A DISSERTATION ON
Development of Primary Cell Culture from Different Tissues of *Channa punctata*, (Bloch ,1793)

SUBMITTED TO THE
DEPARTMENT OF BIOENGINEERING
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FOR THE
DEGREE OF B. TECH-M. TECH DUAL DEGREE
IN BIOTECHNOLOGY

BY
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UNDER THE SUPERVISION OF

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DECLARATION FORM

I, Mayuri Sharma , a student of **B.Tech – M.Tech Dual Degree Biotechnology** (5^{th} year/10^{th} semester), Integral University have completed my six months dissertation work entitled **Development of Primary Cell Culture from Different Tissues of *Channa punctata*, (Bloch ,1793")** successfully from **NBFGR** under the able guidance of **Dr. Murali S Kumar .**

I, hereby, affirm that the work has been done by me in all aspects. I have sincerely prepared this project report and the results reported in this study are genuine and authentic.

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CERTIFICATE

This is to certify that Ms. Mayuri Sharma, B. Tech – M. Tech, Dual Degree Biotechnology (Enrollment No. 1800100377), Department of Bioengineering, Integral University, Lucknow has carried out dissertation work at ICAR-NBFGR under the guidance and supervision of Dr. Murali S, Scientist, ICAR-NBFGR, Lucknow for the period 13th February to 05th August 2023. The title of M. Tech dissertation was **"Development of primary cell culture from different tissues of *Channa Punctata* (Bloch, 1793)"**.

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I wish her good luck and bright future.

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TO WHOM IT MAY CONCERN

This is to certify that I Mayuri Sharma, a student of **B.Tech.-M.Tech.** (Dual Degree) Biotechnology (5th Year/10th Semester) Integral University has completed her six months dissertation work entitled "Development of Primary Cell Culture from Different Tissues of Channa punctata, (Bloch ,1793))" successfully. She has completed this work from ICAR- NBFGR under the guidance of Dr. Murali S Kumar the dissertation was a compulsory part of her **B.Tech -M.Tech** Dual Degree Biotechnology program. I wish her good luck and bright future.

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Mayuri Sharma

Date:

List of Abbreviations and Symbols

Abbreviation	Full Name
%	Percentage
°C	Degree Celsius
µm	Micrometer
Bp	Base Pair
DW	Distilled Water
DNA	Deoxyribonucleic Acid
Gm	Gram
Max	Maximum
Mins	Minute
RNA	Ribonucleic Acid
Rpm	Rotation per minute
SDS	Sodium dodecyl sulphate
Sec	Second
TE	Tris-EDTA
U	Unit
V/v	Volume per volume
Cm	Centimetre
Hrs	Hours
Kb	Kilo base pair

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Abstract

Fish cell has been established as a promising tool for studying many key issues of aquaculture and fisheries covering fish growth, disease, reproduction, genetics, and biotechnology. The present study aimed to develop a primary culture system from the fin and muscle tissues of *Channa punctata,* which is an important candidate species for aquaculture. The primary culture was established from tissues through the explant method and the cultured cells were maintained in Leibovitz's- 15 medium supplemented with FBS (Fetal Bovine Serum) and 500 IU/ml penicillin, 500 µg/ml streptomycin, and 2.5 mg/ml Amphotericin B. Culture of fin and muscle tissues were found to be optimum at 28°C incubation temperature along with 20% FBS. The cultured cells were authenticated using DNA barcoding. The COI and 16s sequence analysis confirmed the species of origin. This primary culture of different tissues would play an important tool in studying *in vitro* research for conservation genetics and biotechnological studies.

Keywords: *Channa punctata*, Primary cell culture, DNA barcoding, Cryopreservation, *In vitro*, Conservation genetics

CHAPTER 1

INTRODUCTION

1. Introduction

Cell culture is a biological method that successfully maintains live cells in a controlled environment apart from the animal (Hohne M *et al.,*1990). Short-term (for a few hours or days) or long-term (for a few weeks, months, and even years) cell culture is an option. Wilhelm Roux, who in 1885 was able to maintain chick neural tissue (in a saline solution), is credited with the discovery of cell culture (Wolf K and Quimby MC, 1964). Cell culture can generally be divided into two categories: primary cultures and cell lines. Primary culture is created from clumps of cells or isolated cells from recently removed specimen tissues. Primary cultures are considered finite and are not frequently (Hohne M *et al.,* 1990). *In vitro* maintenance and subcultures with either finite (if cells have the propensity to achieve senescence) .

For the study of numerous aspects of cellular physiology, molecular biology, genetics, immunology, endocrinology, nutrition, comparative biology, and biotechnology in teleosts, the availability of fish cell culture and cell lines is essential (Hightower LE and Renfro JL, 1988).

Fish cell lines have been effectively employed using Leibovitz-15, an amino acid-rich nutritional medium that does not require CO2 buffering (Leibovitz, 1963). Fetal bovine serum (FBS) is used as a supplement with the tissue culture media with a varying concentration range of 5% to 20%. Fish serum has also been used (1%) in combination with FBS in developing fish cell lines (Chen SL *et al*., 2004) (Lakra WS *et al*., 2006). Some other additives such as fish muscle extract, sucrose, and prawn shell extract can also be used for better results. Various growth factors such as mammalian epidermal growth factor (mEGF) (Watanabe T *et al*., 1987) and, basic fibroblast growth factor (bFGF) (Chen SL *et al*., 2004) had been used to stimulate the growth of the fish cell line.

Fish cell culture differ slightly from mammalian cell culture in that a larger temperature range is used for incubation. Osmolality must also be increased for fish of marine origins. Fish cells can be kept alive for a long time with little maintenance since they have lower metabolic rates than eurythermal cells (Lannan CN *et al*., 1984). To find and isolate fish viruses, a large number of fish cell lines have been developed from fish tissues. For the cellular-level investigation of species-specific responses to viral infection, cell lines from various tissues of various species will be useful. For accurate monitoring of viral illnesses, more cell lines from various organs and tissues of a host species must be

established (Gardenia L *et al*., 2020). This is because some pathogenic viruses are known to be organ or tissue-specific.

The beginning of short-term primary cultures is not reproducible, and the uniformity of the cultures restricts their application (Bols NC *et al*., 1994). The majority of established cell lines come from malignant tumours, spontaneous transformation, or oncogenic immortalization, and these modifications result in constantly reproducing (immortal) cell lines (Freshney RI, 2005). The majority of fish cell lines come from healthy tissues such as the skin, gills, heart, liver, kidney, spleen, swim bladder, and brain. Particularly, the tissues employed in the initial culture are most usually cited as coming from embryos or fins. Due to its strong capacity for regeneration, the fin is the second most frequent tissue used for cultivation after the ovary (Fryer JL and Lannan CN, 1994). For instance, further research can be done to ascertain whether cells have fibroblastic properties if they exhibit the morphology, organization, and culture behaviours of fibroblasts.

Channa punctata, the spotted snakehead is one of the most important fish species of India and neighbouring countries including Bangladesh, Nepal, Myanmar etc. It is called as snakehead because of having certain snake-like characters including elongated and cylindrical bodies, presence of large scales on the head and somewhat flattened head with eyes located in a dorso-lateral position on the anterior part of the head. It is a common freshwater fish which is abundantly found in ponds, beels and canals. This is partially air-breathing freshwater fishs and are considered predators and piscivorous. Disease, siltation, pollution, poisoning, loss of habitat, over exploitation, destructive fishing, introduction of alien species are the major threats to this species.

