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An in-depth analysis of Indian fish cell culture

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Conflict of Interest

There is no conflict of interest for authors to declare.

Ethical Statement

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Abstract

Cell culture is considered a mainstay in *in-vitro* research both in human and veterinary biomedicine, with its vast applications in cytogenesis, cellular physiology, toxicity, genomics, immunological studies, vaccine development, and host-pathogen interaction. In addition, cell culture finds its use in toxicological studies and diagnostic virology, where it is still considered the gold standard. Cell culture is also used as a replica model for various biological studies as it mimics the host organism's cellular architecture and reduces variability due to various factors present under *in-vivo* conditions. The last two and a half decades in India have witnessed extensive use of cell culture in deciphering various phenomena under *in-vitro* conditions. To this end, a number of primary cell cultures and cell lines have been developed and characterized using karyotyping, isozyme analysis, and molecular techniques. The current review intends to highlight the developments, applications, and future prospects of fish cell culture in the Indian subcontinent.

Keywords: Fish, *In-vitro*, Cell culture, Cell line

Introduction

The process of taking cells, tissues, or organs from a fish and culturing them in a controlled environment that encourages growth and division is known as "fish cell culture." An *in-vitro* environment typically consists of an appropriate tissue culture vessel (made up of glass or plastic material) filled with the medium (either liquid or semisolid), which provides the inoculated cells with the nutrients required for survival and division at an incubation temperature and growth factor similar to other mammalian cell cultures. Fish cell culture has emerged as an important laboratory working model for research in aquaculture problem domains such as fish viral pathology, physiology, pharmacology, industrial wastes, pesticides and metal toxicity, cell

carcinogenesis, and transgenes (Hasoon et al., 2011; Yadav et al., 2012; Taju et al., 2013; Barman et al., 2014; Dubey et al., 2015; Nambi et al., 2017; Chowdhry et al., 2019).

Many cell cultures have been initiated and developed from fish in India to study the pathology of various viral diseases in fish. Viruses are by far the most dangerous emergent pathogens, which continue to cause tremendous loss to the aquaculture industry (I. Ahmed et al., 2008; Majeed et al., 2013; Swaminathan et al., 2016c & 2022; Behera et al., 2018). Also, fish cell culture in India has found its vast application in studying environmental toxicology, cell cycle analysis, and transfection (A. Majeed et al., 2015; Swaminathan et al., 2020). Earlier attempts at fish cell culture development in India trace back to the 1990s by Bright Singh et al., (1995), Lakra & Bhonde (1996), and Sathe et al., (1997). The Department of Biotechnology, Government of India, has been instrumental in advancing fish cell culture research in the country. According to the ICAR-National Bureau of Fish Genetic Resources (NBFGR) annual report 2020, the National Repository of Fish Cell Lines (NRFC) in India has developed a total of 71 fish cell lines until 2020. Few authors have made reasonable attempts to review developments in fish cell culture (Hameed 2010; Lakra et al., 2011c; Meena et al., 2018; Goswami et al., 2018 & 2022b; Thangaraj et al., 2021). Earlier, Lakra et al., (2011c) reviewed a comprehensive registry of 283 finfish cell lines created in total from the year 1995 to 2010, and according to him, there were 483 fish cell lines developed until 2010. Meena et al., (2018) made an assessment of existing finfish cell lines in India. A recent review of the last decade (2010-2020) given by Thangaraj et al., (2021) clearly confirms that India is in second place behind China in the initiation and development of cell lines, as out of 280 cell lines developed from fish all over the world during this time period, leading country China produced 78, India 65, followed by countries like Korea, Japan, Canada, Taiwan, Portugal, and the USA, etc. In this review, he also suggested that the total number of fish cell lines developed until 2020 is 783.

Although these authors have reviewed the developments in fish cell culture, the focus has been mainly on the global perspective, with the exception of Meena et al., (2018). However, to the best of our knowledge, an in-depth review on Indian cell culture has not been made yet. Therefore, the present review mainly focuses on current applications, advances, and future prospects of Indian fish cell culture. Furthermore, the review intends to present a comprehensive registry of Indian fish cell culture.

1. Fish *in-vitro* cell culture

In-vitro fish cell culture exists either as primary cell culture or a continuous cell line (Bejar et al., 1997; Rathore et al., 2001; Smita et al., 2012; Bain et al., 2013). Fish cell culture has been initiated in a variety of fish and tissues. In general, explant and tissue dissociation are commonly used methods to initiate fish cell culture. A pictorial representation of the same is presented in figure 1. In the explant method, tissue is finely chopped into small pieces of 1 mm3 and placed in cell culture flasks containing cell culture media. This approach has proved successful in initiating cell culture (Lakra et al., 2010a & b; Kumar et al., 2019). On the other hand, the tissue dissociation method of cell culture uses proteolytic enzymes like trypsin and collagenase on the explant tissue, leading to the centrifugation of the cell suspension and its subsequent placement into a culture flask containing cell culture media. Sandbacka et al., (1999) have reported the development of a primary cell culture using this approach. However, both of these methods require an anchorage-dependent solid surface for cell adhesion, attachment, and growth. Polydimethylsiloxane (PDMS), an adaptable, translucent, and biocompatible polymer that is gas permeable but mainly resistant to water, is used to make cell culture flasks that maintain cell cultures in a humid environment with 5% CO_2. Various researchers have reported that initial outgrowth from explant leads to the confluent primary cell culture, which can be then passaged and maintained in a cell culture laboratory (Hameed et al., 2006; Lakra et al., 2006a,b; Ahmed et al., 2009; Babu et al., 2011; Goswami et al., 2012a; A. Majeed et al., 2013; Chaudhary et al., 2014). Furthermore, the disassociation of cells from the culture flask is attained by use of a trypsin-EDTA (ethylenediamine tetraacetic acid) enzyme solution. Trypsin functions by breaking down the extracellular matrix and cell junction proteins, whereas EDTA's function is to chelate with free Ca2+ and Mg2+ ions, which are essential for the structural organization of the extracellular matrix. Typically, 0.25% trypsin and 0.2% or 0.02% EDTA solution, typically prepared in PBS, are used to dislodge the fish cellular confluent monolayer (Parameswaran et al., 2007; Babu et al., 2011; Dubey et al., 2014; Murali et al., 2020). However, an excessive use of trypsin-EDTA can jeopardize the viability of the dislodged cells, as they develop into new cell colonies during subculturing.

