flow rates [22]. This technique was used to sequence major histocompatibility complex (MHC) peptides from virus-infected cells [23] and also for identifying the peptides from *Trypanosoma cruzi* processed and presented by the MHC class I pathway [24].

1.5 Bioinformatic analysis

Bioinformatic analysis has become inevitable for handling the large amount of data generated by proteome studies. The development of protein databases has greatly boosted the protein identification. Some of the popular protein databanks include SWISS-PROT, PIR, Protein Research Foundation, and Protein Data Bank. Searching against the databases allows quick and accurate identification of a large number of proteins. Nevertheless, the quality of data obtained from MS, quality of database, and the search method used to influence the success of database searching [5].

Peptide mass fingerprinting (PMF): It was developed in 1993 and was the first method for protein identification from MS data. In PMF, the protein is identified based on comparison of the masses of peptides obtained from the proteolytic digestion of the target protein to that of the theoretical values predicted from in silico digestion of proteins in a database. This is an easy method to perform and is fast and fully automated. In addition to these advantages, there are some disadvantages as well. One of the major limitations is the ambiguity in protein identification due to peptide mass redundancy.

Sequence query/sequence tag search: In this method, partial amino acid sequence (sequence tag) is obtained by interrogating MS/MS spectrum that does not cover the entire peptide and therefore lacks specificity to match only the original protein in a database. As a result, identification is achieved by combining the tag with other information, such as enzyme specificity and peptide mass.

MS/MS ion search: In this method, in addition to matching peptide masses, masses of the fragments are also matched. After the in silico digestion, the predicted peptides are subjected to in silico fragmentation according to the Roepstorff–Fohlman scheme 17, and the predicted fragment masses are compared to the experimental values. Technically, this is a peptide identification method, and proteins are identified by inference.

Spectral library search: This approach utilizes the actual peptide spectra from MS/MS data. Unlike sequence databases, whose entries may be obtained automatically or semiautomatically by the annotation of genome sequences, this sort of information must be acquired in advance.

De novo peptide sequencing: This is a protein identification strategy wherein at first, peptide sequence data are obtained de novo from MS/MS data followed by searching all the peptide sequences in suitable databases. Advantages of this method include the possibility of exploring the peptide information across both DNA and protein databases its usage for organisms that do not have well-annotated databases [5].

2. Proteomic approaches in salmonid fish health research

During the recent past, there is an emerging interest among the researchers to decipher the salmonid fish health by employing proteomic tools to better understand the host–pathogen interaction, pathogenesis, and disease processes. Among the various salmonid diseases, quantum of work has been done on enteric redmouth (ERM) and myxozoan parasites and their effect on salmonid health.

2.1 *Yersinia ruckeri*

ERM disease or yersiniosis is an important acute or chronic disease in salmonids caused by the gram-negative rod-shaped enteric bacteria, *Yersinia ruckeri*. This opportunistic bacterium was originally isolated from rainbow trout (*Oncorhynchus mykiss*) from the United States during 1950s, and presently, the bacterium is reported globally from intensive fish culture systems across Europe, North America, South America, Australia, South Africa, the Middle East, and China. Among the host species, rainbow trout is identified to be the most susceptible fish species to *Y. ruckeri* infection particularly during early life stages (fry and fingerling). The typical clinical signs of the disease include subcutaneous hemorrhages in and

around the mouth and throat of the infected fish (which earned the name ERM disease); petechial hemorrhages on the surfaces of the liver, pancreas, pyloric caeca, swim bladder, and lateral muscles; enlargement; darkening of the spleen; and reddened lower intestine with opaque yellowish fluid [25].

Proteomic techniques have enabled the researchers to better understand the microbial pathogenesis, factors affecting the virulence of the pathogen, and host response during infection. Various proteomics works have been undertaken in *Y. ruckeri* to generate baseline information about the bacterium and its host [26–30]. Two strains of *Y. ruckeri* have been described, a motile strain that can secrete lipase (biotype 1) and a nonmotile strain that cannot secrete lipase (biotype 2). Importantly, the vaccine developed against biotype 1 could not offer protection against the recently discovered biotype 2 strain. Comparative proteomics was performed between biotype 1 and 2 strains under normal and iron-limited conditions [26]. Iron is an important nutrient for infectious bacteria that can help in host recognition and virulence. A total of 1395 and 1447 proteins of *Y. ruckeri* were identified under normal and iron-limited conditions, respectively. About 61 differentially expressed proteins were identified between normal versus iron-limited conditions in four different isolates of *Y. ruckeri* (Fig. 18.3). Among them, 35 and 26 proteins were up- and downregulated, respectively. Interestingly, many upregulated proteins had a biological function of iron-ion binding, transport, and siderophore biosynthesis. However, proteomics was employed specifically to understand the outer membrane protein (OMP) of *Y. ruckeri* [27]. OMPs have a major role in virulence and host–pathogen interaction during bacterial infections. Initially, a total of 141 OMPs were predicted using bioinformatic tools, of which 77 were identified as “core” proteins. Subsequently, about 84 unique OMPs were identified in *Y. ruckeri*, employing both gel-free and gel-based proteomic approaches. Some of these OMPs are known to be involved in disease pathogenesis and could be potentially targeted for developing vaccines.

Several researchers have investigated the host proteome response during *Y. ruckeri* infection [28–30]. A shotgun proteomic approach was done to investigate the proteome changes in the major lymphoid organs such as the head kidney and spleen of rainbow trout affected by ERM disease [28]. About 34 and 85 proteins were differentially expressed in the head kidney and spleen, respectively. Carbon metabolism, ribosome, and phagosome pathways were predicted to be enriched in the spleen of the infected fish using protein–protein interaction network analysis (Fig. 18.4). The intestine serves as an important site of host–pathogen interaction during ERM disease. Shotgun proteomics of the intestine in rainbow trout affected by ERM disease resulted in 437 differentially expressed proteins, of which 205 and 232 proteins were up- and downregulated, respectively [29]. This experiment suggests that the proteome of the intestinal mucosa of the fish host is significantly modulated by *Y. ruckeri* infection. Researchers also investigated the proteome changes in Chinook salmon (*Oncorhynchus tshawytscha*) cell line during *Y. ruckeri* invasion [30]. Many surface adhesion proteins were found to be significantly modulated during *Y. ruckeri* invasion. Taken together, proteomics serves as an excellent tool to understand both the host and the pathogen interaction during *Y. ruckeri* invasion.

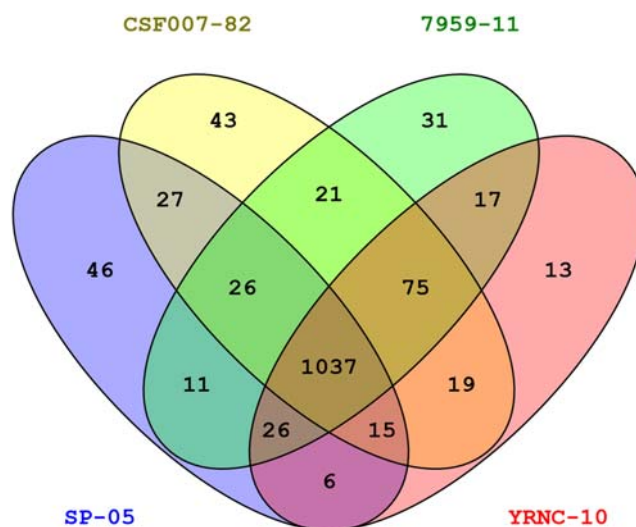


FIGURE 18.3 Differentially expressed proteins identified in *Yersinia ruckeri* strains. Four bacterial strains were used belonging to biotype 1 (SP-05 and CSF007-82) and biotype 2 (7959-11 and YRNC-10). G. Kumar, K. Hummel, M. Ahrens, S. M. Ledouble, T. J. Welch, M. Eisenacher, E. R. Fazeli, M. Matbouli, El Shotgun proteomic analysis of *Yersinia ruckeri* strains under normal and iron-limited conditions. *Vet. Res.* (2016), 47, 100.

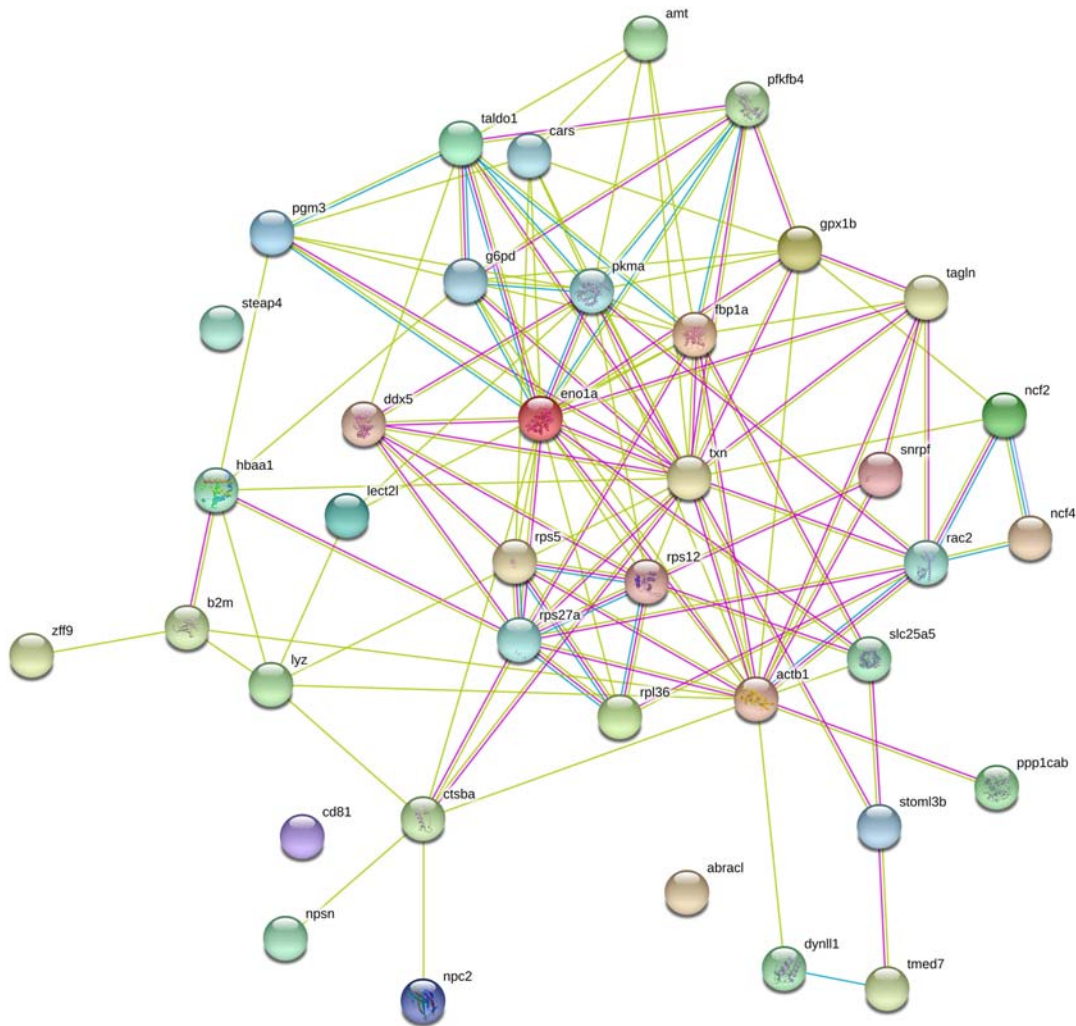


FIGURE 18.4 Protein–protein interaction network of 38 upregulated proteins (excluding uncharacterized proteins) in the spleen of infected rainbow trout during enteric redmouth disease. The network was constructed using the string based on zebrafish data. G. Kumar, K. Hummel, K. Noebauer, T.J. Welch, E. Razzazi-Fazeli, M. El-Matbouli. *Proteome analysis reveals a role of rainbow trout lymphoid organs during Yersinia ruckeri infection process. Sci. Rep.* 8 (2018) 13998.