C. punctata was originally described by Hamilton (1822) based upon the specimen captured from the river Ganges and its tributaries. The fishes of this species have been given different names, i.e., *Ophicephalus karruwey* Lacepède, 1801; *Ophicephalus lata* Hamilton, 1822 *Ophicephalus indicus* McClelland, 1842*; Ophiocephalus affined* Günther, 1861 all of which are now considered as the synonyms and have been withdrawn in favour of C. *punctata* following the priority rule. It has a great demand in market because of its high nutritional value. The culture of snakeheads is still not common due to the scarcity of quality seed supply and lack of knowledge on their feeding and breeding techniques. Study of food and feeding habits of fishes have

manifold importance in fishery biology. It is known as piscivorous, carnivorous and cannibalistic fish.

Snakeheads have been regarded as good food and aquarium fish, and can fetch higher prices in the market. These have long been regarded as a valuable food fish by the Asian people and have been popular in most of the markets. *C. punctata* is considered as important food fish in Andhra Pradesh, India (Talwar PK and Jhingran AG, 1992). Some people believe them to have medicinal or healing properties healing properties, making them prized as food, particularly to people with illnesses or recovering from surgery. In such situations, the fish are killed just before cooking, the thinking being that healing properties are lost if the fish are killed sooner. Availability of cell cultures, especially muscle and fin can be advantageous to evaluate cell-based food concept.

In this background, the present study attempts to develop primary cell culture from different tissues of *Channa punctata*, (Bloch ,1793) with the following objectives:

Objectives

1. Development of primary cell culture from different tissues of *Channa punctata*.
2 .Maintenance of the developed primary cell culture .

CHAPTER 2

Review of literature

2. Review of Literature

Over time, a huge number of cell lines derived from various fish tissues have been created. The chapter discusses the creation of fish cell lines, their historical context, current national and international conditions, the techniques employed in cell culture, the media and supplements used in cell culture, and their characterization, uses, and preservation.

2.1 Animal Cell Culture- Historical Background

Sydney Ringer 1882 laid the foundation of the *in vitro* animal tissue culture. He developed a balanced salt solution that is close to that of the animal's body fluids which was the first instance of *in vitro* cultivation.

Animal cell culture was first successfully undertaken by Ross Harrison in 1907. Harrison cultivated frog nerve cells in lymph fluid and he observed the growth of nerve fibres in vitro for several weeks. This initiative by Harrison paved the way for the development of Animal Cell Culture. Harrison grew frog nerve cells in lymph fluid and tracked the development of nerve fibres in a dish for several weeks. Harrison's initiative set the stage for the advancement of animal cell culture. The superiority of plasma over lymph fluid for the growth of animal cells was discovered by Montrose T. Burrows. Carrel was the first to grow mammalian somatic cells in 1912. The first mammalian cell line, known as "Hela cells from the uterine cervical cancer patient," was created in 1951 by George O. Gey and his colleagues (Yao T and Asayama Y , 2017). The first fish cell line, known as RTG-2, was created from a Rainbow Trout Gonad and maintained at 19°C for two years. The cultures were established in cord serum media. Animal cell culture is an exciting area of research that generates a pronounced avenue to unravel and exploit novel strategies having profound applications (Freshney RI , 2010). Standardisation and commercialisation of cell culture techniques drew the benefit of reliable media and sera and control of contamination with antibiotics as well as clean-air equipment which have broadened the scope of this field from a laboratory tool to a large scale industry. Large-scale culture applications have led to the manufacture of automated equipment, and today's high-end cell culture robots are able to harvest, determine cell viability and perform all liquid handling. Hence there has been a growing importance for cell-based assays, particularly in the pharmaceutical industry. Tissue culture has also been used for diagnosis and toxicology. The toxic effects of pharmaceutical compounds and potential

environmental pollutants can be assayed in vitro. Toxicological investigations using cell culture have been extremely useful for clarification of action mechanisms of toxic substances with specialised cell systems; clarification of the effects on basic cell functions mainly with fibroblasts and epithelioid cells and in metabolism investigations (Paganuzzi-Stammati A *et al.,* 1981). Developments with human embryonal stem cell cultures (Thomson J.A *et al.,* 1998; Webbe DJ and Minger SL, 2004) have made possible the study of brain or nervous tissue, that will differentiate into neurons and may provide useful and specific models for neuronal diseases (Ebert *et al.,* 2009). The prospect of transplantation of cultured cells has generated a whole new branch of culture, that of tissue engineering (Atala A and Lanza RP, 2002) (Vunjak-Novakovic G and Freshney RI, 2006) encompassing the generation of tissue equivalents by organotypic culture, isolation and differentiation of human embryonal stem (ES) cells and adult totipotent stem cells such as mesenchymal stem cells (MSCs), gene transfer, material sciences, construction and utilisation of bioreactors, and transplantation technology. With the success in human induced pluripotent stem cells (iPSCs), cell culture has far-reaching applications as in the production of new disease models and in drug development as well as application in transplantation medicine once technical limitations (for example, mutation through viral integration) are eliminated. In biomedical field, cultured cells are already used routinely for a variety of applications, for example Genzyme‟s Epicel (cultured epidermal keratinocyte autografts) for burn patients and Carticel (cultured autologous chondrocytes) for cartilage repair, as well as at in vitro fertilisation (IVF) clinics where the zygote is cultured usually for a few days prior to implantation in the mother‟s uterus (Clarke S and Dillon J, 2011). Mario Capecchi, Martin Evans and Oliver Smithies won the Nobel Prize for Physiology and Medicine in 1997 for their work using embryonic stem cells from mice to introduce specific gene modifications (knockout mice). The Nobel Peace Prize for Physiology and Medicine was awarded in 2012 for groundbreaking discoveries in stem cells to John B. Gurdon and Shinya Yamanaka. While Gurdon in 1962 introduced the concept of cloning, it was Yamanaka (Takahashi K and Yamanaka S, 2006) who discovered that reprogramming can be accomplished by just four specific transcription factors in the egg, the cell could return to its primitive or stem cell form. They demonstrated the induction of pluripotent stem cells from mouse embryonic or adult fibroblasts by introducing just four factors, Oct3/4, Sox2, c-myc, and Klf4, under ES cell culture conditions. These iPS cells are capable of differentiating into various cell types, such as beating cardiac muscle cells, neurons and pancreatic cells (Zhou GZ *et al.,* 2009).

Cell culture is becoming the next big thing in pharma research, as laboratories rush to meet the demand for stem cells and biologics. The global cell culture market was worth USD 3.4 billion in 2011, and it is expected to grow at a compound annual growth rate (CAGR) of 9.3% between 2011 and 2018 to reach USD 6.3 billion in 2018.

2.2 History of Fish Cell Culture

Fish cell cultures have been in use for over 3 decades (Schirmer K, 2006) and, though originally developed to support the growth of fish viruses (Wolf K,1988) for studies in aquatic animal viral diseases, fish cell lines have grown tremendously in number covering a wide variety of species and tissues of origin. While various applications have been reported, their potential role in science and technology are just beginning to be exploited. Fish immunology (Bols NC *et al.,* 2001), physiology, genetics and development (Ganassin RC., *et al*., 2000), toxicology (Babic H *et al.,*1991; Segner H, 1998; Fent K, 2001;(Davoren M and Fogarty AM, 2006), ecotoxicology (Castaňo A *et al.,* 2003), endocrinology (Bols NC and Lee LEJ, 1991), bio-medical research (Hightower LE and Renfro JL, 1988), disease control (Villena AJ, 2003), biotechnology and aquaculture (Bols NC, 1991) are some of the areas in which fish cell lines have proven as invaluable resources.

Teleost cell lines are the second most abundant among animal cell lines, mammalian cell lines being the most numerous (Wolf and Quimby, 1969). The first successful attempt for fish tissue culture was made by Osowski H(1914), who cultured explants of trout embryos and fry in Ringer‟s solution and frog lymph.