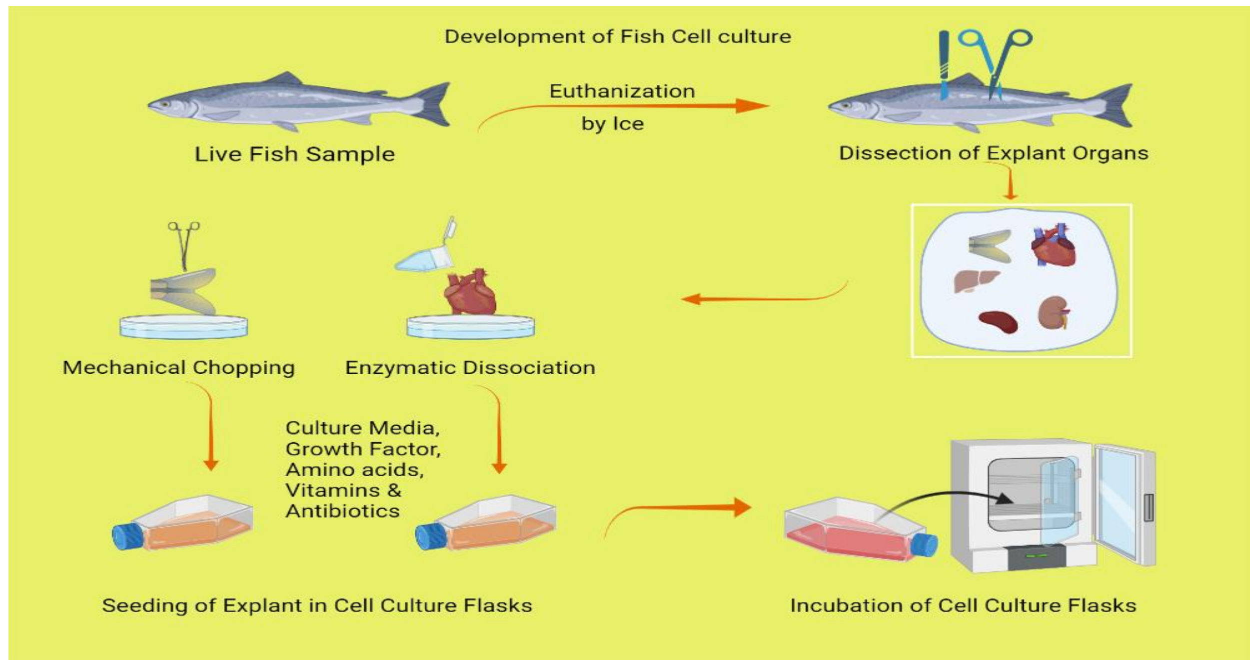

Figure 1. Steps involved for *in-vitro* cell line initiation from fishes.

Cell culture media is usually composed of glucose, sodium bicarbonate, essential amino acids, and vitamins; however, it always requires, in small amounts, a growth supplement, which acts as a required input for the division of cells. Fish cell cultures, like other *in-vitro* eukaryotic cells, have their basic need of nutrients, which are taken from cell culture media, which offer essential nutrients as well as necessary supplemented growth regulators. Eagle's Minimal Essential Medium [EMEM] serves as an all-purpose cell culture medium for the growth of all vertebrates, while other commonly used media include Glasgow Minimum Essential Medium [GMEM], a modified version of EMEM, Hank's Minimal Essential Medium [HMEM], and Leibovitz-15 [L-15]. EMEM is the most commonly used growth medium, but it requires CO2 buffering during incubation, whereas L-15 does not require CO2 buffering during incubation because it is an amino acid-rich medium and is most commonly used in fish cell culture both nationally (Chaudhary et al., 2013; Singh et al., 2019) and internationally (Ku et al., 2010; Riggs et al., 2019). Lakra et al., (2011c) in their review mentioned that most of the fish cellular *in-vitro* cultures have been grown using L-15 since 1994.

The commonly used growth supplement in fish cellular *in-vitro* culture is fetal bovine serum [FBS], but its concentration has a significant effect on the growth of primary cell culture and cell line, which usually varies from 5% to 20%, and there is inhibition of cell growth at higher

concentrations of FBS (Wang 2006). While FBS is commonly used as a growth supplement, researchers also use mammalian epidermal growth factor [mEGF] (Pandey 2013) and basic fibroblastic growth factor [bFGF] (Chen et al., 2003) to initiate or stimulate the growth of primary cell cultures and cell lines. Cell cultures after being treated by these mitogens (mEGF & bFGF), which are bioactive molecules that initiate cells to differentiate and divide (Zhao et al., 2019), have a significant proliferative response, as bFGF is a potent mitogen produced from lymphoid cells of *Penaeus monodon* (Hsu et al., 1995), embryonic stem cells of sea perch and embryonic cells of *Paralichthys olivaceus* (Chen et al., 2003 & 2004), and *Oryzias latipes* (Hong et al., 1996). A fish fibroblastic cell line known as PCF was generated by Goswami et al., (2014) after using the caudal fin explant of the dark mahseer, *Puntius* (Tor) *chelynoides*. Cell proliferation was seen to be more rapid by the time of bFGF (5 or 10 ng/ml) dose inoculation than in the absence of it in normal conditions, as cell proliferation sharply diminished when bFGF was absent. Sticking or clinging factors, such as fibronectin, laminin, poly-L-lysin, or gelatin, and collagen, are also used for the growth of primary cultures and cell lines to increase the anchorage of cells to the substratum (Vancha et al., 2004). In addition to growth media and growth factors, we supplement the media with essential amino acids and vitamins for cell growth, as these are crucial for vital cell processes. However, we use antibiotic-antimycotic solutions (Vierck et al., 2000) in cell culture to control the growth of microbes, as the normal immune surveillance of an organism is absent in an artificial culture environment. Optimum temperature and growth factor concentration have the defining role in *in-vitro* cell culture growth of fish, as it has been previously shown to affect the development and establishment of fish *in-vitro* cell cultures by many researchers (I. Ahmed et al., 2009; Goswami et al., 2012b; Dharmaratnam et al., 2020).