2.2 Myxozoan parasites

Myxozoans are evolutionarily degenerate microscopic cnidarians that have a complex obligate parasitic life cycle involving an intermediate vertebrate host (fish, amphibians, reptiles, birds, and mammals) and a definitive invertebrate host (annelids and bryozoans). About 2200 species of myxozoans have been described till date, and this number is likely to increase further due to the increased research efforts on this obligate parasite [31]. Myxozoan diseases such as proliferative kidney disease caused by *Tetracapsuloides bryosalmonae*, whirling disease caused by *Myxobolus cerebralis*, and salmonid enteronecrosis caused by *Ceratonova shasta* are responsible for the serious economic losses in salmonid aquaculture. Moreover, these diseases are also responsible for the decline of wild salmonids in their natural habitat [32]. The rising water temperature due to global warming is known to significantly influence the pathogenicity and spread of myxozoan parasitic infection. Hence, there is a growing research interest on the myxozoan parasites, particularly to decipher them using omics technologies such as genomics, transcriptomics, and proteomics.

Compared to free-living cnidarians (corals, jellyfish, sea anemones, etc.), myxozoan parasites have evolved to extremely reduce their size, and at the same time retained their nematocyst (stinging organelle) in the form of a polar capsule. In the free-living cnidarians, the nematocyst is used for defense and to capture the prey. However, in the case of myxozoan parasites, the polar capsule aids in host attachment and infection. Till date, nematocyst proteomes of *C. shasta*,

Myxobolus honghuensis, *Myxobolus wulii*, and *Thelohanellus kitauei* have been generated [33,34]. In case of *M. honghuensis*, *M. wulii*, and *T. kitauei*, a customized comprehensive proteomic reference database has been developed for the nematocyst proteome, whereas no functional information about the protein is available [33]. However, in case of *C. shasta*, a detailed baseline functional information about the proteome has been generated [34,35].

The myxozoan *C. shasta* is an important parasite that affects and causes large-scale mortalities in the wild and cultured salmonids. *C. shasta* is native to the freshwater bodies of the Northwestern Pacific region of the United States. The portal entry of *C. shasta* in the fish host is through gills and it reaches the intestine via the circulatory system. Later, the development of parasite in the intestine causes hemorrhage and severe inflammation that eventually leads to enteronecrosis. Proteomics was employed to study the evolutionary adaptation of polar capsule to parasitism in *C. shasta* [34]. A customized dielectrophoresis-based microfluidic chip was used to segregate the polar capsules and the proteomic data were generated using MS/MS. A total of 112 proteins were identified in the polar capsule of *C. shasta*, and they were classified under different categories as shown in Fig. 18.5. Further analysis of these proteins from the polar capsule of *C. shasta* revealed that the polar capsule of the myxozoan parasites is homologous to the nematocysts of free-living cnidarians, and the parasite has retained the basic structural and housekeeping proteins originally present in the free-living cnidarians. However, the parasite has lost the toxin proteins in its polar capsule.

Similarly, comparative proteomics was done in *C. shasta* between the myxospore stage released by the invertebrate host and the developmental stages in the fish host using a Q Exactive Plus mass spectrometer [35]. A total of 2123 proteins were identified in *C. shasta*, of which 2084 were common in both life stages. Furthermore, 39 and 169 proteins were unique in the myxospore and the developmental stages in the fish host, respectively (Fig. 18.6). Proteins involved in metabolism and ribosomal and cytoskeleton proteins were upregulated in the developmental stages, whereas expressions of unknown proteins and nematocyst proteins were high in the myxospore. Moreover, the results suggest that the mobility in the myxozoan developmental stages in the fish host facilitated by lamellipodia and filopodia is attained via dynamic proteomic changes in the actin cytoskeleton. At the same time, the mature myxospores are reinforced by F-actin stabilization supported by the actin network (Fig. 18.7). Thus, proteomic tools can be efficiently used to generate baseline information about the myxozoan parasite and the host–pathogen interaction. More research focus is needed to understand the host proteome response during myxozoan infection.

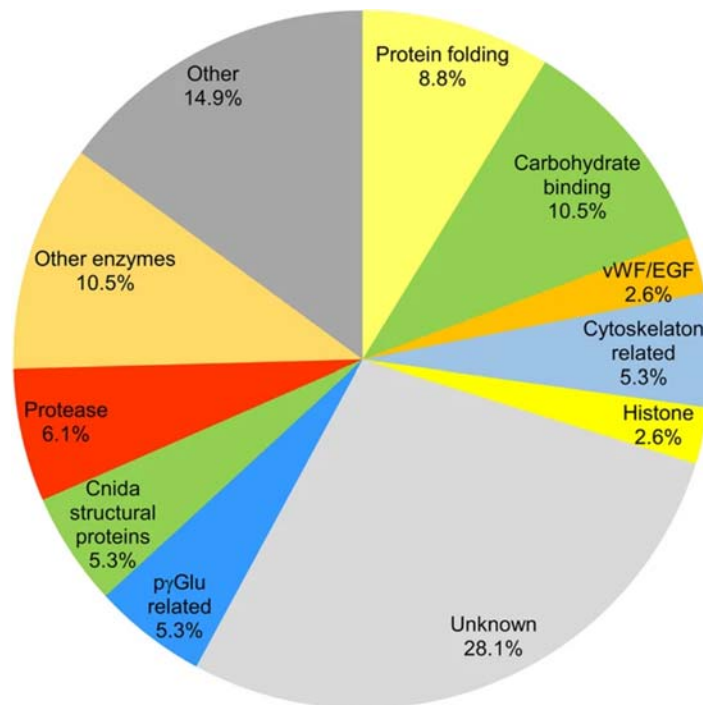


FIGURE 18.5 Classification of proteins identified in the polar capsule of *Ceratonova shasta*. G. Piriatskiy, S. D. Atkinson, S. Park, D. Morgenstern, V. Brekhman, G. Yossifon, J. L. Bartholomew, T. Lotan, Functional and proteomic analysis of *Ceratonova shasta* (Cnidaria: Myxozoa) polar capsules reveals adaptations to parasitism. *Sci. Rep.* (2017), 7, 9010.

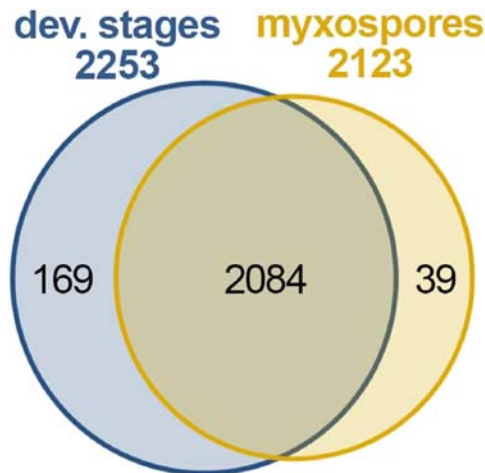


FIGURE 18.6 Proteins present in the mature myxospores and the developmental stages isolated from the fish host. V. Brekhman, M. Ofek-Lalzar, S.D. Atkinson, G. Alama-Bermejo, K. Maor-Landaw, A. Malik, J.L. Bartholomew, T. Lotan. *Proteomic Analysis of the Parasitic Cnidarian Ceratonova shasta (Cnidaria: Myxozoa) Reveals Diverse Roles of Actin in Motility and Spore Formation*. *Front. Mar. Sci.* (2021), 8, 632700.

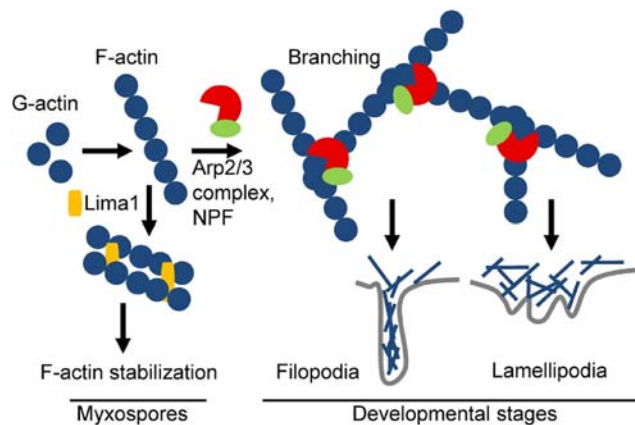


FIGURE 18.7 Arp2/3 and nucleation-promoting factors (NPFs) nucleate F-actin to produce branched actin that favors the formation of lamellipodia and filopodia that can facilitate mobility in the developmental stages. However, in the case of mature myxospores, the process of nucleation is inhibited and F-actin is more stabilized. G. Piriatskiy, S. D. Atkinson, S. Park, D. Morgenstern, V. Brekhman, G. Yossifon, J. L. Bartholomew, T. Lotan, *Functional and proteomic analysis of Ceratonova shasta (Cnidaria: Myxozoa) polar capsules reveals adaptations to parasitism*. *Sci. Rep.* 7 (2017), 9010.

3. Conclusion

It is evident that the application of proteomic approaches in salmonid health research shall help to develop effective vaccines and treatments for the devastating diseases. However, compared to other food production sectors like agriculture and veterinary sciences, only a few proteomic studies have been done in aquaculture. Moreover, many times scientists have to depend solely on the zebrafish proteomic database for annotation and protein–protein interaction analysis. Hence, we recommend that curated proteomic databases have to be developed specially for salmonids and their pathogens in order to advance the salmonid health research.

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Proteo-metabolomic technologies in context of fishery/aquacultural research and applications

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1. Introduction

Aquaculture, the fastest growing commodity in the global food-producing sector, provides an excellent source of nutrients and plays an important role in food security. In aquaculture, important fish groups belong to orders Cypriniformes, Gadiformes, Perciformes, Pleuronectiformes, and Salmoniformes. The current production has been intensified to meet the demand and is facing several challenges. Several new, sustainable aquaculture development strategies are in progress. These production challenges broadly include nutrition, quality and safety, stress and disease, ecotoxicology, and welfare of aquaculture species. Recent biotechnological innovations are playing a major role in improving the production of fish. Biotechnology-driven tools allow identification of traits in fish to increase productivity and improve quality.

Novel omics-based approaches in biotechnology can contribute to improving various aspects of the aquaculture value chain. As an aftermath of deciphering human genome database, the scientific community is working to access the information at molecular levels and their interactions between RNA, protein and metabolic level. Proteins are the expressed products of genes associated over almost all physiological functions of cellular life, ranging from the catalysis of biochemical reactions within intermediary metabolism to the processing and incorporation of internal and external signals. Metabolomics is the study of metabolites (such as amino acids, fatty acids, and carbohydrates) in cells, tissues, or biological fluids. It primarily facilitates assessment of changes in metabolite levels. The amount of available information on this technique is a bit limited to arrive at a comprehensive understanding of the changes in the organisms, but further developments are bound to enable better understanding via this. In recent years, omics applications in the fishery sector has made it convenient to acquire bioinformation in many studies, especially the screening of key pathways or major genes for the regulation of economically important traits.

In fish, omics such as genomics, transcriptomics, proteomics, and metabolomics has revealed importance in biological phenomena. Where aquaculture is concerned, high-throughput omics approaches have been utilized in various aspects, such as whole genome sequencing, which is continuously developing with improving sequencing platforms. Among fishes, Japanese puffer fish is the first fish whose whole genome was sequenced, and as per the recent reports (till 2019), genome sequence was available for ~300 fish species on NCBI or public repositories [1]. Recently, genome maps of *Labeo rohita* (rohu) [2] and *Clarias magur* (magur) [3] have also been reported. Despite the early success of genomic data in food fish, information on the fish proteome and metabolome is marginal. Moreover, the genome sequence information is currently available only for a few food fishes, which makes it more difficult to annotate the proteome-level data. Even targeted mass spectrometry (MS) methods are at times difficult to implement, as reference proteomes of nonmodel species are not readily available.

Development of various mass spectrometric methods in proteomics for identification of protein such as matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), which allow the soft ionization of a peptide, has paved a way for increasing proteomics research. Various such instances are available in literature, which represent the potential of proteomic technologies in providing proteomic resources for future fish research. In a recent work by Nissa

et al. [4], a comprehensive proteome and posttranslational modification map of multiple tissues of *L. rohita* has been reported. The same group also developed huge peptide repository in the form of PeptideAtlas build [5]. This is a valuable proteomic resource for *L. rohita* and first of its kind for any food fish. Metabolomics represents the global assessment of metabolites in a biological sample and reports the closest information to the phenotype of the biological system. Metabolomics has gained importance in biotechnological applications concerning food quality prediction, food safety, and understanding the mechanism of infection.