RTG-2 cell line of rainbow trout, *Salmo gairdneri gonad* origin initiated in 1960 was the first permanent fish cell line to be developed (Wolf K and Quimby MC,1962. (Fryer JL ,1964) made a quantitative study of requirements for dispersing tissues and culturing cells from embryonic Pacific salmon and rainbow trout hepatoma and established five different lines of cells from these tissues. Five single-cell clone lines were developed from normal primary cultures of rainbow trout pituitary glands and passaged for over 150 times. The expression of gh and prl genes in the single-cell clone lines is responsive to induction by E2, dexamethasone, and o, p‟–dichlorodiphenyltrichloroethane (Chen MJ *et al*., 2010).

2.3 Fish Cell Culture in India

Fish tissue culture has advanced in a very short span of time from an esoteric art to a workaday tool of many uses. A few pioneering works in India include, a cell line (MG-3) from gills of mrigal, *Cirrhinus mrigala* and a cell line (RG-1) from gills of rohu, *L. rohita* (Sathe PS *et al.,* 1995; 1997) both cell lines were characterised with respect to isoenzyme pattern and chromosome number. The profile of three isoenzymes were tested (viz., lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and glucose 6 phosphate dehydrogenase (G6PDH) and RG-1 cell line only differed from MG-3 cell line in mobility pattern of G6PDH. Both cell lines crossed 20 passages and had a chromosome number of 50 growth medium with Leibovitz‟s L-15 as the base, supplemented with fetal bovine serum (FBS) (10% v / v), fish muscle extract (10% v/ v) , prawn muscle extract (10% v/v), lectin (concanavalin A, 0.02 µg mL^{-1}), lipopolysaccharide (0.02 µg mL^{-1}), glucose D (0.2 µg mL^{-1}), ovary extract (0.5% v/v) and prawn haemolymph 0.5% has been formulated with 354±10 mOsm for the development and maintenance of a cell culture system from the ovarian tissue of African catfish, *C. gariepinus* (Kumar GS *et al.,* 2001). The cell culture was passaged 15 times after which they ceased to multiply and consequently perished. The cell cultures were maintained for 12–15 days without fluid change between the passages.

(Lakra WS *et al.,* 2006) reported the development of a diploid cell line (TP-1) for the first time from golden mahseer, *T. putitora* which has potential application in biodiversity conservation of the species (Lakra WS *et al*., 2010). Thirty-day-old fry were used to prepare a primary cell culture employing trypsin digestion. The optimal conditions of nutrition and incubation for growth of the dissociated cells were determined. The thymidine uptake assay was employed to investigate the effect of different concentrations of fetal calf serum (FCS) and fish muscle extract (FME) on the growth of TP-1 cells and to study the cell proliferation rate at different time intervals. It was found that L-15 supplemented with 20% FCS and 10% FME at an incubation temperature of 28°C resulted in optimal growth. The concentration of FCS was reduced to 10% after 10 subcultures. On reaching confluency, the cells were subcultured with a split ratio of 1:2 and grew to confluency in 4 days. The morphology of the cells were fibroblast-like, The cells were successfully cryopreserved and revived at passage numbers 5, 8, 15 and 18. The cells were characterised for chromosome number (2n=100) at 10 and

20 passages. The cell cycle analysis by FACS (Fluorescence Activated Cell Sorter) revealed that most of the cells on the first and fourth day of culture were in S-phase, indicating a high growth rate.

A lot of fish cell lines have been developed in India since the last few years. Kumar MS et al., (2021) established an eye muscle cell line from snow trout, *Schizothorax ricjardsonii*. Kumar MS et al., (2021) established a caudal fin cell line from hill trout, *Barilius bendelisis* for the first time. Multiple cell lines of Indian catfish, magur has been developed (Neha S et al., 2021; Neha S et al., 2018).

C. catla cell lines have been developed from eye muscle (SICE) (Ishaq Ahmed VP *et al.,* 2008) which showed strong positive reaction to epithelial markers viz., pancytokeratin, cytokeratin 19 and the proliferative marker Ki67. The cell cycle analysis by fluorescence-activated cell sorter revealed that most of the cells on the second day of culture were in S-phase, indicating a high growth rate.

The National Bureau of Fish Genetic Resources (ICAR-NBFGR) in Lucknow established the NRFC as part of a research project with funding from the Department of Biotechnology, Government of India, New Delhi. NRFC at 81 cell line accessions has the world's largest collection of fish cell line accessions.The goal of NRFC is to act as a national referral centre for fish cell lines in the nation as well as to receive, authenticate, preserve, and provide fish cell lines to researchers and academics for Research and Development projects.

The NRFC has a state-of-the-art facility for the development, characterization, and maintenance of cell lines. Fish cell lines developed across the country have majorly been deposited in the NRFC and are being maintained at the cryopreservation facility. The facility is fully functional for catering to the needs of researchers and academicians in the country.

The National Repository of Fish Cell Line (NRFC), NBFGR, Lucknow, is currently home to roughly 81 fish cell lines from different fish species in India. Each of these cell lines was donated by a different researcher. Many different tissues, including the ovary, fin, swim bladder, heart, spleen, liver, eye muscle, brain, and skin, have been used to create fish cell lines. Fish biotechnology research and fish conservation have benefited from the creation of new cell lines from the species *Etroplus suratensis* (Babu

V *et al*., 2012), *Clarias batrachus* (Murli SK *et al*., 2020), *Catla catla* (Chaudhuri DK *et al*., 2014), *Channa striata* (Majeed SA *et al*., 2014), Puntius *chelynoides* (Goswami M *et al*., 2014) and *Wallago attu* (Dubey A *et al*., 2014), *Clarias magur* (Singh N *et al*., 2021).

(Rathore G *et al*., 2007) developed primary cell culture from fin explants of *Labeo rohita* and the study reports a successful development of primary cell culture from fin explants of *L. rohita* and subsequent subcultures up to eight passages. Explant tissues of the fin were seeded and incubated at 30°C in DMEM supplemented with 10% FCS. On day 5 of seeding, the differentiation of the cells was visible, and a complete monolayer was obtained on day 20. A cell count of $2x10^6$ cells was sufficient to produce a monolayer in five days on the 8th passage.

Lakra WS *et al*., 2010, emphasized an experimental strategy in developing and characterizing two new cell lines from the fin and heart tissues of common carp *Cyprinus carpio* and two new cell lines (CCF and CCH) were established. The cells were optimally maintained in Leibovitz-15 medium supplemented with 10% fetal bovine serum (FBS) and 10 ng/ml of basic fibroblastic growth factor (bFGF). The effects of temperature, concentration of FBS, and bFGF on the growth of CCF and CCH cells were examined in the established common carp cell lines, CCF and CCH from fin and heart, respectively, would provide an enhanced capability for viral detection and identification in the aquatic animal species in India.

Lakra WS and Goswami M, 2011, conducted a study entitled "Development and characterization of a continuous cell line PSCF from *Puntius sophore*". The cell line was optimally maintained at 28°C in Leibovitz 15 (L-15) medium supplemented with 10% foetal bovine serum (FBS). The origin of the cell lines was confirmed by the amplification of 581 and 655 bp fragments of 16 S rRNA and cytochrome oxidase subunit I (COl) of mitochondrial DNA (Mt DNA) genes, respectively. The PSCF cells were successfully transfected with Green Fluorescent Protein (GFP) reporter plasmids and the expression of GFP gene in the cells indicated the potential utility of the cells in gene expression studies.

Babu VS *et al.,* 2011, established and characterize fin cell line from Indian walking catfish, *Clarias batrachus*, In Leibovitz's L-15 media supplemented with 15% fetal bovine serum, the cell line expanded effectively (FBS) and has undergone more than 110 subcultures since it first started in 2007. The fin cell proliferation accelerated as the

FBS concentration rose from 2% to 20% at 28°C with 15% concentration being the ideal level for growth or 20%, or at a concentration of 5%, with poor growth. The study showed that fish Noda virus and INV-ab infection was detected in the cells and was confirmed by cytopathic effect and reverse transcriptase polymerase chain reaction.