2. Scenario of *in-vitro* fish cell culture in India

In India, the first attempts at fish cell culture were made in 1995 on *Heteropneustus fossilis* (Bright Singh et al., 1995) and then on *Cirrhinus mrigala* (Sathe et al., 1995) using kidney and gill explants, respectively. Figure 2 shows us the number of *in-vitro* fish cell cultures developed during the time interval of every five years, and it also depicts us that this field started its growing speed from 2005 onwards in India.

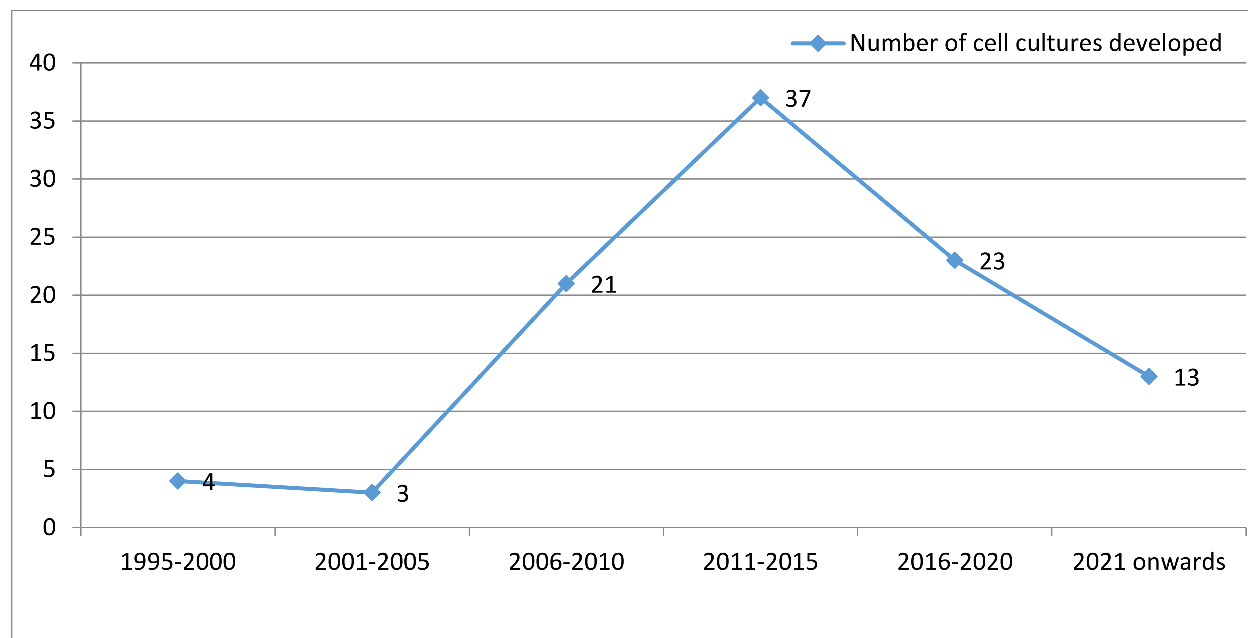

Figure 2. Graphical representation of *in-vitro* cell cultures, showing us the numbers developed from fishes after every five years in India.

However, the current fish cell cultures in our country are highly advanced and developed due to the application of various characterization techniques such as karyotyping, immunocytochemistry and COI, 12SrRNA, 16SrRNA, and microsatellite gene analysis (Parameswaran et al., 2006a & b; Lakra et al., 2011a & b; A. Majeed et al., 2013). These developed cell cultures are also utilized to study various aspects such as fish pathology, toxicology, transfection, cell cycle analysis, and phagocytosis (Lakra et al., 2006a & b; Swaminathan et al., 2010; Awasthi).

Table 1. Different cell cultures derived from fish species in India with characterization and application.

S.No.	Species name, tissue used & cell line designation	Characterization technique	Application	Reference
1	*Heteropneustus fossilis,* kidney & -	-	-	Bright Singh et al., 1995
2	*Cirrhinus mrigala,* gill & MG-3	Isozyme analysis	-	Sathe et al., 1995
3	*Labeo rohita*, cadual fin & -	-	-	Lakra & Bhode, 1996
4	*Labeo rohita,* gill & RG-1	Isozyme analysis	-	Sathe et al., 1997
5-6	*Clarias gariepinus,* gill; kidney & -	-	-	Rthore et al., 2001
7	*Clarias gariepinus,* ovary & -			Kumar et al., 2001
8	*Lates calcarifer,* kidney & SISK	Immunophenotyping and microsatellite markers	Viral susceptibility testing and cytotoxicity test of bacterial extracellular products	Hameed et al., 2006
9	*Tor putitora,* fry & TP-1	-	Cell cycle analysis	Lakra et al., 2006a
10-11	*Lates calcarifer*, fry; fingerling & LCE; LCF	-	-	Lakra et al., 2006b