The progress of multiomics research has prompted the development of new tools and web-based applications that enhance thorough interpretations and correlation of biological molecules. The combination of proteomics with other omics approaches has expanded the repertoire of tools needed to study diet formulation, disease diagnosis and welfare, adulteration, brood stock, and breeding programs. With the recent advancement of next-generation sequencing and MS, there is upcoming integration of omics in biological features in order to study a biological system as a whole. An untargeted proteomic and metabolomic approach using liquid chromatography (LC) coupled with Orbitrap high-resolution (HR) MS was used to detect the adulteration in *Pangasius hypophthalmus* [6]. In chilled storage of grass carp, degrading bacteria were analyzed using integrated proteometabolomic method for new preservation methods [7]. For many culture species, initial larval production yields are greatly hampered by high mortality rates due to scarce knowledge regarding their fundamental study on genetic, environmental, nutritional, or disease-related factors. In embryogenesis of zebrafish, metabolome is highly dynamic and can be used to successfully predict development stage, identify provisional requirements for energy acquisition and growth, and reveal coordinated flux in gene and protein expressions. The proteins and metabolites would also help in the comprehensive understanding of molecular communication and provide new insights into the molecular mechanism of a fish. This chapter focuses on applications or potential of omics technologies with special emphasis on metabolomics and proteomics in fishery or aquaculture sector required for a sustainable production.

2. Basic terminologies related to proteomics and metabolomics

Proteins are the biomolecules consisting of amino acids as the building blocks and are essential to life. Proteins are involved in several functions inside the body such as those related to immune mechanisms, cell signaling and regulation, transport, structure, metabolism, and many more [8]. Proteome indicates all the proteins present in a cell, tissue, or organism at a given time, and proteomics is an umbrella term used for the study of proteome. The proteome is not static like the genome, so it is studied at a given time and can be unique to specific types of cells or tissues within the same organism [9].

However, the term metabolome indicates the complete set of metabolites, i.e., small molecules present in a cell, tissue, organelle, biofluid, or a whole organism. Metabolites includes both endogenous molecules like amino acids, nucleic acids, carbohydrates, sugars, amines, and vitamins and exogenous molecules like drugs, toxin, and food additive. Metabolomics is the systematic study of all chemical processes involving metabolites, with the goal of identifying chemical fingerprints that specific biological activities produce through the analysis of their small-molecule metabolite profiles [10]. Metabolomics includes the techniques and methods that can be utilized for the measurement of metabolites in cells, tissues, and body fluid samples [10]. The metabolic profile can provide a detailed picture of how a cell functions. Here a brief overview is provided for the experimental strategies used in fish or aquaculture research along with examples of its application for understanding different aspects as disease metabolism or identification of biomarkers.

3. Proteomic and metabolomic workflow

Proteomics techniques that are generally used for expression profiling include gel-based and gel-free proteomic separation, protein chromatography, and MS. Furthermore, depending upon the objective, discovery or targeted proteomic pipeline can be employed in a proteomic experiment. Discovery proteomics is also called as untargeted proteomics in which the main aim is to comprehensively identify all the protein in a sample. It is employed when there is no specific hypothesis or a simple hypothesis like there is a difference between the different sample cohorts such as control and test samples. In such an experiment, there is no prior information on what proteins are present in a sample. However, targeted proteomic analysis begins with a hypothesis and only a target panel is monitored in the samples. For an LC coupled with tandem mass spectrometry (MS/MS)-based proteomic workflow, initial sample preparation could be the same for discovery and targeted proteomics and it may vary during the data acquisition in MS. A general proteomic workflow for sample preparation and analysis is shown in Fig. 19.1A.

Advancements in MS- and nuclear magnetic resonance (NMR)-based technologies have paved a way for obtaining more data and metabolomic information for biological samples. Metabolomics approach can be used for screening the

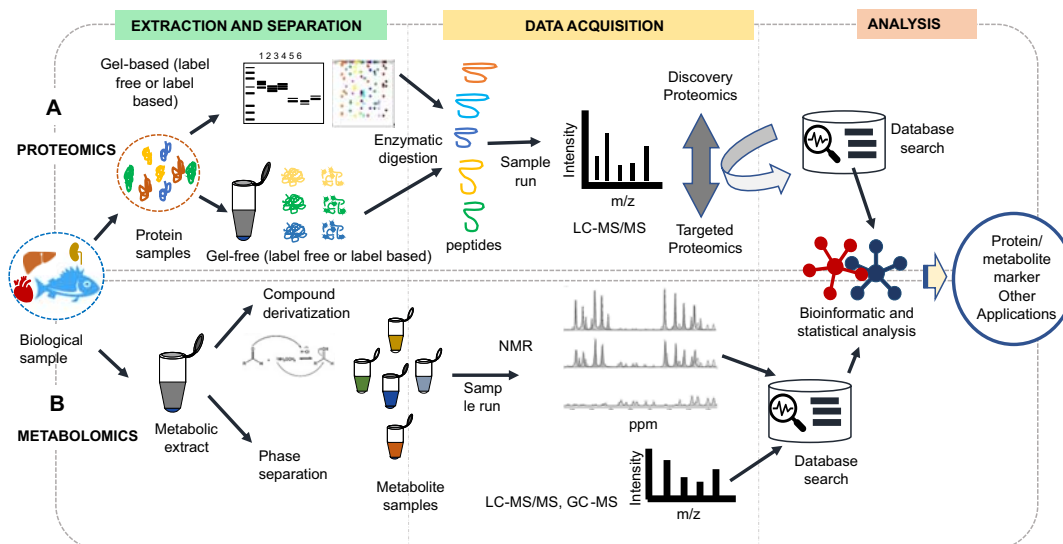


FIGURE 19.1 Schematic representation of general proteomic and metabolomic workflows. (A) Proteomic workflow showing that biological samples are processed for protein extraction followed by gel-based (one-dimensional electrophoresis [label-free] and difference in gel electrophoresis [label-based]) or gel-free approach (label-based/label-free) to obtain peptides, which are subjected to liquid chromatography–tandem mass spectrometric (LC–MS/MS) analysis for data acquisition. The data acquired can be through targeted or untargeted manner, which is further analyzed (statistically) to obtain a biological conclusion. The analysis can lead to the validation of some hypothesis or biomarker that can be further utilized for various applications. (B) Metabolomic pipeline showing sample separation by derivatization or polarity-based phase separation, followed by data acquisition by LC–MS/MS, gas chromatography (GC)–MS, LC–high-resolution mass spectrometry, nuclear magnetic resonance, and analysis of the acquired data to give significant metabolites, finally leading to a biological conclusion and application.

metabolite profile or for detecting the changes occurring at the metabolite level in a biological system under the influence of an internal or external stressor [11]. Recent aquaculture research using metabolomics techniques has revealed its enormous potential for tackling problems in all elements of the production line, from hatchery production to postharvest quality control [12]. Generally, two types of metabolomic workflows can be utilized depending upon whether the objective is to perform untargeted or targeted metabolomics. For a general metabolomic experiment, the pipeline includes sample preparation to extract the metabolites followed by data acquisition with analytical methods, identification, and quantification of metabolites and further statistical analysis to make some biological interpretation/conclusion (Fig. 19.1B).

Untargeted metabolomics is the discovery-based analysis of samples and is performed to generate hypothesis or when there is some generic (not very specific) hypothesis such as, some differences in metabolite profile between the experimental conditions [13]. Basically, the untargeted metabolomics aims to entirely profile the samples to identify as many metabolites as possible [13]. With such approach, all possible metabolic networks and pathways are identified, which might be playing important role in a particular (experimental) condition. The findings from such an approach can then be studied in detail to get more insights. However, a targeted metabolomic study design starts with a hypothesis and focusses on monitoring specific number of metabolites. Such an approach is a kind of validation and considers the metabolites that were found to be significant in a discovery analysis or reported in literature as significant biomarkers. The target metabolites might be important players or interactors of a specific metabolic pathway. This can also be referred to as hypothesis testing and it provides high accuracy, specificity, and precision [14].

4. Techniques/approaches used for proteomic analysis

Various techniques used in proteomic analysis of biological samples can be gel based or gel free as briefed below.

Gel-based approaches: Here the purified protein or mixture of protein is run in polyacrylamide gels during electrophoresis. Then the desired gel band/spot is excised and analyzed using MS after doing in-gel protein digestion. The electrophoresis may include one-dimensional electrophoresis (1-DE), two-dimensional electrophoresis (2-DE), or two-dimensional difference in gel electrophoresis (2D-DiGE). Gel-based approaches are the most popular and adaptable methods of global protein separation and quantification. They are well established for screening protein expression on a large scale and comparatively less expensive than gel-free proteomics [15]. 1-DE has been used for understanding disease pathobiology in rainbow trout or effect of differential diets on allergenicity [16,17]. In case of 2-DE, the protein mixture

undergoes electrophoresis in two dimensions, in which the proteins are separated first based on their isoelectric points (pI) followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) for molecular weight–based separation. This technique was introduced by O’Farrell and Klose in 1975, and it provides a good resolution. The general workflow for a typical 2-DE experiment includes extraction of proteins, first-dimensional separation through isoelectric focusing, SDS-PAGE separation, gel staining, protein spot visualization, and band excision followed by MS for protein identification. Thousands of spots corresponding to different molecular forms of proteins with same molecular weight but different pIs can be visualized on the same gel [18]. 2D-DiGE is the advanced form of traditional 2-DE, which comes under the label-based and gel-based technology. The workflow for a DiGE experiment follows the same steps as that of traditional 2-DE, except that in this case, the proteins are first labeled with reactive fluoresce cyanine dyes (Cy2, Cy3, and Cy5) and then isoelectric focusing is performed. The advantage of DiGE over 2-DE is that it overcomes the gel-to-gel variation and provides visualization of proteins from two different samples on the same gel [18]. Gel-based proteomics have been employed to understand different aspects in case of fishes such as effect of ocean warming on fish health and metabolism [19], molecular mechanism of skeletal deformities [20], and authentication of fish species [21].

4.1 Gel-free proteomics approaches

Although the gel-based proteomics are well established, there are few concerns when dealing with large number of samples and/or objective is comparative proteomic analysis of a greater number of sample/cohorts. In gel-free approaches, protein digestion and separation are performed in a gel-free manner. Here after protein solubilization, the protein digestion can be performed using different strategies such as in-solution digestion or filter-based methods such as filter-assisted sample preparation [22], Suspension Trap (S-Trap) [23], and single-pot, solid phase–enhanced sample preparation (SP3) [24]. Filter-based methods generally combine the advantages of in-gel and in-solution digestion methods. Filter-based methods are more compatible with a wide range of salts and detergents, using a filtering device for the digestion into peptides. The eluted peptides can then be desalted prior to MS analysis. In S-Trap protocol, methanol and phosphoric acid are used to from a protein suspension from SDS. Then suspension is then trapped on porous material, and SDS residue is washed away. In case of SP3, contaminants or interfering compounds are removed by using a hydrophilic interaction mechanism. After protein digestion, the peptide sample is separated using LC followed by analysis in mass spectrometer.

Further gel-free experiments can be performed in a label-based or label-free manner. Labeling can be done at amino acid, peptide, or protein level using isotope labels. It includes chemical labeling such as isobaric tag for relative and absolute quantification (iTRAQ), isotope-coded affinity tag, and tandem mass tags (TMTs); enzymatic labeling such as ^{18}O oxygen labeling; and metabolic labeling such as stable isotope labeling of amino acids in cell culture [25]. Label-based approaches allow multiplexing and high-throughput analysis in a proteomic experiment, and quantification is generally based on reporter ions specific for the labeling method. In case of label-free quantification method, the peptide sample is directly subjected to MS and quantification is based on spectral counting or ion intensity [26]. These label-based and label-free approaches are used for discovery or untargeted proteomic analysis. For performing targeted proteomic analysis, currently used techniques include multiple/selected reaction monitoring (MRM/SRM), parallel reaction monitoring, and sequential window acquisition of all theoretical spectra (SWATH) [25].

Proteomic approaches have been utilized to study various aspects of fishes starting from basic biology to understanding disease pathogenesis and authentication or identification of a species as shown in Fig. 19.2A–B.