Yaswanth BS *et al*., 2020, designated cell line as OCF was developed for the first time from the caudal fin of *Amphiprion ocellaris*. The cell line was maintained in Leibovitz's-15 medium supplemented with 15% FBS and was successfully sub cultured up to 34 passages. The OCF cell line was used for testing nervous necrosis virus (NNV) susceptibility Cytopathic effect (CPE) was observed in terms of plaque formation after virus inoculation Nested PCR confirmed the susceptibility of the OCF cell line to NNV. The cell line was successfully cryopreserved by a slow freezing procedure at - 80 °C with a revival efficiency of 70-75%. The study revealed that the OCF cell line would be useful for virological studies In addition, the cell line would play an important role as a fin *in vitro* tool for carrying out toxicological and biotechnological studies.

2.4 International Scenario

Cellulosaurus, (Bairoch A, 2018) finished up in excess of 900 fish cell lines out of 104,421 cell lines from in excess of 590 species till Walk 2023. Cell lines have by and large been made from an assortment of fish tissue tests, like gill, caudal blade, eye, liver, and kidney. Major cell line repositories such as the American Type Culture Collection (ATCC), the European Collections of Cell Cultures (ECACC), and the German Collection of Microorganisms and Cell Cultures (DSMZ) have all received and verified submissions of cell lines from researchers from all over the world. North of 40,000 cell lines, including those from different tissues of 45 fish species, are right now held at the European Assortments of Cell Societies (ECACC). These cell lines address 45 particular species and 50 different tissue types. 43 cell lines from amphibian creatures — out of the in excess of 3,400 cell lines that have been saved at the American Sort Culture Assortment (ATCC) — are at present accessible to scientists around the world. Of those, 19 are fish cell lines (Nagpure NS *et al*., 2016).

Universally, 280 recently settled fish cell lines were created because of work done in finfish cell culture all over the planet throughout recent years, bringing the complete number of finfish cell lines to 783 from 20 unmistakable tissue kinds of 44 different fish families from 21 distinct nations. The fish of the Cyprinid family, which is most often

developed in Asian countries, were the wellspring of the best number of cell lines made in the past decade (Thangaraj RS *et al*., 2021).

Fernandes MN *et al*., 1995, established essential cell culture from gill explants of Rainbow Trout, Oncorhynchus mykiss. Leibovitz L-15 media with l-glutamine and 5, 10, or 20 percent fetal calf serum (FCS) were used to cultivate the explants. The attachment efficiency was serum-dependent, even though a higher FCS concentration did not encourage additional cell outgrowth. The gill explant was valuable for inspecting long haul cell science cycles and diminishing the quantity of culture arrangements.

Saad MK *et al*., 2023 created and examined a continuous Atlantic mackerel (Scomber scombrus) skeletal muscle cell line, or "Mack" cells. New got fish muscle biopsies were utilized to extricate the cells, and autonomous separations from two unique species were completed. Mack1 cells (cells from the underlying confinement) were subcultured in excess of multiple times throughout over a year in culture.

2.5 Cell Culture Media and Reagents

In general, media created for mammalian cell culture are used in the majority of fish cultures. A popular culture medium for the cells of mammals, birds, reptiles, amphibians, and of course fish is Eagle's Minimal Essential Medium (EMEM) supplemented with FBS (Wolf and Quimby, 1966). Hank's MEM (HMEM), Leibovitz L-15 medium (L-15), and Glasgow MEM are other media that are frequently used in fish culture. Fish cell lines have been effectively employed with Leibovitz-15, an amino acid-rich nutritional medium that doesn't require CO2 buffering (Leibovitz, 1963). FBS which is added to the tissue culture media in concentrations ranging from 5% to 20%, is utilized as a supplement. To create fish cell lines, Fish serum (1%) was combined with FBS (Chen TL *et al*., 2004) (Lakra WS *et al*., 2006). For better outcomes, additional additions including fish muscle extract, sugar, and prawn shell extract might be employed. Mammalian epidermal growth factor (mEGF), among other growth factors (Watanabe T *et al.,* 1987) and basic fibroblast growth factor (bFGF) (Chen TL *et al.*, 2004) has been used to stimulate the growth of the fish cell line.

2.6 Contaminants and Test for Detection of Contamination

The most frequent contaminants in cell culture are mycoplasmas (Chen TR *et al*., 1977). One of the simplest and most consistent methods for detecting mycoplasma infections is the fluorescent staining of DNA with Hoechst 33258. This method shows

mycoplasma infections as small particulate or filamentous staining over the cytoplasm. In cell culture, contaminants are difficult to get rid of, the main problem with cell culture techniques is microbial contamination. The operator or the lab apparatus can cause contamination. The CO2 on pollutants in cell culture are bacteria, fungi, moulds, yeast, and mycoplasma. To exclude contamination in cell culture, extreme caution must be used. For aseptic conditions in the workplace, sterilization techniques such as swabbing, burning, dry heat, wet heat, radiation, and filter sterilization should be used for decontamination routines. And other techniques for the screening of mycoplasma are PCR, ELISA assay, immunostaining, and autoradiography (Freshney RI, 2010). Incidences of mycoplasma in primary cultures and continuous cell lines have been reported earlier (Ludovici PP *et al*., 1973).

2.7 Development and Characterization of Primary Cell Culture

Toullec JY *et al.,* 1996, developed primary cell culture from the Penaeid shrimps i.e., *Penaeus vannamei* and *P. indicus*. Tissues from various organs of the shrimp *P. vannamei*, as well as embryos of *P. indicus*, were cultured in different media. Cell outgrowths were observed with ovaries, epidermal regeneration buds, and embryos. Optimal results, i.e., a survival for several months, were obtained in M-199 based medium for ovaries and embryos, and in Grace's medium for regeneration buds. In every case, a 10% fetal bovine serum supplement was given.

Khurshid K *et al*., 2022, reported the establishment of a primary cell culture system from the heart of *Schizothorax esocinus*, an indigenous cold-water fish of the Indian Himalayas. The standardization of the primary cell culture was done using L-15 media at different FBS concentrations and temperature ranges. The primary cell culture system of the heart showed consistent growth at an optimum FBS concentration of 15% and an optimum temperature of 24ºC. Characterization of newly developed primary cell culture was done by karyotyping. The chromosome number of the primary cell culture system was found to be 98 which is consistent with the chromosome number of *S. socius.*

2.8 Application of Fish Cell Lines

Fish cell cultures can be used to study a variety of basic fish research issues, including toxicity, virology, immunology, reproductive biology, carcinogenesis, gene regulation and expression, DNA replication, and repair. Cell lines may be valuable for revealing fundamental truths about development, reproduction, and health. Because cell

lines allow for manipulation, they may also be employed as sources of biochemical products rather than whole organisms.

2.8.1 Virology

While fish viral vaccines are still in the development stage, purified viruses can be utilized as vaccines and are anticipated to be the first health product to be obtained from piscine cell cultures (Sindermann CJ, 1990).

Zeng W *et al*., 2021, established a permanent cell line from the heart of *Mircopterus salmoides* (MSH) that has been subcultured over 70 times and showed optimal growth at 27 ◦C using Medium 199 supplemented with 10% fetal bovine serum. The MSH cells showed susceptibility to *M. salmoides* rhabdovirus (MSRV), Largemouth bass rana virus (LMBV), Infectious spleen and kidney necrosis virus (ISKNV), Spring viremia of carp virus (SVCV), Tilapia Lake virus (TiLV) and Grass carp reovirus genotype I (GCRV-I) but were resistant to nervous necrosis virus (NNV). The newly formed MHS cell line was highly vulnerable to numerous fish viruses, and as a result, they were able to effectively create an immortal cell line from the heart tissue of *M. salmoides*. This cell line can be used in future genetic, virological, and immunological studies.