12	*Lates calcarifer,* blastula & SISE	Immunocytochemistry	Viral susceptibility testing for cytopathic effect and transfection with GFP reporter Gene	Parameswaran et al., 2006a
13	*Lates calcarifer,* spleen & SISS	Immunocytochemistry	Viral susceptibility testing and transfection with GFP reporter Gene	Parameswaran et al., 2006b
14	*Labeo rohita,* cadual fin & -	-	-	Rthore et al., 2007
15-16	*Chanos chanos,* heart & SIMH *Epinephelus coincides,* eye & SIGE		Viral susceptibility testing and transfection with GFP reporter Gene	Parameswaran et al., 2007
17	*Catla catla,* eye & SICE	12S rRNA gene	Viral susceptibility testing and transfection with GFP reporter Gene	I. Ahmed et al., 2008
18	*Epinephelus malabaricus,* gill & -	-	-	Sobhana et al., 2009
19	*Catla catla,* heart & SICH	12S rRNA gene and immunocytochemistry	Cytotoxicity test of bacterial extracellular products, genotoxicity, cell cycle analysis and transfection	Ahmed et al., 2009
20-21	*Labeo rohita,* eye & LE *Catla catla,* brain & CB	12S rRNA gene and immunocytochemistry	Cytotoxicity test of bacterial extracellular products and transfection	I. Ahmed et al., 2009
22-25	*Labeo rohita* ,swim bladder; heart; fin & RSB, RH and RF	COI and 16SrRNA genes	-	Lakra et al., 2010a
26-27	*Cyprinus carpio,* fin; heart & CCF; CCH	COI and 16SrRNA genes	-	Lakra et al., 2010b
28	*Etroplus suratensis,* caudal fin & PSF	16SrRNA and COI genes	Testing cytotoxicity of bacterial ECP (extracellular products)	Swaminathan et al., 2010
29	*Clarius batrachus,* fin & ICF	Immunocytochemistry , 12S rRNA gene	Viral susceptibility, cell cycle analysis	Babu et al., 2011
30	*Lates calcarifer,* brain & ASBB	Microsatellite markers	Viral susceptibility for cytopathic effect	Hasoon et al., 2011
31-32	*Puntius denisonii,* caudal fin; heart & PDF; PDH	COI gene	-	Lakra et al., 2011a
33	*Puntius sophore,* caudal fin & PSCF	Immunocytochemistry , COI and 16S rRNA genes and microsatellite gene analysis	Transfection	Lakra et al., 2011b
34	*Chitala chitala,* heart & -	-	-	Smita et al., 2012
35-38	*Etroplus suratensis,* eye; brain; gill; kidney & IEE; IEB; IEG; IEK	Immunophenotyping, 16SrRNA and COI genes	Testing cytotoxicity of bacterial ECP (extracellular products), viral susceptibility, cell cycle analysis	S. Babu et al., 2012
39	*Tor tor,* fin & TTCF	Immunocytochemistry , 16SrRNA and COI genes	Genotoxicity and transfection	Yadav et al., 2012
40	*Puntius denisoni,* fin & RTF	16SrRNA and COI genes	Viral susceptibility, Testing cytotoxicity by bacterial ECP (extracellular products)	Swaminathan et al., 2012
41	*Puntius chelynoides,* eye & PCE	16SrRNA and COI genes	Transfection and genotoxicity	Goswami et al., 2012a
42	*Labeo rohita,* blastula & RESC	Immunocytochemistry	Transfection	Goswami et al., 2012b

43	*Catla catla,* adherent blood mononuclear cells & CCM	COI and 16SrRNA genes	-	Chaudhary et al., 2012
44	*Labeo rohita,* gill & LRG	COI gene	Transfection and cytotoxicity testing	A. Majeed et al., 2013
45	*Channa striatus,* kidney & CSK	16S RNA gene	Transfection and viral susceptibility testing	Majeed et al., 2013
46	*Schizothorax richardsonii,* caudal fin & SRCF	Immunocytochemistry, 16SrRNA and COI genes	Testing cytotoxicity of bacterial ECP (extracellular products) and transfection	Goswami et al., 2013
47	*Chitala chitala,* caudal fin & CF	16S rRNA and COI genes	-	Kapoor et al., 2013
48-49	*Puntius fasciatus,* caudal fin & PFF *Pristolepis fasciata*, caudal fin & CFF	16S rRNA and COI genes	Testing cytotoxicity of bacterial ECP (extracellular products) and testing viral susceptibility	Swaminathan et al., 2013
50	*Catla catla,* gill & ICG	12S rRNA gene	Study of cytotoxicity of metal salts	Taju et al., 2013
51	*Catla catla,* thymus & CTE	Immunophenotyping, 16SrRNA and COI genes	Transfection, testing cytotoxicity of bacterial ECP (extracellular products), viral susceptibility and cell cycle analysis	Chaudhary et al., 2013
52	*Wallago attu,* caudal fin & WAF	Immunocytochemistry, COI and 16S rRNA genes	Transfection, cytotoxicity and genotoxicity	Dubey et al., 2014
53	*Heteropneustes fossilis,* embryo & HFB-ES	COI gene, Cyt $_b$ gene, and DNA profiling of microsatellite genes	Study of stem cell oct-4 gene regulation and alkaline phosphatase enzyme activity	Barman et al., 2014
54	*Puntius chelynoides,* caudal fin & PCF	Immunocytochemistry, 16SrRNA and COI genes	Testing cytotoxicity of bacterial ECP (extracellular product), transfection and genotoxicity	Goswami et al., 2014
55	*Channa striatus,* gill & CSG	16S rRNA gene	Transfection and cytotoxicity	Majeed et al., 2014
56	*Catla catla,* thymus & CTM	COI and 16S rRNA genes	Cytotoxicity, cell cycle analysis and respiratory burst activity	Chaudhary et al., 2014
57	*Lates calcarifer,* caudal peduncle & SBCP2	-	Viral susceptibility	Jhon et al., 2014
58	*Labeo rohita,* thymus & LRTM	12S rRNA gene	Respiratory burst activity study	Rebello et al., 2014
59	*Labeo rohita,* head kidney & LRPM	-	Demonstrated the property of phagocytosis	Awasthi et al., 2015
60	*Channa striatus,* heart & CSCVE	Immunocytochemistry and 16S rRNA gene	Cytotoxicity, cell cycle analysis and transfection	A. Majeed et al., 2015
61	*Channa striatus,* thymus & CST	Immunocytochemistry, 16SrRNA and COI genes	Testing cytotoxicity of bacterial ECP(extracellular product), transfection and metal toxicity	Sood et al., 2015
62	*Wallago attu,* muscle & WAM	Immunocytochemistry, COI and 16S rRNA genes	Transfection, Pesticide cytotoxicity,	Dubey et al., 2015
63	*Cyprinus carpio* koi, fin & CCKF	Immunocytochemistry, COI and 16S rRNA genes	Testing cytotoxicity and CPE(cytopathic effects) of bacterial ECP and viral strains	Swaminathan et al., 2015