4.2 Techniques/approaches used for metabolomic analysis

Advances in scientific understanding are propelled by technological advancements. In metabolomics research, a variety of analytical methods are used, among which MS and NMR spectroscopy are most commonly used. Each of these techniques has its own strengths and limitations [14]. MS-based metabolomics is an effective technique for even low-molecular-weight metabolites as it provides both selectivity and sensitivity. NMR spectroscopy is used for analyzing the metabolites in tissues or biofluids and it does not involve any additional step of derivatization, preselected measurement parameters, or separation [11]. However, instead of so much improvement in the sensitivity, it remains a weak point of NMR methods compared to MS-based methods. In short, it can be inferred that these two methods (NMR and MS) complement one another, and combining them can provide more complete information in the realm of metabolomics.

4.3 Nuclear magnetic resonance for metabolomics

NMR is a spectroscopic technique that works on the principle of energy absorption and re-emission of nuclei of atoms in response to changes in an external magnetic field [10]. NMRs are only active for a few atoms, including ^1H , ^{13}C and ^{31}P .

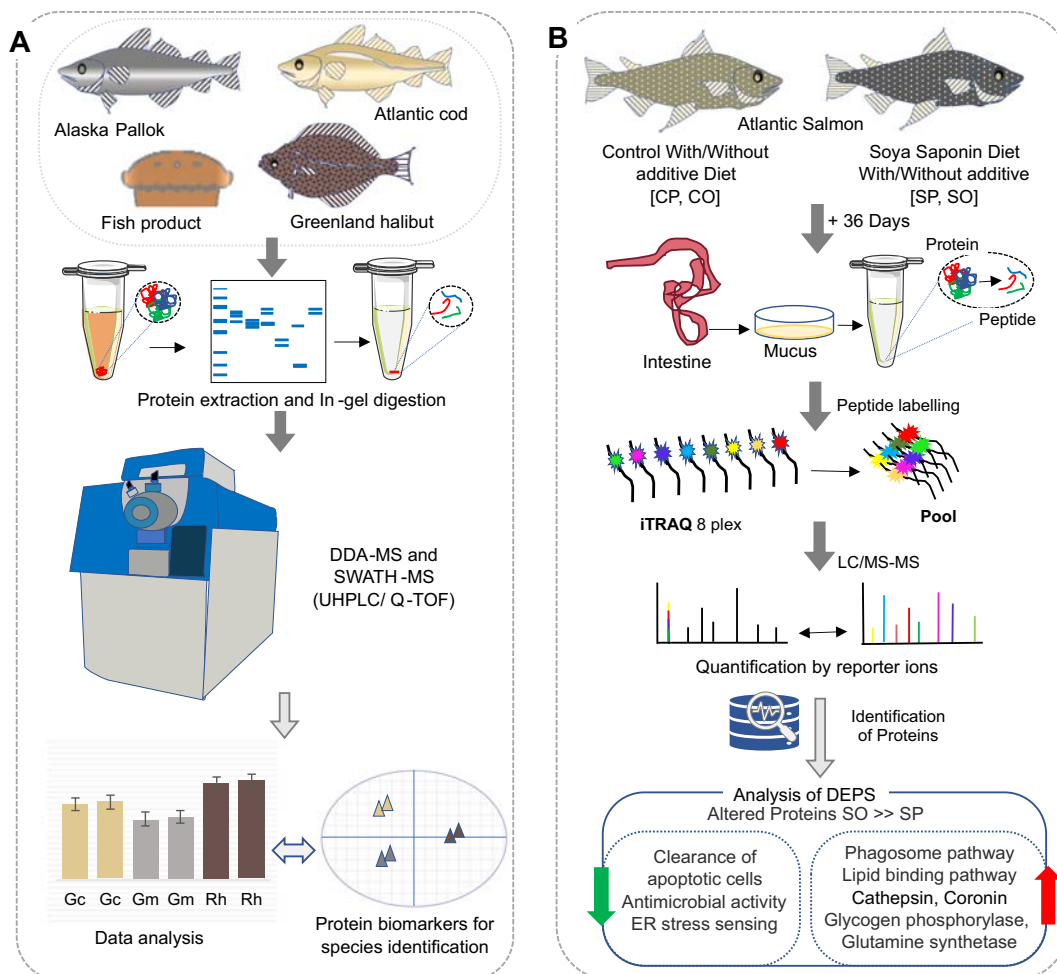


FIGURE 19.2 Examples of studies in fishery research using mass spectrometry-based proteomic approach. (A) Representation of the work done by Chien et al. [27] to identify the protein biomarkers for species identification and authentication of fish product using ultrahigh-performance liquid chromatography-quadrupole time-of-flight-mass spectrometry (UHPLC-QToF-MS). The study was performed using three fish species, Alaska pollock (*Gadus chalcogrammus*), Atlantic cod (*Gadus morhua*), and Greenland halibut (*Reinhardtius hippoglossoides*), and corresponding fishery product. After protein extraction, in-gel enzymatic digestion of protein was performed to obtain peptides that were subjected to sequential window acquisition of all theoretical fragment-mass spectrometry (SWATH-MS). The acquired data were analyzed and proteomic markers were identified and validated for species identification and authentication. (B) Schematic representation of the work by Kiron et al. [28], where the group studied the intestinal inflammation in Atlantic salmon (*Salmo solar*) by analyzing the dietary effect of inflammation inducer and health additive. Two control groups of fishes consisted of fish diet with health additive (CP) and without health additive (CO) and the test groups diet consisted of soy saponin (inflammation inducer) with a health additive (SP) and without a health additive (SO). After differential feeding of 36 days, the intestinal mucus samples were collected followed by protein extraction and in-gel digestion to obtain the peptides. Isobaric tag for relative and absolute quantification (iTRAQ 8-plex)-based proteomic approach was used to obtain the mass spectrometry (LC-MS/MS) data followed by protein identification and quantification with reporter ions. The comparative analysis of differentially expressed proteins (DEPs) revealed more alteration of proteins in SO group than SP group. Several downregulated proteins were related to the biological processes like clearance of apoptotic cells, antimicrobial activity, and endoplasmic reticulum (ER) stress sensing. Pathways like phagosome and lipid binding were upregulated with upregulation of various proteins like cathepsin, coronin, glycogen phosphorylase, and glutamine synthetase.

NMR generates spectral data that can be used to quantify metabolite concentrations and characterize their chemical structure. The most frequently used approach for metabolomics under this category is proton NMR (^1H) spectroscopy. In this technique, a liquid sample is inserted into a tube of around 5 mm diameter. Radio frequencies are pulsed in a range that covers all energies that are required for the excitation of the selected nuclei [14]. Depending upon the chemical environment, nuclei absorb energies at a particular radio frequency. The absorbed energy is released and the signal generated is measured known as free induction decay (FID). Then using a Fourier transformation, the conversion of FID happens from time domain data to frequency domain data, resulting in an NMR spectrum that has absorption energy displayed against the peak intensity [14]. Furthermore, the technique can have variations depending upon the type of sample. In case of liquid samples, the NMR spectroscopy can be performed using conventional liquid NMR spectroscopy. However, tissues that are

in semisolid form, such as connective tissue and tumor tissue, may be subjected to variations in magnetic susceptibility and their HR spectra cannot be acquired by conventional methods [29]. In such situations, solid-state NMR HR magic angle spinning is generally used for spectrum acquisition. In a study, done by Cai et al., HR proton NMR spectroscopy was performed for metabolomic analysis of intact muscle tissue (salmon), a whole fish (Siamese algae-eater), and intact eggs (shishamo smelt). This study explored the feasibility of HR proton NMR based on intermolecular multiple-quantum coherence technique [29]. The NMR spectroscopy has been reported for metabolomic analysis of different fish samples related to fisheries or aquaculture applications. With an aim of developing functional foods, proton NMR spectroscopy has been employed to collect the spectral information of water-soluble fish muscle extract. The spectral information was used to explore the distribution of macromolecules like collagens and lipids in the sample [30]. The technique has been used in different application in fish studies such as those related to dietary effects on growth [31,32]. The technique has been used to fingerprint the metabolomic differences in different life stages in (young smolt and adult) Atlantic salmon (Fig. 19.3A).

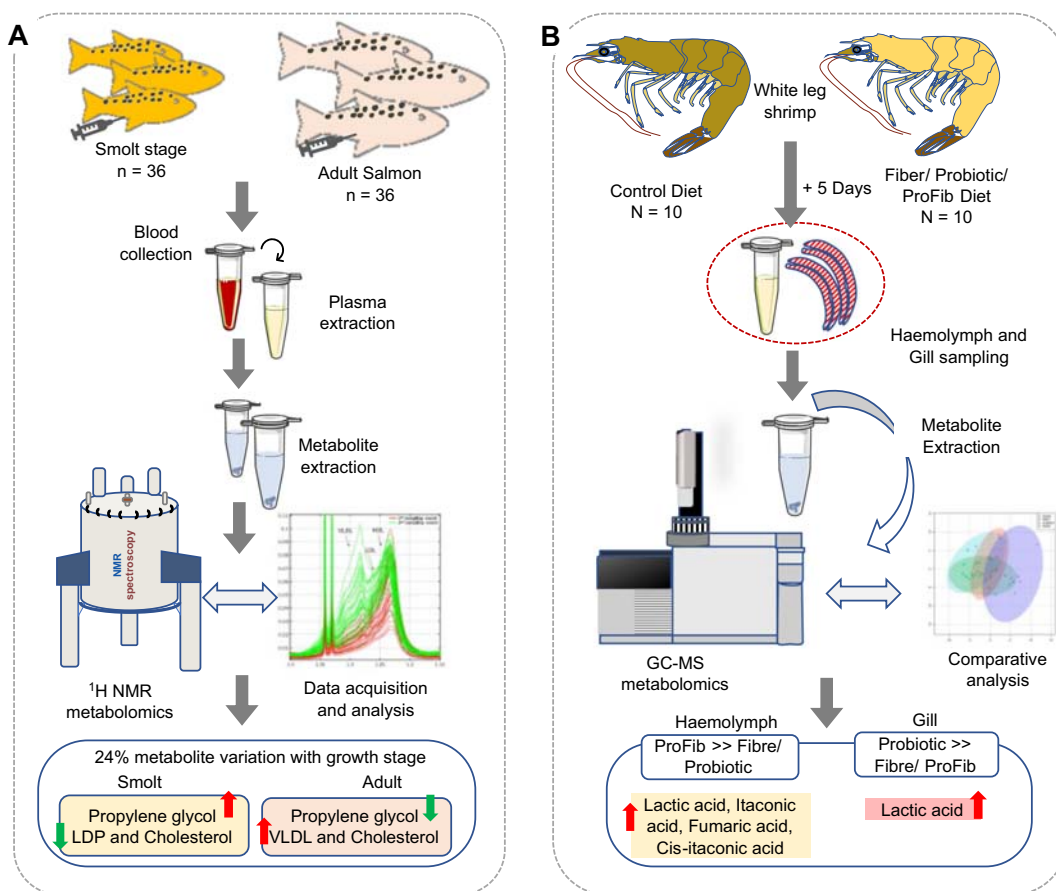


FIGURE 19.3 Examples of case studies in fishes using NMR and GC-MS-based metabolomics approach. (A) Representation of the work done by Aru et al. [33] to fingerprint the metabolome in young (smolt) and adult stages of blood plasma in Atlantic salmon using nuclear magnetic resonance (NMR) spectroscopy. A total of 36 samples each from two life stages were collected to extract the plasma followed by ¹H NMR spectroscopy-based data acquisition. The data were further processed and analyzed for the identification and quantification of metabolites. Around 24% of the metabolites were found to be differentially present in smolt and adult stages. Smolt stage has higher level of propylene than adults, whereas adult has comparatively higher level of very low-density lipoproteins (VLDL) and cholesterol in their plasma. (B) Representation of the work done by 'Alfaro et al. [34] to characterize metabolite profiles of the gill and hemolymph of *Penaeus vannamei* (whiteleg shrimp) using gas chromatography–mass spectrometry (GC-MS) approach. The shrimps were exposed to three different conditions (10 shrimps in each condition) including a diet of probiotic *Vibrio alginolyticus*, cellulose fiber diet, combination of probiotic and fiber diet (ProFib), and a control diet. The probiotic was added in water and fiber was given as a feed additive. Following this diet for 5 days, gills and hemolymph samples were collected and subjected for GC-MS-based data acquisition after metabolite extraction. The comparative metabolite analysis of different conditions revealed that for the hemolymph, a total of 27 metabolites showed alteration, and the ProFib diet showed the highest variation than other treatment conditions, whereas gill metabolites showed higher changes in probiotic group. Increased metabolites in the hemolymph include lactic acid and citric acid cycle intermediates like itaconic acid, fumaric acid, and cis-itaconic acid, while the gill showed significant increase of lactic acid in treated group.