2.8.2 Immunology

The development of fish-derived cell culture methods is crucial for understanding the basic principles of fish immunology (Faisal M and Ahne W, 1990). There are potential factors to take into account while creating vaccines against significant fish infections, as well as in comparative immunology and immune system research. The effects of numerous chemicals, including antibiotics, on the regulation of immune system cells, have been studied using *in vitro* systems.

Zhi S *et al*.,2023, developed a novel immortal cell line CCM (Yellow River carp muscle cells) derived from the carp muscle. CCM has been passed over 71 generations for one year. To their knowledge, this is the first muscle cell line in Yellow River carp and the first study on the immune response signal pathways of Yellow River carp based on the muscle cell line. CCM cell line provides a more rapid and efficient experimental material for fish immunology research.

2.8.3 Toxicology

Fish cell cultures have been employed in toxicological studies as an *in vitro* model biological system for assessing the effects of various chemicals, pesticides, and industrial wastes as well as in the study of carcinogenesis for examining cell transformation by fish viruses, chemical agents, and the interaction of viruses and chemical carcinogens (Goswami M *et al*., 2014).

Kalman J *et al*., 2001, presented work on the potential of fish hepatocytes (topminnow fish hepatoma cell line, PLHC-1) and macrophages (carp leukocyte cell line, CLC) to study the toxicity and intracellular fate of helical-ribbon carbon nanofibers (CNFs) and graphene oxide (GO) used in a variety of intermediate industrial products was evaluated, allowing a first ranking of GRMs according to their cytotoxicity. Cells were exposed to a concentration range of 0-200 µgml-1 of GRMs for 24 and 72 h and cell viability was assessed by measuring mitochondrial activity (Alamar Blue assay), plasma membrane integrity (5-carboxyfluorescein diacetate acetoxymethyl ester assay) and lysosomal function (neutral red uptake assay). Results showed that both the cell type and the choice of endpoint determined the toxicity of GRMs. In both cell lines, CNFs appeared to have higher toxicity than GO, and the highest degree of graphitization in fibres was associated with lower toxicity. These findings contribute to the understanding of the toxicity and behaviour of these GRMs in living systems, therefore aiding in designing safer materials for the environment.

2.8.4 Biomedical Research

In terms of contemporary research tools, cell cultures have various benefits. In general, cultures make it possible to study cellular events in a defined and controlled environment, free from the complexity and variability of systemic or broader physiological factors. For an experiment on a specific species, cell lines are the sole routinely accessible supply of biological material (Bols NC *et al*., 2005).

Hightower LE and Renfro JL (1988) reviewed a paper on application of fish cell lines to biomedical research. To spark more interest in the lower vertebrates as practical alternatives to mammalian cell culture in biomedical research, they briefly summarise several of the successful models in this study along with recent advancements in fish cell culture. Epithelial ion transport, endocrinological investigations, the cellular stress

response (heat shock response), thermotolerance, cancer biology, and environmental toxicology are among the subjects studied.

2.8.5 Vaccine Development

Attenuated viral vaccines can be developed by repeatedly passing the wild-type virus through susceptible fish cell cultures, which has proven to weaken the virus (Noga and Hartmann, 1981). Virus attenuation has been achieved in the case of the koi herpesvirus vaccine in the koi fin cell culture (Ronen A *et al*., 2003) and the Channel Catfish Virus (CCV) vaccine in *Clarias batrachus* kidney cell line (Noga EJ and Hartmann JX, 1981). Cyprinid Herpesvirus (CyHV3) was attenuated by serial transfer (20 passages) of the virus in Koi fin (KF- 2) cells and found to be very effective against CyHV3 infections (Noga EJ and Hartmann JX,1981).

Zeng W *et al.,* 2016, presented work on grass carp, the immunological results and effectiveness of an inactivated HuNan1307 vaccination were assessed. The grass carp PSF cell line was used to create the GCRV isolate HuNan1307, which was then inactivated with 1% bpropiolactone for 60 hours at 4°C. The inactivated GCRV vaccination was administered to grass carp, who were then challenged with the isolate HuNan1307. The findings demonstrated that the inactivated vaccination required a minimum dose of 105.5 TCID50/0.2 mL to produce immunological protection. All grass carp who received the inactivated vaccine exhibited a high litre of serum antibodies as well as a neutralizing antibody that is specific to the GCRV. The data in this study suggested that the inactivated HuNan1307 vaccine may represent an efficient method to induce immunity against GCRV infection and the induced disease in grass carp.

CHAPTER 3

Materials and Methods

3. Materials and Methods

3.1 Location

National Repository of Fish Cell Line, ICAR-NBFGR, Lucknow**.** The present work was carried out in Cell Culture Facility of National Repository of Fish Cell Line, ICAR-NBFGR, Lucknow, Uttar Pradesh.

3.2 Collection of Specimen

Fig.1(a)

Fig.1(b)

Fig.1(c)

Fig.1(a) Location of Specimen (Khurram Nagar Fish Market, Lucknow. Fig.1(b) Body weight of *Channa punctata* fish. Fig.1(c) Length of *Channa punctata* fish.

Normal and healthy live specimens of *Channa punctata* (body weight 6.31 g total length:5.9 cm) originally collected from Khurram Nagar fish Market, Lucknow (Figure 1) Uttar Pradesh were transported to the Indoor fish Hatchery of ICAR-NBFGR and were fed with pelleted feed @5% Body Weight in well-aerated water.

3.3 Essential Materials Needed for Cell Culture

Cell culture flasks: Specially treated T25 (25 square cm are for growth) and T75 (75 square cm are for growth) cell culture flasks are needed to grow the cells.

Leibovitz-15 (L15) media: L15 media is commonly used for fish cell lines. Normally media of 4-5ml for T25 and 9-11 ml for T75 flask is needed.

Supplements: FBS serum and L-glutamine are commonly used supplements. Normally 5-20% FBS is used depending on the needs of the cell line.

Trypsin: It is a proteolytic enzyme used in passaging procedure to help detach cells from the flask surface. Usually, it is used along with EDTA in 0.25% concentration.

PBS: Phosphate buffer saline is a physiological saline used to wash cells. During passaging it is used to remove traces of serum so as to enable trypsin to act.

Antibiotic-antimycotics: Antibiotic-antimycotic mixtures at 1X concentration are used to prevent contamination. It should be noted that prolonged use is not recommended.

3.4 Essential Conditions Needed for Cell Culture

Temperature: Cells need same temperatures as the organism from which it was removed. For fishes, the preferred temperature is 16-28 ° C depending on the species.

pH: When conditions are too acidic or basic, cell damage and death occurs. Cells grow best at neutral pH. It is required to monitor pH regularly as pH will change as cell utilize nutrients and release by products.

Humidity: Low humidity can result in evaporation of water from growth medium resulting in cell lysis. A pan of water place at the bottom of incubator is sufficient to maintain enough humidity.

3.5 Essential Equipment Needed For Cell Culture

CO_2/BOD Incubator: Incubators are required to maintain optimum temperature and environment for cells. Mammalian cell culture media is based on a carbonate-bicarbonate buffering system due to which 5% CO_2 is needed for cells. Fish and insect cell culture media are based on a pyruvate buffering system and hence are not dependent on CO_2.

Laminar flow hood: Laminar flow is required for a safe and contamination-free environment to work on

Inverted and fluorescent microscope: Inverted microscope is required to observe the cells in the culture flask while a fluorescent microscope is required for immunotyping and related studies.