64	*Danio rerio,* eye & DrRPE	12S rRNA gene and immunocytochemistry	Transfection and phagocytic activity assay	Nambi et al., 2015
65	*Danio rerio,* muscles & DRM	Immunocytochemistry, COI and 16S rRNA genes	Transfection and cell cycle analysis	Kumar et al., 2016
66	*Pterophyllum scalare,* caudal fin & AFF	Immunophenotyping, COI and 16S rRNA genes	Susceptibility of bacterial extracellular products also viral susceptibility testing	Swaminathan et al., 2016a
67	*Horabragus brachysoma,* caudal fin & HBF	Immunocytochemistry, COI and 16S rRNA genes	Susceptibility of bacterial extracellular products and nodovirus	Swaminathan et al., 2016b
68	*Danio rerio,* gill & DrG	12S rRNA gene	Cytotoxicity testing, oxidative stress and apoptosis related gene expression	Nambi et al., 2017
69	Channa striatus, kidney & SSK	COI gene	Transfection and cytotoxicity testing	Chaudhary et al., 2017
70	*Pangasianodon hypophthalmus,* caudal fin & PHF	COI and 16S rRNA genes	Transfection, cytotoxicity testing and virus susceptibility testing	Soni et al., 2018
71-73	*Pomacentrus caeruleus,* fin; liver; caudal peduncle & PC1F1Tr; PC1L1Ex; PC1CpTr	-	-	George et al., 2018
74-75	*Oreochromis niloticus,* brain; liver & OnlB; OnlL	Immunophenotyping, COI and 16S rRNA genes	Tilapia virus susceptibility testing	Thangaraj et al., 2018
76	*Clarias magur,* testis & Testis-Derived Cell	COI and 16S rRNA genes	-	Singh et al., 2019
77	*Astronotus ocellatus,* caudal fin & AOF	Immunophenotyping, 16S rRNA and COI genes	Testing cytotoxicity of bacterial ECP and virus susceptibility testing	Kumar et al., 2019
78	*Danio rerio,* caudal fin & DrF	Immunophenotyping and 12S ribosomal RNA	Cell viability testing	K. Sivalingam et al., 2019
79-81	*Oreochromis mossambicus,* gill; heart; eye & TG; TH; TE	COI and 16S rRNA genes	Virus isolation	Nanthini et al., 2019
82	*Gambusia affinis,* bone & BGA	Immunophenotyping and COI gene	Virus susceptibility testing	Vijayakumar et al., 2020a
83	*Gambusia affinis,* skin & SGA	Immunophenotyping and COI gene	Transfection and virus susceptibility testing	Vijayakumar et al., 2020b
84	*Danio rerio,* caudal fin & DRCF	Immunocytochemistry, COI and 16S rRNA genes	Transfection	Meena et al., 2020
85	*Carassius auratus,* caudal fin & FtGF	COI and 16S rRNA genes	Virus susceptibility testing	Dharmaratnam et al., 2020
86	*Amphitrion ocellaries,* caudal fin & OCF	Immunocytochemistry, COI and 16S rRNA genes	Nervous necrosis virus susceptibility testing	Yashwanth et al., 2020
87	*Clarias dussumieri,* caudal fin & ClDuF	Immunocytochemistry, COI and 16S rRNA genes	Transfection	Swaminathan et al., 2020
88	*Schizothorax richardsonii,* eye muscle &	Immunocytochemistry	Transfection and cytotoxicity	Murali et al., 2020

	SREM-1	, COI and 16S rRNA genes	testing of metals	
89-91	*Oncorhynchus mykiss,* eye; spleen; kidney & RTE; RTS; RTK	Immunophenotyping and COI gene	Cyprinid herpesvirus-2(CyHV-2), Tilapia lake virus(TiLV) and Fish nodavirus(FNV) susceptibility testing	Suryakodi et al., 2021
92	*Oreochromis niloticus,* heart & OnH	Immunocytochemistry and 16S rRNA gene	Transfection and virus susceptibility testing	Yadav et al., 2021
93-97	*Trachinotus blochii,* Heart; fin; gill; kidney; eye & SPH; SPF; SPG; SPK; SPE	Immunocytochemistry and COI gene	Virus susceptibility testing and gene expression	Abdul et al., 2022
98-99	*Pseudetroplus maculatus,* fin & OCF *Etroplus canarensis,* fin & CPSF	-	Virus susceptibility testing	Thangaraj et al., 2022
100	*Schizothorax esocinus,* heart	-	-	Khurshid et al., 2022
101	*Danio rerio,* skin & DRS	Immunocytochemistry , COI and 16S rRNA genes	Transfection, testing cytotoxicity and CPE(cytopathic effects) of bacterial ECP	Sathiyanarayanan et al., 2023
102	*Schizothorax niger,* heart	-	Cytotoxicity	Dar et al., 2024
Total number of fish cell culture developed = 102				

Table 1 is the complete registry of fish cell cultures in India, which shows that a total 102 different fish cell cultures have been developed so far; some of them were developed as primary *in-vitro* cell cultures, whereas most of them were grown as cell lines. Fins are by far the most commonly employed tissues in the generation of fish cell cultures in India; however, heart, gill, eye, kidney, and thymus have also been utilized to a greater number, followed by the brain, blastula/embryo, fry/fingerling, muscle, spleen, liver, and others (Figure 3).