4.4 Mass spectrometry for metabolomics

MS is a technique that measures the molecular weights of molecules. Main components of MS include the ion source, the mass analyzer, and the detector. Metabolites present in the samples get ionized at the ion source, and electrospray ionization and electron ionization are the majorly used ionization techniques for metabolites. Many thousands of metabolites can be detected and quantified simultaneously using MS-based metabolomics methods. However, the dynamic range and chemical diversity of the metabolome might make it difficult to reliably perform the identification and quantification measurements [35]. A separation step is usually performed before MS-based metabolomics to minimize the complexity of the biological sample that helps in the analysis of distinct sets of molecules. LC and gas chromatography (GC) columns, often known as LC-MS and GC-MS procedures, are the most prevalent separation techniques in MS technology [12].

For identification and quantification of small molecular metabolites (<650 Da), GC-MS-based metabolomics is ideal. These include sugars, small acids, hydroxyl acids, catecholamines, alcohols, amino acids, sterols, drugs, toxins, and fatty acids. The metabolites monitored with GC-MS are thermally stable and volatile or become thermally stable and volatile after modification in functional group [36]. Once the metabolite sample is ready, it is injected into the mobile gas known as carrier gas, which is then passed through the stationary phase. An inert/unreactive gas acts as mobile phase, which flows through a small diameter tube called column, inside the GC instrument. The column is coated with the material that may have variable affinities for various components present in the sample. Variable affinity of the different molecules with the column helps them in separating while they are flowing through the column, which is responsible for their differential elution resulting in a chromatogram [12]. This approach of GC-MS has been employed for the assessment of freshness in fish fillets through analysis of polar fractions of metabolites during cold storage [37]. The same approach has also been used to analyze the effects of different diets in *Penaeus vannamei* (whiteleg shrimp) (Fig. 19.3B).

LC-MS or high-performance LC-MS works on a similar principle to that of GC-MS, but in this case, sample heating at high temperature is not done. In case of GC, the metabolites are separately based on boiling point followed by retention with polarity, whereas in case of LC, polarity and metabolite size are considered. Here the sample flows in liquid mobile phase at high pressure through the column packed with material having affinity for the metabolites in the sample. LC-MS/MS has a number of advantages to GC-MS, including the ability to identify and analyze a wider spectrum of chemicals with little sample preparation [38]. Metabolomic profiling using LC-MS approach was utilized to explore the mechanism of fatty liver formation in tilapia fish [39].

5. Current proteomic and metabolomic studies for fishery/aquaculture and authors' perspective

With systems level understanding proving to be indispensable in growth, survival, and reproduction of every living being, omics approaches, especially proteomic and metabolomic studies, have lent novel insights into the nuances of aquaculture. The most recent studies on various aquaculture species have shed light on immune and environmental stress response in fish, effect of diet and health status, and development of quality control mechanisms.

Targeted proteomics and metabolomics are alternative methods to immunoassay, aiding discovery of interesting proteins and metabolites and their corresponding peptides or molecule significant in the samples. In a targeted proteomic assay, proteins of potential interest are detected with specificity, reproducibility, and accuracy. Targeted assays are essential for sets of highly expressed proteins and metabolites based on the expression value of the fish. PeptideAtlas is a bunch of peptides that can be used as an efficient resource for designing a targeted experiment or validating the protein/peptide target related to a shotgun experiment. One of the significant advantages of the targeted assay is to acquire exact expressed protein in a given sample. SRM, also called MRM, is recently the most commonly used targeted approach. For one of the aquaculture species, *L. rohita* (rohu), a new PeptideAtlas build has been reported [4]. PeptideAtlas is a user-friendly online portal for researchers who can freely access both protein and peptide-related information. PeptideAtlas contains all identified proteins and peptides that can be helpful for discovery experiments as well as designing targeted assays in rohu or related fish species.

In metabolomics, various differential extraction methods can ensure capture of the metabolome from the samples. Because the metabolome is extremely sensitive to the internal and the external condition of the organism, it becomes necessary to study even the minute differences in the metabolome, where the utility of sensitive separation techniques like liquid or GC coupled to high resolution MS or proton NMR spectroscopy is absolutely indispensable. The differing polarities of various metabolites and their detection enables targeted monitoring. The metabolome in certain cases is even subjected to further reactions like oxidation or reduction to enable a deeper analysis.

Currently available literature has proven the potential of these omics technologies with respect to their applications in sustainable fisheries or aquaculture as represented in Fig. 19.4.

A few examples of these applications are summarized in Table 19.1 and can be overviewed under the following heads.

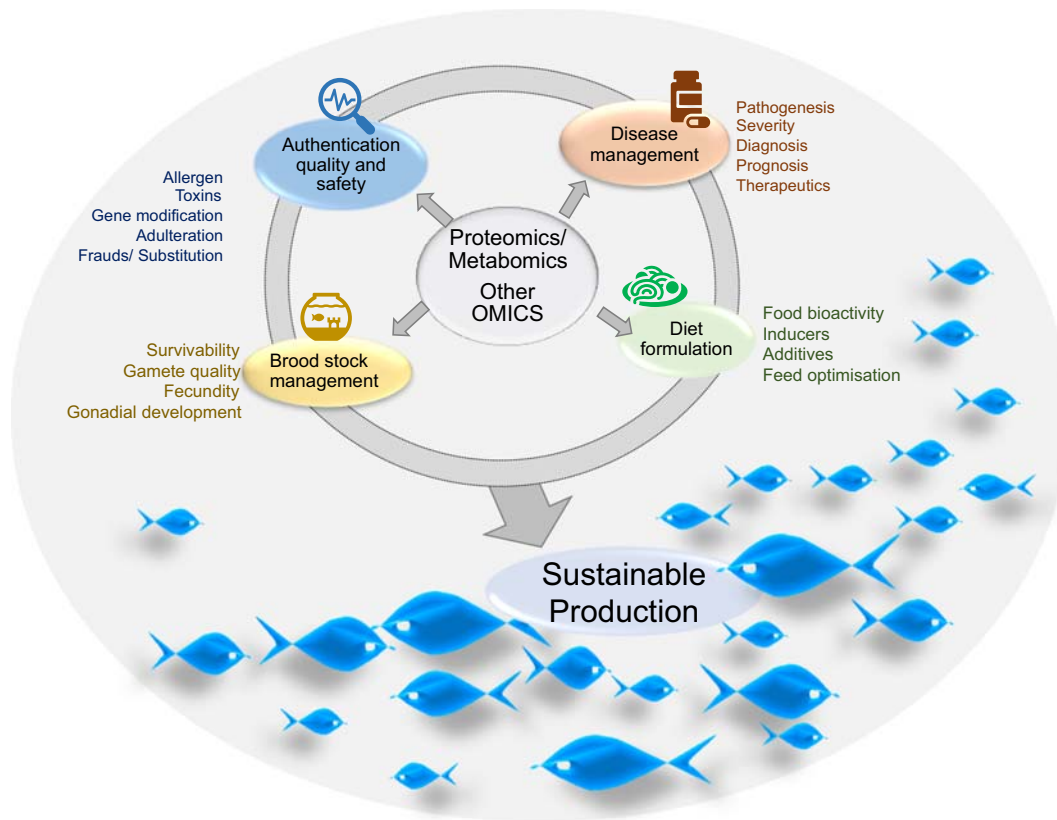


FIGURE 19.4 A schematic representation of the utility of omics approaches for sustainable aquaculture growth. Integration of metabolomics, proteomics, and other omics technologies can be utilized to access multiple factors in order to achieve sustainable fish production. Such approaches can be utilized for improving and optimizing diets for larvae and brooders, for analyzing bioactivity of food components, and accessing the effects of food additive or specific inducers (diet formulation). Other areas include the improvement of species health and welfare by understanding disease pathogenesis, understanding the severity factors, identifying diagnosis and prognosis markers, and improving therapeutics (disease management). These omics approaches can be utilized for species authentication to maintain the quality and safety such as by monitoring allergens, toxins, genetic modifications, adulteration, and frauds (authentication, quality, and safety). Such approaches can help in studying and improving survivability, gamete quality, gonadal development, and fecundity to help in brood stock management.

TABLE 19.1 Examples of proteomic and metabolomic studies for fishery/aquaculture.				
Category	Technique used	Species (tissue)	Aim of study	References
A. Dietary formulation				
Proteomics	LFQ	Atlantic salmon (<i>Salmo salar</i>): distal intestine and skin mucus	Immune and proteomic responses for soyabean meal	https://onlinelibrary.wiley.com/doi/full/10.1111/anu.13248
Proteomics	LC-MS/MS	Chinook salmon (<i>Oncorhynchus tshawytscha</i>): brain, liver, and intestine	Proteomic investigation of high feed intake and low feed intake Chinook salmon (<i>O. tshawytscha</i>)	https://www.sciencedirect.com/science/article/abs/pii/S0044848622000291
Metabolomics	¹ H NMR	Rainbow trout: plasma, liver, muscle	Assessment of metabolism and related growth and quality traits in trout fed spirulina-supplemented plant-based diets	https://doi.org/10.1016/j.aquaculture.2022.738033

TABLE 19.1 Examples of proteomic and metabolomic studies for fishery/aquaculture.—cont'd

Category	Technique used	Species (tissue)	Aim of study	References
Metabolomics	LC-MS	Genetically improved	Studying metabolic changes in genetically improved farmed tilapia (<i>O. niloticus</i>) with fatty liver induced by a high-fat diet	https://doi.org/10.1111/are.14929
		Farmed tilapia (GIFT, <i>Oreochromis niloticus</i>): liver tissue		
Metabolomics	¹ H NMR	European seabass (<i>Dicentrarchus labrax</i>): plasma tissue	Semiautomated analysis of fish plasma by ¹ H NMR metabolomics—applications to aquaculture	https://doi.org/10.1016/j.aquaculture.2022.738028
Metabolomics	LC-MS	Grass carp (<i>Ctenopharyngodon idellus</i>): flesh	Impact of flavonoid-enriched diets on the growth and flesh quality of grass carp (<i>C. idellus</i>) based on metabolomics	https://doi.org/10.1111/anu.13381
Metabolomics	NMR	Red drum, (<i>Sciaenops ocellatus</i>): liver	Assessment of the effect of combinations of natural feed items on juvenile red drum, <i>S. ocellatus</i>	https://doi.org/10.3390/jmse10040547
Metabolomics	NMR	Rainbow trout (<i>Oncorhynchus mykiss</i>): plasma, digesta metabolome	Impact of plant-based diet on plasma and digesta metabolome	https://doi.org/10.3390/metabo11090590
B. Disease response				
Proteomics	LFQ	Atlantic salmon: plasma	Identification of novel biomarkers of inflammation	https://doi.org/10.1016/j.dci.2021.104268
Proteomics	iTRAQ	Antarctic icefish (<i>Chionodraco hamatus</i> and <i>Notothenia coriiceps</i>): skin mucus	Immunity proteomic profile	https://doi.org/10.1111/jfb.14908
Metabolomics	GC-MS	Whiteleg shrimp (<i>Penaeus vannamei</i>): hemolymph and gills	Evaluation of immune stimulatory products for whiteleg shrimp (<i>P. vannamei</i>) by metabolomics	https://doi.org/10.1016/j.fsi.2021.12.007
Metabolomics	LC-MS	<i>Takifugu rubripes</i> : blood serum	Potential metabolomic biomarkers in blood serum of <i>T. rubripes</i> challenged with <i>Cryptocaryon irritans</i>	https://doi.org/10.1111/are.15289
C. Ecotoxicity and stress response				
Proteomics	Label-free MS	Rainbow trout	PAH toxicity in trout alevins	https://doi.org/10.1016/j.scitotenv.2022.154846
Proteomics	TMT	Hybrid grouper (♀ <i>Epinephelus fuscoguttatus</i> × ♂ <i>Epinephelus lanceolatus</i>): liver	Temperature and waterless stress	https://doi.org/10.1007/s00709-021-01654-w
Proteomics	2-DE	Chinese rare minnow	Larval exposure for cadmium and mercury	https://doi.org/10.1016/j.aquatox.2022.106134
Metabolomics	Proteome: LC-ESI-MS/MS; metabolome: GC-MS	Rainbow trout (<i>O. mykiss</i>) alevins: cardiac tissue	Changes in cardiac proteome and metabolome following exposure to the PAH retene and fluoranthene	https://www.sciencedirect.com/science/article/pii/S0048969722019398