Centrifuge: A good clinical centrifuge is required for pelleting cells for cryopreservation and removing cryoprotect during revival and a lot of other purposes too.

Hemacytometer: Hemacytometer is required for cell counting during viability testing and routine cell culture experiments.

Liquid nitrogen cans: They are required to store cells in vials for long-term storage.

Water bath: A water bath is needed to warm the cells instantly during the revival procedure.

Freezer: A good freezer is needed to store cell culture reagents and materials for usage. Autoclave: A suitable autoclave is needed to sterilize all cell culture materials to reduce the chance of contamination.

3.6 Development of Primary Culture

3.6.1 Preparation of Donor Fish

As contamination is the major problem in cell culture experiment , adequate care was taken to minimize the possible routes of contamination. The donor fish i.e., *Channa punctata* was kept in well-aerated sterile water containing penicillin (500 IU/ml) and streptomycin (400 g/ml) with an anti-fungal amphotericin B (10 pg/ml) without feeding for 24 hr. to reduce the possibility of contamination from faces and regurgitated feed.

3.7 Decontamination

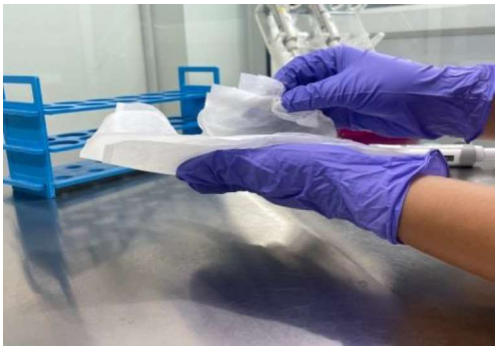
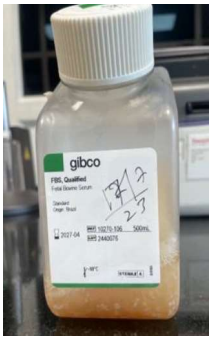


Fig.2 Fish surface wiped out with 70% ethanol for decontamination of external organ.

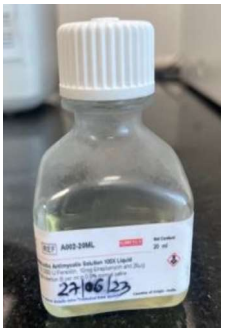
External decontamination is necessary before dissection. The donor fish (*Channa Punctata*) was then sacrificed by euthanasia using clove oil, then the surface was wiped out with 70% ethanol for decontamination of external organs such as fin, Skin, scales etc. before dissection (Figure 2).

3.8 Preparation of Primary Culture Media

(a) Leibovitz's L-15



(b) Fetal Bovine Serum(FBS)



(c) Antimycotic Antibiotic Solxn

(d) 1X Media

Fig.3 Constituents of Primary Culture Media

For the preparation of primary culture media, we use (L-15) Leibovitz's 15 (Himedia India) 16ml (FBS) Fetal Bovine Serum 20% (4ml) (Gibco Brazil) (Figure 3(a,b)) to prepare 20 ml of final volume. After preparation of total 20 ml primary growth media add Antibiotic e.g., Antibiotic Antimycotic Solutions 100x Liquid (Himedia

India) 100ul (Figure 3(c) into the primary culture media (Figure 3 (d)). The complete media preparation was done under the sterilize condition into the laminar flow.

3.9 Preparation of Primary Culture of Fin

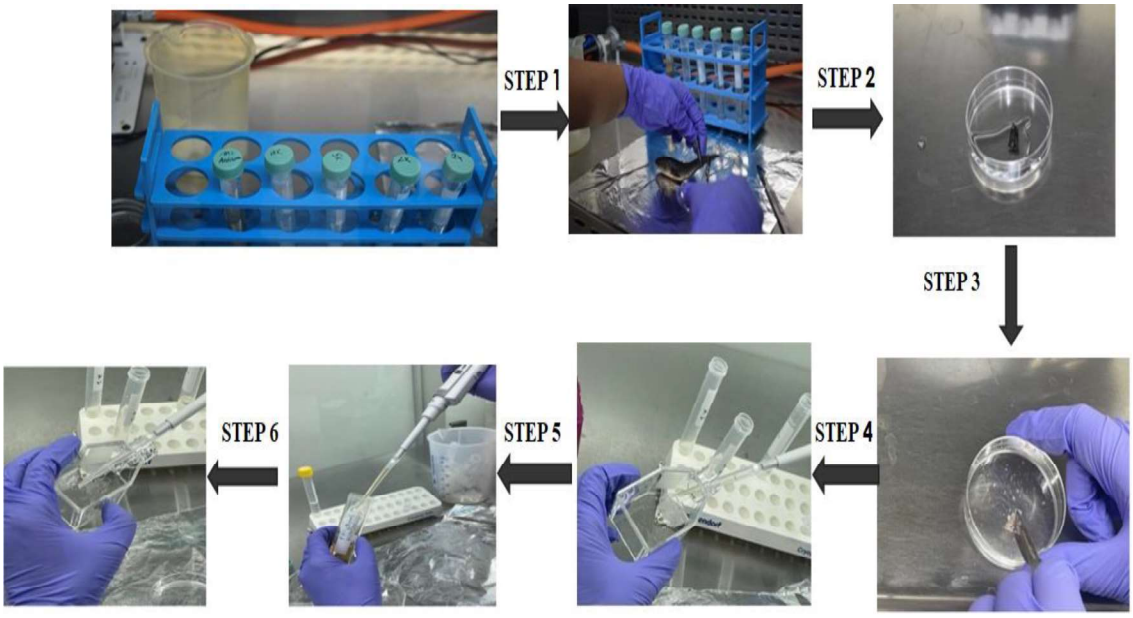


Fig.4 Step-Wise Representation of Preparation of Primary Culture of Fin

Before killing fish, use clove oil to sedate it. Fish needs to be thoroughly cleaned with 70% ethanol before dissection. A fin tissue was aseptically collected in sterile petri plates containing phosphate-buffered saline (PBS), 500 IU/ml penicillin, and 500 Mg/ml streptomycin. A sterile surgical blade was used to chop the tissue parts into small fragments in a petri dish. Then, the tissue pieces were moved to a new petri plate and washed repeatedly with 1 ml of PBS, (HiMedia, India) containing 10x, 5x, 2x, and 1x Antibiotic- Antimycotics mixture washed, and the pieces were allowed to be settled down before removing the supernatant.

The tissue pieces 25–30 were placed per T25 flask (ThermoFisher Scientific, USA) and the embedded tissue in the flask was air dried for 2-3 minutes after that add 1 ml of Fetal Bovine Serum (FBS), 100–200 IU/mL of penicillin, 100–200 g/mL of streptomycin, and 0.25 ug/ml of amphotericin B, then tissue attached flask was allowed to incubate at 28 °C in BOD Incubator for 21-23 hours. If the pieces have adhered, then add 4 ml of freshly prepared 1x Media (L-15+20% FBS+ Antibiotic) and then again incubate

at 28°C in the BOD Incubator. Using an inverted microscope (Nikon, Japan), tissue explants were observed for the growth of cells and the chances of contamination. Media was replaced according to the observation of cells, mostly media was replaced around 50%-70% for every 3-to5 days with L-15 media containing 20% FBS+ penicillin G 1600 units/mL, streptomycin 1,600 µg/mL, and amphotericin B 40 µg/mL and incubated at 28°C in BOD Incubator. The monolayer was formed around 20th Day.

3.10 Preparation of Primary Culture of Muscle

Before killing fish, use clove oil to sedate it. Fish can be thoroughly cleaned with 70% ethanol before dissection. Fin tissues was aseptically collected in sterile petri plates containing Phosphate Buffered saline (PBS), 500 IU/ml penicillin, and 500 Mg/ml streptomycin. A sterile surgical blade was used to chop the tissue parts into small fragments in a petri dish. Then, move the tissue pieces to new petri plates and washed repeatedly with 1 ml of PBS ,HiMedia ,India containing 10x, 5x, 2x, and 1x Antibiotic-Antimycotics mixture washed, and the pieces was allowed to be settled down before removing the supernatant.