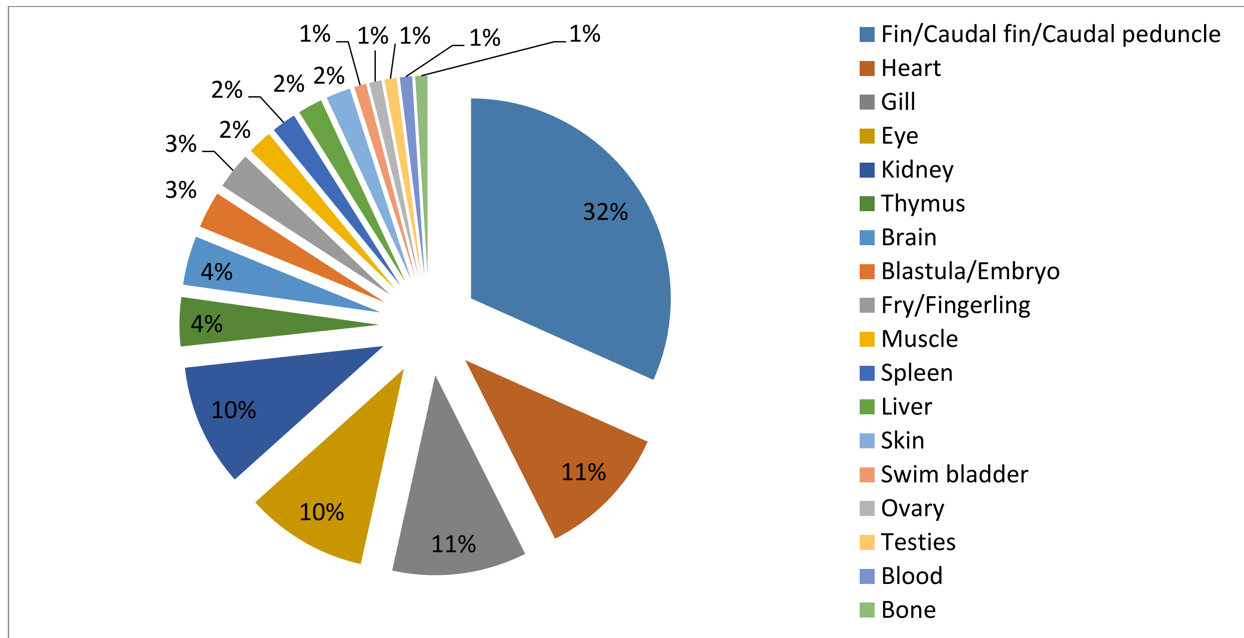

Figure 3. Different explants percentages used for initiation of different *in-vitro* cell culture in India from fishes.

Out of 102 fish cell cultures in India, 32% are developed from fins, which clearly demonstrates the superior regeneration capacity of fish fin tissue cells. *In-vitro* fish cell cultures grown from varied fish organs provide different avenues of application, like cell cultures developed from fins are usually used for the study of fish virology and toxicology (Swaminathan et al., 2015; Kumar et al., 2019; Yashwanth et al., 2020); however, cell cultures developed from eye muscle, kidney, bone, and heart (Murali et al., 2020; Majeed et al., 2013; Vijayakumar et al., 2020a) were also used for the same application. A cell line produced from the embryo of *Heteropneustes fossilis* was used to study the activity of the alkaline phosphatase enzyme and Oct-4 gene expression (Barman et al., 2014). Two cell lines derived from the thymus glands of *Catla catla* and *Labeo rohita* were used to study the respiratory burst activity (Chaudhary et al., 2014; Rebello et al., 2014). The head kidney serves as a hematopoietic organ, and researchers have used a cell line from the *Labeo rohita* head kidney to demonstrate the phagocytosis property (Awasthi et al., 2015). However, researchers have also used a fish cell line from the eye of *Danio rerio* for the same assay (Nambi et al., 2015). Cell line derived from a vulnerable species, *Schizothorax richardsonii*, eye muscle can act as a material for germplasm conservation in the future and its wide applications in toxicity testing of the local aquatic environment as well as pathological and transfection studies (Murali et al., 2020).

3. Fish *in-vitro* cell culture characterization techniques

3.1 Immunocytochemistry

Epithelial and fibroblastic cells observed under a microscope are confirmed by immunotyping with specific types of cell markers. Monoclonal antibodies, i.e., antigen-specific antibodies produced for cytokeratin, fibronectin, and vimentin, are more commonly utilized to detect epithelioid and fibroblastic cells (Langner et al., 2011; Swaminathan et al., 2016a, b; Meena et al., 2020). Hameed et al., (2006) used pan-cytokeratin and cytokeratin 19 marker antibodies to confirm the epitheloid nature of SISK cells in Lates calcarifer. Langner et al., (2011), also used monoclonal antibodies against pan-cytokeratins (CK1,4,5,6,8,10,13,18 & 19 which are filamentous proteins of epithelial cells) to identify epithelial cells in addition to that he also used monoclonal antibodies against the nestin (which is the marker for both stem cell as well as for neuro-epithelial cell), glial fibrillary acidic protein (GFAP) which acts marker for ectoderm cell, alpha smooth-muscle actin (alpha-SMA) acts marker for mesoderm cell, octamer binding transcription factor 4 (Oct-4) marker present in the nucleus of stem cell, stage-specific embryonic antigen-1 (SSEA-1) marker present on the stem cell surface, and MF20 which is a marker for muscle cell myosin protein. The study concluded that epitheloid stem cells were present under culture because antibodies against stem cell markers (GFAP, SSEA-1, and alpha-SMA) showed positive interactions at different concentrations, while Nestin, Oct-4, and MF20 showed no interactions.

3.2 Chromosomal analyses

Identification of the chromosomal number of an organism or cell is critical for detecting genetic make-up, and it is generally carried out through the process of karyotyping. It is the process by which metaphase cells are arrested for the visualization of chromosomes under a microscope (Bertollo et al., 1986; Oliveira et al., 2009). Cell culture is very advantageous for chromosome investigations because it can generate a larger number of metaphase chromosome plates than preparation from direct tissue. Chromosomal analysis aids in the development of cytotaxanomy, which is then used to study the ancestral linkage of closely related species (Ganai et al., 2014; Sember et al., 2021). According to Goswami et al., (2013), the diploid number of chromosomes from the *Schizothorax richardsonii* cell line at its metaphase plate was 98, respectively.

3.3 Molecular Characterization

It is a widely applied term in fish cell culture, and it means using different molecular markers to certify the growth of a particular fish cell culture. The most commonly used molecular markers for the characterization and verification in the case of the fish *in-vitro* cell culture growth are the COI gene, 16S rRNA, microsatellite DNA, 18S rRNA, and 12S rRNA (Lakra et al., 2010a; Rebello et al., 2014; K. Sivalingam et al., 2019). Amplification of the COI gene, 16S rRNA, microsatellite DNA region, 18S rRNA, and 12S rRNA, followed by their sequence analysis, is carried out to certify the identity of the cell line produced from the particular piscine species. Lakra et al., (2011b) confirmed the origin of the *in-vitro* cell line that was developed from *Puntuis sophore* through a characterization study of the 16S rRNA and mitochondrial COI gene. The PCR product for the cell line results in 655 and 581 base pairs for the COI and 16S rRNA, respectively. Following that, a comparison of the COI and 16S rRNA sequences generated from cells revealed a 100 to 99 percent match with the already available *P. sophore* sequences. PS-113, 116, 65, 85, 93, 145, 131, 122, and 127, a collection of nine polymorphic microsatellite loci created at the institute using cross-species amplified primers, were also used to identify cell line at the individual level. In 16 randomly selected samples, the matching probability (PM) was computed. All of the cell line's alleles were carried by the contributor species *P. sophore* and were from the cell line that actually had been grown. The power of discrimination for the loci set was quite strong (PM $=1{\cdot}06026\times10-21$). Rebello et al., (2014), after amplifying the extracted DNA with primers based on the genomic sequence of the rohu 12S rRNA, 273bp anticipated PCR products were produced. The gene sequences were also 99 percent identical to the *Labeo rohita* mitochondrial DNA sequences in GenBank.