Continued

TABLE 19.1 Examples of proteomic and metabolomic studies for fishery/aquaculture.—cont'd

Category	Technique used	Species (tissue)	Aim of study	References
Metabolomics	LC-MS/MS (DDA); HCD scan	Pacific white shrimp (<i>Litopenaeus vannamei</i>): intestine, hemolymph	Toxic effects of ammonia and thermal stress on the intestinal microbiota and transcriptomic and metabolomic responses of <i>L. vannamei</i>	https://doi.org/10.1016/j.scitotenv.2020.141867
Metabolomics	Reversed-phase chromatography, hydrophilic interaction chromatography/MS	Flounder (<i>Paralichthys olivaceus</i>): serum	The effect of temperature on flounder (<i>P. olivaceus</i>) via metabolomics and lipidomics	https://doi.org/10.1016/j.jtherbio.2022.103203
Metabolomics	LC-HRMS	Common carp (<i>Cyprinus carpio</i>): liver	Metabolome adaptation and oxidative stress response of common carp (<i>C. carpio</i>) to water pollution	https://doi.org/10.1016/j.envpol.2022.119117
Metabolomics	GC-MS	Mandarinfish (<i>Siniperca chuatsi</i>): serum	Metabolic differences of the farmed mandarin fish <i>S. chuatsi</i> in recirculating ponds aquaculture system and pond	https://doi.org/10.1038/s41598-020-63252-9
Metabolomics	UHPLC/MS-MS	European seabass (<i>D. labrax</i>): liver	Effect of temperature on the growth and development of juvenile European seabass (<i>D. labrax</i>)	https://doi.org/10.1016/j.scitotenv.2021.145155

5.1 Quality control of aquaculture species

Fish quality is of paramount importance in the development of aquaculture. Adulteration is one of the major issues in the food industry. Both proteomics and metabolomics approaches have been utilized for quality control check—based applications. The misidentification of species and product repackaging constitute the most common food frauds. Texture is the foremost quality indicator of fish for consumers, and better texture makes fish easy to process into high-quality products, for example, in pangasius adulteration by pork, which can be noticed by proteomic- and metabolomic-level studies [40].

In a study by Li and colleagues [41], a 2-DE coupled to MALDI—time of flight (ToF)/ToF MS was used to study the impact of hydroxyl radical oxidation on freshness indicator proteins of *Coregonus peled* upon storage at 4 °C. Around 382 differentially expressed proteins were identified, of which around 20 proteins were quantified and studied to reveal creatine kinase, malate dehydrogenase, and glyceraldehyde 3-phosphate dehydrogenase as potential biomarkers for protein oxidative damage, differentially active depending on the extent of storage time.

A TMT label—based quantitative proteomic approach was used to investigate the effect of dual-frequency sequential ultrasonic thawing on the meat quality of quick-frozen small yellow croaker (*Larimichthys polyactis*). The study revealed 75 differentially abundant proteins in the fish meat, of which 11 proteins were highly correlated and could be considered as biomarkers for analyzing texture profile, color, water-holding capacity, loss in thawing, and change in pH of thawed meat. Thus, this study also brings to light the utility of high mass accuracy spectrometers, as well as multiplexing power of chemical isobaric label application in analyzing the proteome and posttranslational modifications.

Stella et al. [42] applied untargeted metabolomics approach to identify markers of freezing or thawing in skinless European sea bass (*Dicentrarchus labrax*) fish fillets. In this study, 60 samples, each fresh and frozen/thawed, were analyzed by liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) in both positive and negative ion modes to find alteration of two metabolites, eicosapentaenoic acid and docosahexaenoic acid (DHA), as reliable predictors of fish fillet quality. Even fresh and frozen/thawed Atlantic salmon (*Salmo salar*) had similar alterations.

In another study done by Black and group [43], they developed a real-time metabolomic profiling approach via rapid evaporative ionization mass spectrometry (REIMS) capable of identifying species and the catch method robustly, without the need for any sample preparation. A total of 478 samples of five fish species, cod, coley, haddock, pollock, and whiting,

were subjected to REIMS analysis after chopping each sample 8–12 times (3–5s gap), using an electrosurgical knife. The species identification was near instantaneous with 99.98% accuracy.

Another study [44] utilized a combination of targeted and nontargeted metabolomics by LC–MS/MS and HRMS, respectively, to check for use of unauthorized dyes in fish. Rainbow trout was used for validation. The LC–MS/MS method included an oxidative step for most of the dyes, which was developed to target the analysis of 14 oxidized residues belonging to different families of dyes. Comparative analysis of nontargeted metabolomic fingerprints of farmed fish treated with malachite green and Victoria pure blue BO dye revealed some direct or endogenous metabolites, which can be further validated as potential biomarkers.

An ultra-performance LC–quadrupole ToF/MS–based metabolomic method coupled with transcriptomics was utilized by a group of researchers [45] to reveal the metabolomic impact of short-term micro-flowing purification system for different treatment periods (0 d, 1 d, 5 d, and 9 d) in crucian carp (*Carassius auratus*) and its subsequent influence on the flesh quality. The important roles that the tricarboxylic acid (TCA) cycle, ornithine cycle, purine metabolism, and amino acid catabolism play in determining the flesh quality of crucian carp were revealed in this study.

5.2 Fish disease and immune response

Fish health is one of the most important aspects of the welfare of aquaculture, which is vulnerable to all changes in the favorable and unfavorable environment. Any infectious disease in consumable fish may travel up the food chain and eventually affect humans [25]. Therefore, measures taken to improve fish health are of paramount importance, leading to farming of healthy fish, and thereby protecting the consumer's health. Characterization of the proteome and metabolome profiles can aid in understanding the pathogenesis and immune response of various fish species, thus yielding understanding of the overall health status of the aquaculture species.

An MS-aided plasma proteomic approach by a group of researchers [46] isolated immune biomarkers in Atlantic salmon (*S. salar* L.) blood when healthy fish samples were compared to *Aeromonas salmonicida* subsp. *salmonicida*–infected fish. The results of this study reveal numerous novel and robust biomarkers of the acute-phase response and inflammation in fish model at different time points after infection. Increase in haptoglobin, FcγR1, LRG1, and C4 and a consequential decrease in levels of fibrinogen, CD209, and CD44 after *A. salmonicida* infection are a few of the noteworthy results from the study.

Another group [47] used LC coupled with tandem MS iTRAQ technology to investigate the immune proteome profile of the skin mucus of Antarctic fish *Chionodraco hamatus* and *Notothenia coriiceps*. About 4444 proteins were identified in total, from which 227 differentially expressed proteins were found upon comparing profile of both species. Further analysis via KEGG, protein–protein interaction analysis, and “response to stress” GO category gave candidate biomarkers like C3, CASP1, ASC, F3, and C9, enabling better understanding of the fish immune system.

A metabolomic approach based on GC-MS was employed by Alfaro and team [34] to study the metabolite profiles in the hemolymph and gills of whiteleg shrimp (*P. vannamei*) exposed to four immune stimulatory treatments comprising cellulose fiber administered with feed, probiotics supplemented with *Vibrio alginolyticus* in water, both cellulose fiber and *V. alginolyticus* combined, and a control treatment. The best immune stimulation was achieved with the combination of cellulose fiber and *V. alginolyticus*. The increase in itaconic acid in the hemolymph and lactic acid in both the hemolymph and gill tissues of immune-stimulated shrimp suggests that possible use of these metabolites was as immune response biomarkers (Fig. 19.3B).

An untargeted LC-MS approach was used to profile potential blood serum biomarkers in *Takifugu rubripes* challenged with *Cryptocaryon irritans* in a study by Liu et al. [48]. This work was carried out using 10 infected and healthy samples each for LC-MS analysis, resulting in identification of 6 highly specific biomarkers for infection. These candidates included retinoic acid, progesterone, xanthosine monophosphate, hepoxilin B3, tamoxifen, and prostaglandin I2. Additionally, five severely affected pathways were identified including retinol metabolism, steroid hormone biosynthesis, purine metabolism, arachidonic acid metabolism, and drug metabolism involving cytochrome P450.

5.3 Ecotoxicity and stress response

Pollutants are usually hindrances to healthy growth and development of fish. The recent emerging contaminants like microplastics, nanoparticles, and organic chemicals can undergo bioaccumulation due to their persistence in aquatic animals like fishes. This have potential toxicity, affecting molecular antioxidant mechanisms, disorganizing food safety, and affecting human health in the process [49]. Temperature or thermal stress is another noteworthy perturbation to growth and survival of aquaculture species. Numerous proteomic and metabolomic studies have shed light on the mode of action and the effect of the various pollutants and environmental stresses on various aquaculture species.

In a study done by Eriksson et al. [50] on developing rainbow trout alevins exposed to polycyclic aromatic hydrocarbon (PAH), retene and fluoranthene, proteomic and metabolomic approaches were employed to test for the effect of the PAH exposure. The cardiac proteome data were acquired via LC–ESI–MS/MS and the metabolome data by GC-MS. The response to the PAH-induced toxicity was observed to manifest via disruption of different pathways causing impaired cellular integrity, oxidative stress, CYP1A inhibition, and induction of diverse phase I and phase II metabolic reaction, among others.

In another study [51], the impact of different Cd (0.5 and 2.5 mg/L) and Hg (0.1 and 0.3 mg/L) concentrations on Chinese rare minnow (*Gobiocypris rarus*) larvae was studied for 96 h, utilizing a quantitative proteomic and transcriptomic approach.

The effect of heat and ammonia on the intestinal transcriptome, microbiome, and metabolome of Pacific white shrimp (*Litopenaeus vannamei*) was studied by Duan and group [52]. The intestinal metabolomic data were acquired by LC–MS/MS HCD scans and analyzed to isolate 10 stress-related metabolite markers, namely L-lactic acid, gulonic acid, DHA, L-lysine, gamma-aminobutyric acid, methylmalonic acid, trans-cinnamate, N-acetylserotonin, adenine, and dihydrouracil. Thus, disruption of the intestinal amino acid metabolism is the most noticeable in ammonia and thermal stress response, along with increase in intestinal Firmicutes and decrease in Bacteroidetes abundances.

An in-depth metabolomic and lipidomic analysis of the temperature-dependent perturbations in flounder (*Paralichthys olivaceus*) by Liu et al. [53] revealed potential stress-induced biomarkers in the serum. This study utilized reversed-phase chromatography and hydrophilic interaction chromatography coupled with MS along with a multivariate statistical analysis to obtain 495 metabolites and 459 lipids and arrive at 53 significant differential metabolites. A similar study done by Zhang and group [54] on European sea bass (*D. labrax*) liver extract utilized ultrahigh-performance liquid chromatography–MS/MS to test for growth under different temperatures. The temperature-dependent alteration of energy metabolism pathways as well as the compensatory growth effect of European sea bass following a low temperature stress could be discerned from this study. It was also concluded that amino acid metabolism plays a crucial role in the compensatory growth.

LC-HRMS was used by Koubova et al. [55] to study the metabolic and stress response of common carp (*Cyprinus carpio*) to different levels of water pollution. Alternative shifting or restocking of fish from clean water to treated wastewater ponds, and a subsequent biomarker analysis of the fish liver, kidney, intestine, and gill tissues coupled to LC-HRMS profiling of liver pharmaceutically active compounds (PhACs), resulted in detailed stress response study. Upon short term (14 days) exposure to pollutants, the antioxidant response was enough to reverse the pollution-induced PhAC accumulation in the liver, while long term (60 days) exposure to pollutants caused irreversible changes in the fish metabolome.

In an attempt to develop an efficient, economic, and ecofriendly farming system for highly sensitive farmed fish *Siniperca chuatsi*, Xio and colleagues [56] studied the effect of recirculating aquaculture system (RAS) on the metabolic profile of the fish. Farming via RAS system was proven to be beneficial for the fish metabolism, reducing the concentration of stress-induced metabolites in the serum. Biosynthesis of unsaturated fatty acids, lysosome, and tryptophan metabolism pathways were observed to be enriched in the *S. chuatsi* farmed in RAS.