The tissue pieces 25–30 were placed per T25 flask (ThermoFisher Scientific ,USA) and the embedded tissue in flask was air dried for 2-3 minutes and after that we priming the tissue, add 1 ml of Fetal Bovine Serum (FBS), 100–200 IU/mL of penicillin, 100–200 g/mL of streptomycin, and 0.25 ug/ml of amphotericin B, then tissue attached flask was allowed to incubate at 28 °C in BOD Incubator for 21-23 hours. If the pieces have adhered, then add 4 ml of freshly prepared 1x Media (L-15+20% FBS+ Antibiotic) and then again incubate at 28°C in BOD Incubator . Using an inverted microscope (Nikon, Japan), tissue explants were observed for growth of cells and chances of contamination. Media was replaced according to the observation of cells, mostly media was replaced around 50%-70% for every 3 to 5 days with L-15 media containing 20% FBS+50IU/mL penicillin, and 50ug mL/1 streptomycin and 0.06 ug mL/1 amphotericin B, and incubate at 28°C in BOD Incubator. The monolayer was formed around 20th Day.

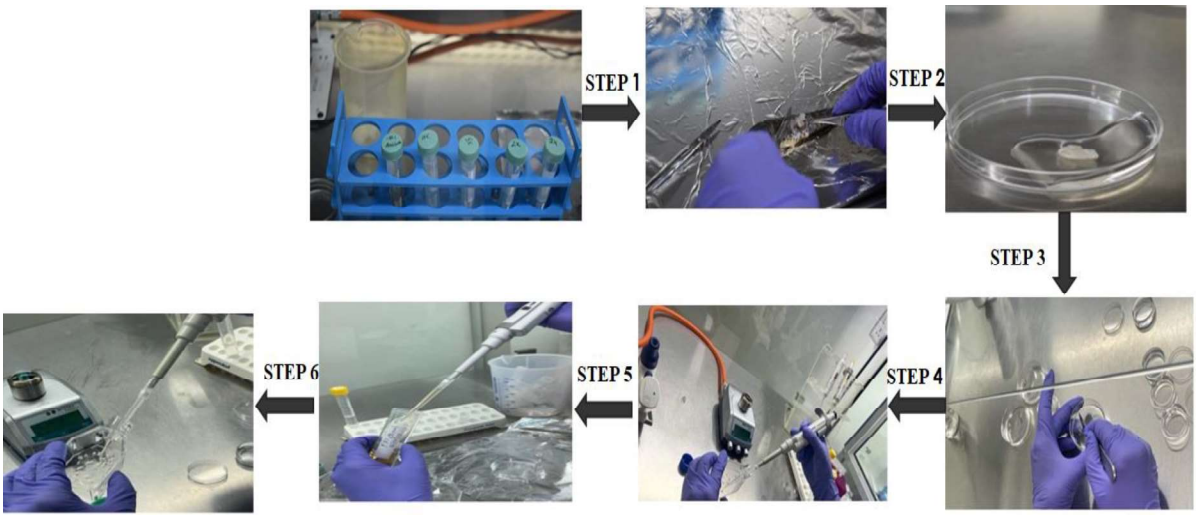


Fig.5 Stepwise Representation of Preparation of Primary Culture of Muscle

3.11 Morphological Observation

The flasks were observed daily for the cell radiation from the tissue, and contamination using an inverted microscope (Nikon, Japan).

3.12 Maintenance of Primary Culture Fin and Muscle

The dispersed cells adhere to the culture substrate and proliferate, whereas dead cells cannot adhere to the flask and hence float. Dead cells were washed off during the subsequent medium exchange. The optimum pH and incubation temperature maintained were 7.4 and 28°C for the culture of fish cells. The medium was changed after 3 days of explant preparation.

3.13 Molecular Characterization

3.13.1 Genomic DNA Isolation

Genomic DNA from the cultured cells and the muscle tissue of *Channa punctata* was isolated using the phenol-chloroform method (Sambrook J and Russell DW, 2001). First, old media from the culture flasks were removed and then the cells were washed with 2 ml PBS and the cells were detached by trypsinization. Then the detached cells were suspended with 2 ml PBS and centrifuged at 10,000rpm for 10 minutes. Discard the supernatant and add 600 µl Lysis buffer + 30 µl SDS + 20µl of Proteinase K to each tube and incubate at 37º C overnight. After cell lysis, an equal volume of Tris-saturated phenol was added to the lysate and mixed well until an emulsion formed. The tubes were centrifuged at 10,000 rpm for 10 min and the aqueous phase was collected in a fresh tube.

An equal volume of phenol: chloroform-isoamyl alcohol was added and then mixed by inverting the tube several times till the emulsion formed and centrifuged at 10,000 rpm for 10 min at 4ºC. The aqueous phase was transferred to a separate tube and an equal volume of chloroform-isoamyl alcohol (24:1), was mixed well and then centrifuged at 10,000 rpm for 10 min at 4ºC. Volume 0.2 (80µl) of isopropanol was added to the tubes and kept in the freezer for 30 minutes and then again centrifuged at 10,000 rpm for 10 minutes. The supernatant was removed and for washing the pellet, 500 µl of 70% alcohol and centrifuged at 10,000 rpm for 8 minutes. Finally, remove the alcohol and tilt in tissue paper for air drying. DNA was then stored at -20°C for further use.

3.13.2 Amplification of 5' COI Mitochondrial Gene

The universal pair of primers FishF1 and FishR1 (Ward RD *et al*., 2005) were used to amplify the mitochondrial cytochrome C oxidase I (COI) gene(Table 1). The Mt DNA gene COI was amplified in a 15 µl reaction; the composition details are provided in Table 2. The thermal cycler (Himedia) was used for PCR amplification. Details of the thermal regime and cycle parameters are provided in Table 3. PCR products were visualized on 1.0% agarose gel after staining with Ethidium bromide (EtBr) and documented using a gel documentation system (Gene Genius, Syngene, India).

Table 1: List of primers used for Amplification of Mitochondrial COI region

S. No	Mitochondrial Region	Primer Name	Primer sequence (5'-3')	Length (bp)	Reference
1	COI	Fish F1	TCGACTAATCATAAAG ATATCGGCC	26	Ward *et al*., (2005)
		Fish R1	ACTTCAGGGTGACCGA AGAATCAA	26	

Table 2: Composition of PCR master mix

Components	Volume/Reaction (µl)
Nuclease Free Water	9.60
PCR buffer (10x with $MgCL_2$)	3.00
dNTPs (2.5mM each)	0.60
Primer Forward (10mM)	0.40
Primer Reverse (10mM)	0.40
Taq. Polymerase (0.75U)	0.50
Template DNA (100ng/µl)	0.50
Total volume	**15 µl**

Table 3: Thermal Regime for Cytochrome C Oxidase I (COI) gene

Steps	Conditions		Cycles
	Temperature	Time	
Initial Denaturation	94 ºC	3 mins	1 Cycle
Denaturation	94 ºC	30 sec.	35 Cycles
Annealing	54 ºC	30 sec.	
Extension	72 ºC	1 min	
Final Extension	72 ºC	7 mins	1 Cycle
Hold	4 ºC	Forever	-

3.13.3 Qualitative Estimation of DNA by Agarose Gel Electrophoresis

Agarose gel electrophoresis is a method used in molecular biology for qualitative and quantitative estimation of DNA and to separate nucleic acids based on molecular weight. The DNA is a negatively charged molecule and moves towards positive through

an agarose matrix. The gel preparation followed by gel electrophoresis was carried out using the following procedure.