3.4 Growth studies of serum and temperature

Serum concentration used and temperature range at which cell cultures are incubated are two important parameters for the study of attachment and growth of cells in cell culture as per their cell physiology requirements. The most commonly used serum in fish cell culture development is fetal bovine serum (FBS), which is typically added to cell culture media. FBS contains various growth factors that aid in cell attachment and growth by division in tissue culture to generate primary cell lines and continuous cell lines (Hameed et al., 2006; Ahmed et al., 2009; Babu et al., 2011; Goswami et al., 2012a & b; Chaudhary et al., 2014; Kumar et al., 2019). Different serum concentrations are used in different cell cultures, and the most common range applied is 5%–

20%. Lower and higher serum concentrations can inhibit cell attachment and growth. In different cell cultures of different fish species, different temperature ranges support the growth of cells as per their cell physiology needs. Swaminathan et al., (2016b), in *Horabragus brachysoma*, found that temperature and serum levels were both important factors in freshly dividing cells to achieve optimal growth in L-15 cell culture medium. The cells continuously divided in the 24-32ºC temperature range, but not at 20 or 35ºC. At optimal growing temperature (28ºC), FBS concentration was increased from 5 to 20%; at 5% FBS, cell growth performance was poor, with small growth at 7.5% FBS; however, moderate growth was reported at 10% FBS, and optimal growth was observed at 20% FBS.

4. Advantages of cell culture development in fishes

4.1 Pathogenic studies

Cell cultures in fish have gained a lot of value, as they are mostly used to study and investigate the pathogenic effects of microorganisms such as viruses, bacteria, and fungi that cause the most lethal diseases in fish and ultimately lead to a lot of profitable losses in aquaculture (Chang et al., 2001; Kawato et al., 2017). Cell cultures have helped us to understand how microorganisms affect organisms at the cellular level (Goswami et al., 2013; Huang et al., 2014; Sood et al., 2015; Yashwanth et al., 2020). Many studies support *in-vitro* testing of bacterial toxins and viruses on a cellular level to determine the cell damage caused by pathogens. Goswami et al., (2013) used bacterial ECP (extracellular products) from *Aeromonas* sp. to study the impact on SRCF cells. During this study, he observed that this bacterial toxin caused the cytotoxic effects after 12 h of inoculation in cell culture: cells developed a round shape pattern, shrinkage, some cells showed detachment from the substratum, and finally nothing remained attached to the flask's bottom from the monolayer of cells. In the modern era of intense and extensive fish culture to meet the food needs of the growing global population, the already identified and emerging virus has posed a significant threat to cultivable fish culture species. However, *in-vitro* fish cell culture is often used for virus isolation (Chang et al., 2001; Rathore et al., 2012; Sahoo et al., 2016; John et al., 2023).

4.2 Toxicology

Pesticides, heavy metals, and pharmaceutical drugs are common aquatic pollutants that are hazardous to aquatic animals (Singh et al., 2008; Taju et al., 2014; Ramesh et al., 2018;

Bhattacharya et al., 2019). Previously, scientists subjected fish to acute toxicity testing in laboratory conditions to confirm the impact of these aquatic contaminants, employing and slaughtering hundreds of thousands of fish samples. Fish cell cultures have replaced acute toxicity testing, and these cell cultures will be crucial in the future for determining the extent of these pollutants' impact on fish cells, as well as for reducing the number of fish used in scientific experiments (Das et al., 2014; Sood et al., 2015). I. Ahmed et al., (2009) investigated the cytotoxicity caused by ZnCl2, CuSO4, and CdCl2 on a noval cell line grown from *Catla catla* gill explants. Majeed et al., (2014) confirmed that the fish cell line is an efficient laboratory tool for the study of pesticide toxicity in fish, which he developed from an air-breathing fish, *Channa striatus*. However, Dubey et al., (2015) produced the muscle fish cell line of *Wallago attu*, which is a freshwater catfish in India. He used it for the study of the cytotoxicity of malathion and chlorpyrifos, which are both organophosphate pesticide compounds.

4.3 Genomic and transfection studies

The role of genes in developmental biology, immune response, stress tolerance, and disease development are practical applications, and genome-based investigations aid in the development of novel therapeutic technologies. Li et al., (2015) developed fin cell lines (diploid, triploid, and tetraploid) from various polyploidy pond loach, thereby expanding the number of available cell lines for various research purposes. These cell lines serve as models for studying the growth and genetics of polyploidy fish. Fish cell lines, apart from genomic research, can also be used to carry out the study of gene regulation and expression in cells by the process of transfection to produce transgenic cell lines. The GFP plasmid is the most commonly used vector in fish cell culture transfection; it is usually used to study transgenic and genetic manipulation.

4.4 Immunological studies

The spleen, head kidney, thymus, gills, and skin are all the organs that play a role in fish immune defense. Cell cultures from these organs can be employed for immunological studies, and these cell cultures can be challenged by a pathogen to evaluate the cell immune regulation (Chaudhary et al., 2012, 2013, 2014, & 2017). The head kidney contains immune cells, such as macrophages, which are reticulated cells from which macrophage cell lines can be generated, much like they can be developed from the spleen and thymus. Chaudhary et al., (2014) produced a cell line from *Catla catla* thymus named CTM, which has proven beneficial for researching the role of

macrophages in thymocyte differentiation and their subsequent maturation, as well as for producing macrophage-related enzymes and different cytokines. Fish's head kidney serves as an immune organ. Wang et al., (2014) developed a fish cell line from this organ to aid in the study of immune stimulus against viral pathogens, which involved the interaction of three different immune macromolecules: irf3, irf7 (interferon regulatory factor 3 & 7), and mdap5 (melanoma differentiation-associated protein 5).