5.4 Reproduction-related research

iTRAQ-based proteomics was used in sex reversal mechanism in *Acanthopagrus schlegelii*, and 4692 proteins were identified in the fish gonads [57]. Differential expression of the common sex-specific proteins like sperm-associated antigen 6 and cilia; flagella-associated proteins in males; and zona pellucida sperm-binding proteins in females was observed [57]. Proteins involved in the integrin signaling, inflammatory pathways, pyruvate metabolism, CCKR signaling, de novo purine biosynthesis, and ubiquitin proteasome pathway were observed to be upregulated in female gonads, whereas proteins participating in DNA replication, the heterotrimeric G protein, Wnt, and hedgehog signaling pathways were upregulated in male gonads, thereby yielding a detailed fundamental account for further studies on molecular mechanisms of fish sex determination and reversal.

High-throughput proteomic information acquisition by SWATH-MS technique has been utilized for several species. In hepatic tissue of hybrid crucian carp, a total of 2502 proteins were detected in association with metabolism and growth rate [58]. Another SWATH-MS study showed that exposure of fluoxetine and 17 β -estradiol in sea bass scales revealed 213 proteins [59].

A group of researchers [60] studied the changes in metabolome of a critically endangered freshwater crayfish during its mating season via LC-MS. This study aimed to determine the metabolomic differentiation between sexes, their reactive responses to pairing, as well as disturbance of pairing and the convergent response to the disturbance of pairing for males specifically. A few significant metabolites with sex-specific alterations were obtained comprising tyrosine, tryptophan,

arginine, adenosine, acetyl-L-methionine, betaine, carnitine and its derivatives, citric acid, glutamine, pantothenic acid, kynurenine acid, inosine, indole-3-acetic acid, and glutathione. They have various roles ranging from stress response to energy mobilization to growth and yolk deposition in females. Thus, such a targeted metabolomic approach succeeded in identifying several biomarkers for testing hypotheses based on reproductive success in fish species.

5.5 Diet formulation

Optimal nutrition is the foremost requirement for the development of aquaculture. The fish feed takes up almost half the cost for production. The advent of omics-level monitoring systems has ensured data-driven decision-making in deciding upon optimal fish nutrition from a plethora of alternatives. Developing such new species-specific diet is essential for the expanding aquaculture industry as it strives to satisfy the ever-increasing demand for safe, inexpensive, good quality fish and other consumer products. Proteomic and metabolomic studies have been engaged in looking for diet-related perturbations in various model fish species.

Quantitative proteomic methods have undergone various advances that assist in overcoming experimental challenges associated with farm or wild fish. Evidently, 1-DE and 2-DE have been the most frequent proteomic techniques used in fisheries research. In organ-specific protein profiling of rohu, 1-DE-based protocol was exploited for 17 tissues along with embryo and plasma. The effect of the inclusion of *Tenebrio molitor* (TM) larvae in the meal for three farmed fish species—gilt-head seabream (*Sparus aurata*), European sea bass (*D. labrax*), and rainbow trout (*Oncorhynchus mykiss*)—was studied using 2-DE by Mente and colleagues [61]. The protein profile of the hepatic extracts from control (0% TM inclusion) and sample fish (25%, 50%, and 60% TM inclusion) were compared using analysis of variance. Furthermore, the ubiquitin and cleaved caspase conjugates were quantified via immunoblot analysis to find that TM inclusion induced cellular stress in the liver, in all the three teleost species. The stress response was similar for the rainbow trout and European sea bass, where the stress induced hepatic autophagy, which had an inhibitory effect on apoptosis. However, the pattern of stress exhibited by gilthead seabream was a bit different, with increased levels of both autophagy and apoptosis.

NMR-based metabolomics has been utilized for various studies related to fish feed formulation. A group of researchers [62] used NMR to test for the effect of eight different diets on the liver metabolome of juvenile red drum. Changes in 19 metabolites were monitored among the different dietary treatments. γ -butyrobetaine, N-formimino-L-glutamate, sarcosine, and beta-alanine were among the most differentially expressed metabolites. NMR was also used by Tavares and colleagues [63] to test the impact of 0%, 2.5%, and 5.0% glycerol-supplemented diets in the plasma metabolome of European sea bass (*D. labrax*). A commercial fishmeal-based pelleted diet was used as a control. A comparative analysis using two independent approaches were employed for the identification and quantification of plasma metabolites by an automated deconvolution analysis using the Bayesil online platform as well as a manual targeted peak fitting analysis (LineFit). This resulted in evaluation of a viable NMR-based automated tool for systematic monitoring of fish plasma profile, which can be used to generate robust variance-based multivariate statistical models.

In the present times, sustainable aquaculture needs development of plant-based feeds for carnivorous fish. ^1H NMR metabolomics studies by Roques [32] revealed that feed supplementation with microorganisms such as *Spirulina* could offer a viable alternative to full plant-based diets that have been proven to be inappropriate for carnivorous species like rainbow trout. A similar NMR-based study by Palma and group [31] based on plasma and digesta metabolome of ARS-Sel and two commercial strains (CS-1 and CS-2) of rainbow trout fed on plant protein and fishmeal diets showed that ARS-Sel is metabolically better adapted to process and utilize nutrients from plant-based diets compared to the commercial strains. The plant protein-induced enteritis onset was also noticeable from the differences in metabolomic profile, especially that of amino acids, thereby offering noninvasive samples to monitor the onset of gastric enteritis.

An untargeted LC-MS-based metabolomic analysis by Tao and colleagues [39] of male genetically improved farmed tilapia (GIFT, *Oreochromis niloticus*) fed a high-fat diet (HFD) for 8 weeks revealed that the juvenile fish fed an HFD accumulated more fat in the liver. There were 36 differentially accumulated metabolites involved in 25 signaling pathways among the control and the sample sets, which indicate that such a fat accumulation severely impairs hepatic lipid metabolism, resulting in blockage of the TCA cycle, disrupting protein and carbohydrate metabolism. These physiological changes may culminate to a lower growth rate in GIFT fed on HFD.

6. Conclusions

A wholesome approach, orchestrated by the integration of metabolomics, proteomics, and other omics technologies, can be utilized to assess multiple factors instrumental in increasing yield and leading to a sustainable fish production system. Improving and optimizing diets for larvae and brooders, analyzing the bioactivity of food components, assessing the effects

of specific food additives or inducers, and other aspects of diet formulation can be studied in depth using a multiomics approach. Proteomics and metabolomics have lent valuable insights into disease pathogenesis, severity factors, identifying diagnosis and prognosis markers, improving therapeutics and species health, and enabling formulation of robust strategies for disease management and fish welfare. These omics approaches can also be utilized for species authentication to maintain the quality and safety of food by monitoring allergens, toxins, genetic modifications, adulteration, and frauds. Such approaches can also help improve survivability, gamete quality, and fecundity to help in brood stock management (Fig. 19.4). Further omics-based studies addressing the impact of environmental stressors on fish and other aquaculture species are also indispensable in the light of anthropogenic degradation of the environment. Therefore, almost every challenge to the development of aquaculture can be faced using omics approaches, thereby providing stimulus for further research and corresponding technological advancements to fuel it.

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Biobank and genetic conservation of aquatic resources

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1. Introduction

Biobanks represent an organized collection of genetic resources and their associated information. Recently, these have become an important source for research purposes, not only in humans but also in other researchable areas including veterinary, animals, and plants. In fishes, biobanks can be significant for sustainable utilization. Modern genetic tools enable researchers for long-term storage of any part of animals or plants as well as extracted DNA. Biobanking is an efficient approach in providing security against species diversity loss [1]. However, most of the biobanks are maintaining either whole organism, early life stages, or reproductive cells. Hence, biobanks are called biorepository, which preserve genetic materials. Biobank has several advantages, such as playing an essential and complementary role in germplasm conservation as a part of ex situ conservation; protecting genetic heredity, providing safety measure against disasters, which plays a significant role in sustainable development, and helping to increase food production and associated livelihood enhancement.

Conservation of genetic diversity is important to ensure the adaptation of wild populations in the future; to preserve life history and behavioral and morphological traits for sustainable fisheries; and to effectively utilize the available fish genetic resources for commercial purposes [2]. Worldwide aquatic ecosystems are experiencing severe threats to biodiversity and ecosystem stability. Overexploitation of aquatic resources and other anthropogenic activities reduce fish production and push several endemic species to become threatened/ endangered. A combination of intensive management of the resources, vigilant coordination of efforts, and sound research is required to conserve fish genetic resources. The principal mandate for gene banks is to preserve genetic materials of organisms, especially those under threat or close to extinction. In 2002, India legislated the Biological Diversity Act (BDA) for the conservation and sustainable utilization of bioresources. The act includes procedures to handle various issues pertaining to conserving and utilizing valuable bioresources. In this context, the biobank serves as an important tool in attaining the goals of the BDA. In this milieu, the chapter reviews the scope of the biobank in the conservation of aquatic germplasm resources.

2. Conservation strategies

Conservation also protects wildlife and promotes biodiversity in the ecosystem [3]. By conserving the germplasm, it should be ensured that future generations could also rightfully utilize the natural resources with existent varieties of species. Threatened fish species require a combination of conservation efforts to improve their status. Knowledge of species biology and level of genetic diversity are important components of conservation. Conservation strategies consist in vitro and in vivo methods of management of bioresources. In vitro gene banks preserve sperm and ova at an ultralow temperature, usually in liquid nitrogen (-196°C) for a long time. In vivo gene banks are established to generate a living genetic material that could be further used to establish, maintain, or enhance the endangered stock [4].

Cryobanking is an effective tool to manage populations by preserving genetic diversity [5]. Furthermore, cryobank helps in increasing heterozygosity among wild populations [6]. There are two main ways to conserve biodiversity in the

ecosystem: (1) in situ (within the natural habitat) and (2) ex situ (out of the natural habitat). In in situ conservation, the whole community is conserved in its ecosystem. When habitat degradation could affect in situ conservation, ex situ conservation becomes an option. Gene banks can maintain animals in live conditions or gametes in cryopreserved conditions as a last-line defense against species extinction.

3. Mode of conserving fish germplasm

Management of genetic diversity of a population depends on the status of the population, either population is declining or becoming endangered.

4. Management of declining population

For managing the declining population, the following measures can be taken: (1) effective population size (N_e) should be maintained as large as possible to enable the maximum reproductive contribution; (2) the factors that reduce the effective population size should be determined and controlled; and (3) the barriers that affect gene flow continuity should be identified and managed. It is essential to protect the fish species and habitat from anthropogenic activities to attain these objectives. If the population is critically reduced, captive breeding can be a useful approach. This method is known as supportive breeding [7].

5. Management of endangered species

Captive breeding is a useful approach for the conservation of endangered fish species. The success of a captive breeding program depends on the rapid growth of the population so that the genetic diversity is enhanced. The founder population should be as large as possible to avoid inbreeding. If the population is small, selective mating should be done to avoid inbreeding. Domestication problems can be avoided by maintaining stocks in an artificial environment, such as a hatchery for a brief period [8]. In captive breeding programs, cryopreserved gametes can be effectively used to recover endangered species.

6. Conservation strategies

6.1 In situ conservation

In in situ conservation, the organisms or ecosystem is conserved in their natural surroundings. In this method, fish germplasm is conserved in its natural habitat. This conservation strategy helps maintain the diversity of the species at the gene level and results in evolutionary adaptations. In this method, along with the target species, the host species associated with them are also conserved [9], for example, protected areas.

6.2 Ex situ conservation

In this method, the target animal is maintained outside their natural habitat. Either the organisms are maintained live or their genetic material is maintained with the cryopreservation method.

7. Live germplasm resource centers

Live germplasm resource centers or live gene banks, which are also called fish refugia, maintain endangered/threatened animals in captive conditions (e.g., Indian Council of Agricultural Research [ICAR]—National Bureau of Fish Genetic Resources [NBFGR], India, and World Fisheries Trust, Canada). Such gene banks could be utilized in conservation programs and contribute to the recovery and utilization of genetic diversity. A live gene bank for threatened salmon stocks was established by the Norwegian Directorate for Nature Management for the conservation of the valuable resources of salmon. A live gene bank should have a facility for rearing selected fish brood stock of interest. In Canada, Environment Canada, the Department of Fisheries and Oceans and Parks Canada, and the Nova Scotia and New Brunswick government created a live gene bank for salmon to raise until reaching adulthood in captivity. The main objective of this live gene bank is to prevent the imminent extinction of stocks and restore the wild population [10]. Live gene banks for common carp were developed in Hungary in 1963. The seeds of common carps have been collected from various parts and maintained in that live gene bank [11]. The concept plan for captive propagation and aquaculture is given in Fig. 20.1.

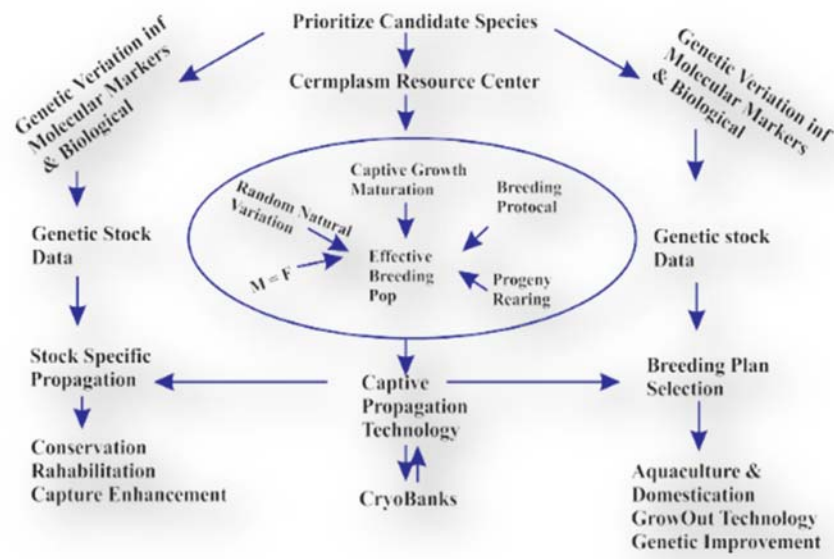


FIGURE 20.1 Concept plan: Development of captive propagation technology for aquaculture and conservation programs [12].

8. Cryobank

Biotechnological development has made it possible for cryopreservation of spermatozoa, embryos, and stem cells. Cryopreservation is the method widely used to protect biological materials over an extended period of time. Cryopreservation of reproductive gametes is a secure method of fish conservation as it facilitates the option to rebuild the novel genetic makeup of an organism. The method provides provision for preserving both the male and female parts of genetic material for the conservation of biodiversity as well as for scientific and aquaculture purposes [13].

9. Sperm preservation

Fish sperm cryopreservation has been successfully accomplished for around 200 fish species [13,14]. Cryopreservation methods have been established for various freshwater and marine fish species, which includes salmonids, carp, rainbow trout, grouper, milkfish, medaka, zebrafish, and cod [15–19]. It is a useful tool in protecting fish biodiversity, where sperm samples of the most valuable males have been preserved and used for producing offspring in hatcheries.

10. Oocyte and embryo preservation

Ovarian tissue cryopreservation was first performed in mammals such as mice, sheep, and goats [20,21]. This method is the best alternative for oocyte and embryo cryopreservation. Cryopreservation of embryos and oocytes in fish species is a bit difficult. Successful cryopreservation of larvae, eggs, and embryos has been achieved in fish species [22–24]. Ref. [13] have achieved cryopreservation of early stages of zebrafish oocytes by controlled slow freezing. Cryopreservation of invertebrate's oocytes and eggs was extremely difficult [25,26] as it was observed that intracellular crystallization formation happens in shellfish oocytes. To overcome this scenario, Ref. [25] utilized the vitrification technique (ultrapid freezing) to successfully cryopreserve the starfish oocytes.

11. Tissue repository

Historical tissue samples, a kind of genetic wealth, play an important role in establishing baseline information of existent genetic diversity as well as on temporal scale studies, for comparative evaluation on biotic and abiotic stressors. Moreover, these tissue accessions form an important resource for allele mining, too. These need to be safeguarded from different hazards including chemical, biological, fire, and electrical by adopting suitable measures.

12. DNA bank

The germplasm of the organism could be stored in the laboratory in the form of total genomic DNA, cloned DNA fragments, genomic, and cDNA libraries. This technique often requires proper labeling, documentation, preservation, and expertise. The information stored in the DNA bank might be of use in recovering important coding genes and help in future research. Several organizations in the world maintain DNA banking facilities and genetic resources repositories by preserving DNA, RNA, or preserved tissues for biodiversity studies. DNA barcoding studies in the world insist on tissue collection along with voucher collection for DNA extraction. However, the tissues for these studies are usually stored in ethanol, reducing the quality of genetic material over time. Hence, tissues and cells should be preserved so as to allow the broadest range of downstream uses. Cryogenically stored tissue and gametes would allow the rescue of extinct or endangered animals [27].

13. Web-based tools

A dedicated and secured information system facility is required and the copy of the data needs to be cloud stored or stored in any other suitable medium. International guidelines are required to be complied with, to safeguard the inventory information.

14. Fish cell line repository

Cell culture is widely utilized in modern research being a suitable alternative for various biological research in animals as they mimic the host cellular mechanism. Hence, many companies develop cell lines from various tissues of organisms for research purposes. A total of 280 fish cell lines were derived from 20 tissue types from 44 fish families across 21 countries in the decade amounting to 783 total finfish cell lines developed so far. The group Cyprinidae dominates the cell lines developed during the last decade [28].

While the storage of cell lines, stability, and recovery rate need to be assessed, employing different freezing mediums at various passage levels should be tried in order to minimize the loss of cells. An automated programmable freezer can provide efficient cryopreservation based on the requirement for the cell [29]. A simpler cryocan with liquid nitrogen can be used for the storage of fish cell lines. Researchers can obtain characterized cell lines from a repository at minimal cost and effort. Furthermore, the repository may act as a backup to secure the cell lines developed from a laboratory from loss. The cell line repository can provide access for in vitro research and conserving fish germplasm resources [30].

The important cell line repositories distributed across the globe are American Type Culture Collection (ATCC), European Collections of Authenticated Cell Cultures (ECACC), and German Collection of Microorganisms and Cell Cultures (DSMZ), which provide characterized cell lines to the interested researchers. Certain cell lines present in the repository may tend to get affected by misidentification and contamination due to transit to the repositories. An STR profile certificate is essential for each line to provide authenticity [31].

15. Initiatives of ICAR-NBFGR for fish genetic resource conservation through biobank

ICAR-NBFGR made a significant effort for fish germplasm conservation. The institute is working in establishing germplasm resource centers with the following objectives: (1) collection of endangered, threatened fishes from wild and mainlining under captive conditions, (2) study of growth and maturity and survival of the species maintained under controlled conditions, and (3) study of life history traits of these species as a tool for conservation. ICAR-NBFGR, in collaboration with Gauhati University, Assam, created a live gene bank of northeastern fish resources at Guwahati, India, and a few threatened fish stocks have been stocked in regional live gene bank centers at Gauhati University [10]. ICAR-NBFGR also developed species-specific cryopreservation protocols of spermatozoa for around 30 fish species. It supports captive breeding technology and restocking of fish species in the wild natural habitat.

As a significant component of agrobiodiversity, other fishes being maintained as NBFGR, Lucknow, include *L. rohita*, *Catla*, *Labeo rajasthanicus*, *Mystus cavasius*, *Garra gotyla*, *Labeo dero*, *Sperata seenghala*, *Labeo bata*, *Mastacembelus armatus*, and *Rita*. *Pangasius silasi*, a new discovered catfish species from Krishna River, is being maintained in Nagarjuna Sagar Dam, Telangana [32]. Its evaluation is being carried out for harmonizing conservation along with livelihood generation through aquaculture. The cages also maintain other important species such as *Ompok bimaculatus*, *Labeo calbasu*, and *Notopterus notopterus* to monitor individual growth and for brood stock development. Ornamental fish species,

Dawkinsia rubrotinctus and *Pethia setnai* and food fishes, *Clarias dussumieri*, *Horabagrus brachysoma*, *Labeo dussumieri* etc., are produced successfully under captive conditions and being provided to the needy entrepreneurs and farmers for propagation [33].

ICAR-NBFGR established a Germplasm Resource Centre for Marine Ornamental Invertebrates at Agatti Island, Lakshadweep, especially for marine ornamental invertebrates. Exploratory surveys conducted on pristine reef islands have revealed hidden diversity by discovering three shrimp species new to science, *Periclimenella agattii*, *Urocaridella arabianensis*, and *Actinimenes koyas*, and range extensions for *Thor hainanensis* and *Lysmata hochi* [32]. Over 500 individuals of 17 species of marine ornamental shrimps were collected from the wild for germplasm conservation and captive propagation. These fascinating ornamental shrimps exhibit myriad colors, semiotic association with other groups, behaviors, and body forms, making them attractive to hobbyists.

On the concept of harmonizing conservation and livelihood, another initiative has been established, which is live germplasm resource center for ornamental clownfish species. This center at Airoli, Mumbai, was established in collaboration with the Mangrove Foundation, Government of Maharashtra, and has 10 species of clownfish conserved in captivity.

A master breeding facility is also established in the facility for producing the seed that could be provided to the trained beneficiaries to rear them to the marketable size for livelihood enhancement. *Amphiprion ocellaris* and *Amphiprion percula* are currently being mass produced for the use in community aquaculture program [34]. Captive propagation of fishery resources is important in reducing the dependency on natural stocks, thereby safeguarding the germplasm resources and maintaining the ecological balance. Furthermore, captive propagation and providing technical know-how to the local community to rear the organism to marketable size will create additional revenue for the communities of coastal and island regions of the country, in addition to sustainable livelihood development [35].

Sperm cryopreservation protocols have been developed for over 30 fish species including commercially important endemic fishes of the country. This can effectively address the issues of insufficient milt production or variation in the maturity of two sexes for captive breeding of cultivable species [2]. Furthermore, ICAR-NBFGR is conducting cross-breeding experiments across the country using the cryopreserved milt of Indian major carps from native distribution i.e., river Ganga, for enhancing the genetic diversity of the brooders and young ones, thereby improving the growth and reproductive traits.

The National Repository of Fish Cell Lines (NRFC) was established at NBFGR, Lucknow, in the year 2010, which was initially funded by the Department of Biotechnology, Government of India, which is a National Referral Centre of fish cell lines in India and abroad for delivering required fish cell lines for research purposes. About 50 fish cell lines developed from 24 fish species are being maintained in the NRFC. The facility offer services for accepting fish cell lines from various research laboratories, maintaining the cell lines using cryopreservation, and distributing the same to the needy researchers in India.

16. Future perspectives

India being mega fish diversity-rich country and due to the astoundingly diverse morphological characters and their pattern of distribution, the necessity of a comprehensive database on fish diversity has been felt for mainstreaming of fish germplasm. Compilation of data on a geographic information system scale would also enable the massive data handling and decision-making. Species-specific gamete cryopreservation, germplasm resource centers, and broad area in situ conservation programs will aid the conservation of endangered and prioritized species. The attributes such as captive-based culture system in germplasm resource centers, cryobanking of gametes and embryos, tissue/DNA banking, and cell line repositories are to be given greater emphasis for preserving the fish germplasm resources and effective utilization of these genetic resources, in a sustainable way.

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FRONTIERS IN AQUACULTURE BIOTECHNOLOGY

EDITED BY WAZIR SINGH LAKRA, MUKUNDA GOSWAMI, AND VANCE LIONEL TRUDEAU

The latest biotechnological developments in aquaculture to aid in research.

Frontiers in Aquaculture Biotechnology presents a broad spectrum of topics covering different key aspects of aquaculture. With the rising importance of aquaculture research, evidence-based information is integral to advance this field. This a solid resource of information on DNA barcoding for fish species authentication and seafood labeling, cell culture including stem cell culture, in vitro research using fish cell lines including in vitro fish meat, reproductive biotechnology including surrogate technology, gene editing and genetically modified aquaculture species, biofloc technology, omic technologies including proteomics, artificial intelligence, and biobanking. This book will be a valuable resource to students, researchers, and entrepreneurs interested in better understanding of this emerging field of aquaculture.

Key Features:

- Aquaculture remains one of the fastest-growing food-producing sectors. Projections continue to rise as consumption increases (62% by 2030 FAO)
- India #8 in research output via SCIVAL and Canada #9; FWCI 1.28; 2.10 respectively
- This project supports 2021 strategy to grow marine bio, aligns with HCI on AI, and is on my Commissioning plan; Row 10262- FWCI Aquatic Science/fish; Row 1788 - Science Direct Usage Data; Aquaculture
- Strong Editor team and reviewers are all highly respected experts who support the project; two are from core market—all three are from top 10 research output/countries

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