4.5 Vaccine development

Overuse of antibiotics, which leads to antimicrobial resistance, as well as poor buy-in grades, are two key issues in the use of antibiotics in the aquaculture industry. Hence, vaccine research against fish infections is critical. Vaccines are the most efficient way to prevent a variety of viral and bacterial infections (Vimal et al., 2014). Literally, most of the licensed fish vaccines are inactivated microorganisms that are conjugated with adjuvants and are usually administered by injecting into the fish or by immersion, in which the fish is immersed in the water containing the vaccine. However, live vaccines are more effective because their immune response mimics a natural pathogen infection and induces a high antibody response. Subunit or recombinant DNA/RNA particles are examples of vaccines made using modern technologies. Technological improvements have shown significant potential for the future of aquaculture vaccines, providing producers with health benefits as well as increased commercial potential. Cell culture is a useful method for identifying and characterizing fish viruses. EPC cells have been employed to create the pCh-IHN/IPN bivalent DNA vaccine, which protects from IHNV and IPNV (Xu et al., 2017). Bailone et al., (2020) used zebrafish cell lines. Because of their important genetic, anatomical, and physiological similarities with humans, zebrafish cell lines in *in-vitro* conditions have been used to look into the efficacy and safety of human vaccines.

5. Reacent advances and future prospects

5.1 3D cell cuture

The wider applications of cell culture show the importance of cell culture in present research and the need for improved methods and ways to culture the cells in *in-vitro* conditions. Cells are typically grown in 2D plateforms, but there is a need to enhance the dimensions of cell culture, given that cells exist in 3D forms in living organisms. 3D cell culture will give us a better understanding of cellular interactions (Ravi et al., 2015).

5.2 Reconstitute medicine

It implies the utility of body fluids, cell extracts, cultured stem and somatic cells, and organ transplants to treat serious injuries and chronic diseases in order to repair and reestablish normal functioning in situations where the bodies own regenerative responses are insufficient. Reconstitute medicine aims to enhance the body's inherent healing abilities by promoting the growth of new body fluids, cells, and tissues in a specific location. Dash et al., (2010), using feeder-free conditions, developed Embryonic stem-like cells by taking cells from the midway stage of the blastula from *Catla catla*, which were used to provide cardiomyocytes for scientific purposes to rebuild the healthy myocardium. However, embryonic stem-like cells grown from early stages of embryo from *Labio rohita* were utilized as a useful instrument for studying cell differentiation and gene expression (Goswami et al., 2012b). Hong et al., (2014) developed Z428 (a Zebrafish embryonic stem cell line derived from blastula). Cells in *in-vitro* conditions showed the embryonic stem cell phenotypic pattern. Also, cells possessed astonishingly high alkaline phosphatase activity, which is a characteristic trait of embryonic stem cells, as well as spontaneous differentiation in culture. These Z428 cells, upon transplanting into the host's embryonic blastula stage, became part of the embryo's three germ layers and actually produced tissue and organ systems. This secure cell line, Z428, carried pluripotent ES-like cells in both laboratory and transplant conditions. The simple generation and continuation of zebrafish ES-like cells in feeder-free state is a useful addition to the present toolkit for studying the growth and differentiation in the zebrafish model. Kirsner et al., (2020) discovered that acute wounds treated with full-thickness fish skin grafts heal noticeably faster than those treated with dehydrated human amnio-chorionic membrane. Therefore, the research findings on stem cell culture strongly suggest that fish cell cultures have the potential to produce induced pluripotent stem cells (iPSC), similar to those found in mice (Okita et al., 2007), through genetic modification of cell lines. This approach could prove beneficial in the study of reconstitute medicines.

5.3 Cellular laboratory agriculture

Cellular agriculture is the process of producing agricultural goods using cell cultures instead of whole plants or animals. Cultured meat is a newly emerging food technology that allows for the production of various animal flesh products without the need to slaughter the animal. Future

Meat Technologies, Aleph Farms, Mosa Meat, Finless Foods of Emeryville, Higher Steaks, and BlueNalu are companies that develop cultured food products from cultivated cells of animals and plants. BlueNalu is the cellular aquaculture company that developed San Diego's first ever cell-based seafood product in 2018. This increasing attention to cellular *in-vitro* agriculture is just a challenging solution to tackle environmental, global health, and live animal protection. The idea of producing seafood *in-vitro* in bioreactors from fish will gain ground as a solution to handle issues with marine capture and industrial-level aquaculture systems (Goswami et al., 2022a & b).

6. Conclusion

The need for cell culture development is increasing due to their ability to mimic the cellular traits and genotypic similarity of almost all organisms' live body tissue. They can also be used to improve the repeatability of experimental findings *in-vitro* and help resolve the moral dilemma of overusing a modal organism in an experiment. Since 1995, over 102 fish *in-vitro* cell cultures from both freshwater and marine species have been cultured and developed in India, with the majority coming from cultivable freshwater fish species. These fish cell cultures were created from a variety of explanted organs, but among the 102 cell cultures, the fin explant predominates. Only a small number of these cell cultures were created as primary *in-vitro* cell cultures; the majority were created as cell lines. These fish *in-vitro* cell cultures were utilized for different purposes, including gene expression research, cell cycle analysis, transfection, viral susceptibility testing, bacterial ECP testing, and toxicological testing. While India is home to the world's lone and largest fish cell line repository (NRFC), the Department of Biotechnology, Government of India, was the primary driving force behind the advancement of fish *in-vitro* cell culture research in the country. However, further research into innovations in fish cell culture is still required. For example, 3D cell cultures for cellular interaction, iPSC cells for reconstitute medicine, and *in-vitro* bioreactors for the creation of lab meat are all needed. We also need to focus on developing fish vaccines to combat the challenges posed by viral diseases and emerging fish viruses in aquaculture. Additionally, we need to develop satellite repositories throughout our vast nation, which has a diverse fish population.

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