3.13.4 Preparation of 1.0% Agarose Gel solution and Gel casting

The 0.4 g of agarose and 40 ml of TBE were mixed to form a solution. The solution was boiled to dissolve the agarose, preferably in a microwave oven for 1 minute. Then 2.5 µl ethidium bromide (10 mg/ml) was added to the solution carefully once the solution was cooled down to 50°C. The solution was stirred gently while cooling to disperse the ethidium bromide. The cooled solution was then poured into the gel casting tray with a comb. When the gel had cooled down and solidified, the comb was carefully removed. The gel was put, together with the casting tray, into a tank with 0.5 X TBE Slots were placed at the end electrode that had the negative current After the gel had been prepared, a micropipette was used to load about 2 µl of stained DNA. The lid of the electrophoresis chamber was closed and the current was set to 300mA and a voltage of 80 V for 40 minutes was applied. DNA ladder (molecular weight markers) was loaded into the first slot. After markers were added, samples were mixed with bromophenol blue (dye is added to visualize DNA movement) and glycerol and later loaded into the rest of the slots A current was applied and DNA was run at 70 V for 15 to 20 min. The DNA moved towards the positive anode due to the negative charges on its phosphate backbone. The small DNA strands moved faster and large DNA strands moved slowly through the gel matrix**.**

3.13.5 Sequence Analysis

The amplified products were eluted using a gel extraction kit (Qiagen, USA) and sequencing was performed with the same set of primers using ABI Sequencer. Both the forward and reverse sequences were obtained. The forward and reverse sequences were aligned using multiple sequence alignment. The aligned sequences were then compared to known sequence of *Channa punctata* using BLASTn i.e., BLAST nucleotide program at NCBI.

CHAPTER 4

Results

4. Results

Several attempts were made to develop a Primary Cell Culture system from *Channa punctata* Primary cell culture was developed from Fin and Muscle. The primary cell culture system developed from the fin and muscle tissues was characterized for species authentication by DNA barcoding.

4.1 Development of Primary Cultures

The explant technique was used to develop primary cultures from the Fin and Muscle tissues of *Channa punctata* Details about the primary cultures, developed from tissues of *Channa punctata* are given in Table 4.

Table 4: Details of Primary Cell Culture developed from tissues of *Channa punctata*

S. No	Tissue samples	Number of Explant Preparations	Time taken for Attachment	Onset of radiation	The number of explants leads to the primary Cultures
1.	Fin	06	24 h	72 h	2
2.	Muscle	04	24 h	120 h	2

4.2 Development of Primary Cultures from the Fin Tissue

A total of six explants were prepared to develop a cell culture system from the fin tissue. Primary cultures were successfully developed from two explants, whereas four explants got contaminated (Fig.6(a)) and.(Fig.6(b)). Morphological observation under the inverted phase contrast microscope revealed that all the explants prepared from Fin tissue explants were found to be attached properly after 24 hours of explant preparation. The radiation of cells started from the edges of the explants after 72 hrs of tissue explant preparation (Fig no. 7 (a). The cells originating from Fin tissue formed a confluent monolayer around the explant after 21 days Fig no. 7 (b). Microscopic observation revealed that the majority of the cells proliferating from the fin tissue were fairly heterogeneous.

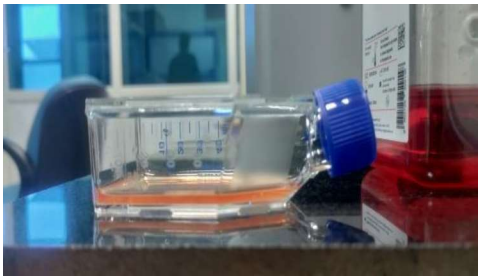


Fig.6(a) Contaminated flask of fin tissue

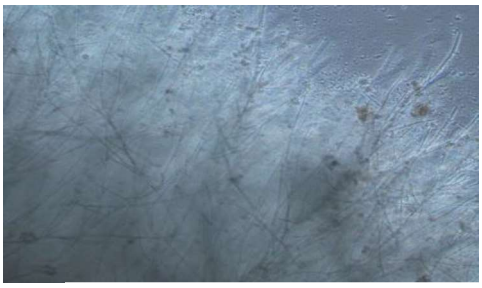


Fig.6(b) Fungal contamination on fin explant (10X) captured using an inverted phase

Figure 6. (a) Show the Contaminated flask of Fin tissue (b) Mycotic Contamination on Fin explant (10X) capture using an inverted phase

Fig.7(a)

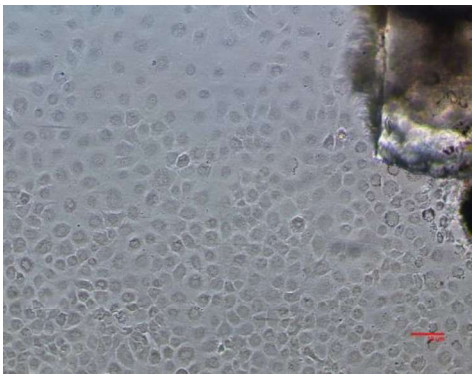


Fig.7 (b)

Fig.7 (a) Inverted phase contrast photomicrographs of the radiation of cells started after 72 hr of explant preparation from the *C. punctata* fin tissue (10X). (b) Inverted phase contrast photomicrographs of the confluent monolayer around the explants after 21 days from the *C. punctata* fin tissue (10X).

4.3 Development of Primary Cultures from Muscle Tissue

A total of four explants were prepared from the muscle tissues. Primary cultures were successfully developed from two explants, whereas two explant got contaminated (Fig.8 (a) and (Fig.8 (b)). Muscle was found to be attached properly after 24 h of explant preparation. The radiation of cells started after the fifth day of explant preparation (Fig.9(a)). A confluent monolayer around the explants was observed after 25 days (Fig. 9(b)). Microscopic observation revealed that the majority of the cells proliferating from the muscle tissue were fairly heterogeneous. Similar results were observed by other

researechers (Chen et al., 2004; (Swaminathan T *et al.,* 2012) and (Kumar MS *et al.,* 2021).

Fig. 8(a) Bacterial Contaminated Flask

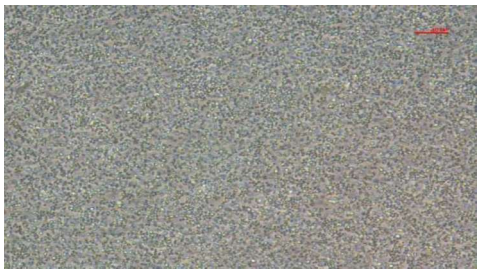


Fig. 8(b) Bacterial Contamination of Muscle tissue (10X)

Figure 8 (a) Show the Contaminated flask of Muscle tissue (b) Bacterial contamination on Muscle explant (10X) captured using an inverted phase

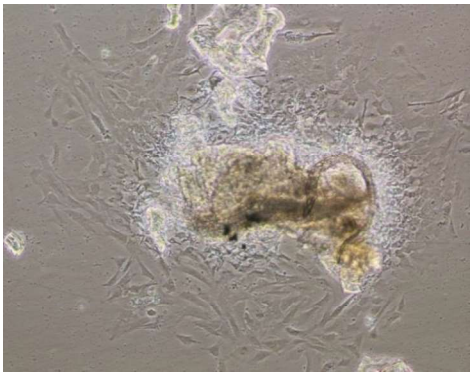


Fig 9 (a)

Fig 9 (b)

Fig.9 (a) Inverted phase contrast photomicrographs of the radiation of cells started after 120 hr of explant preparation from the *C. punctata* Muscle tissue (10X). (b) Inverted phase contrast photomicrographs of the confluent monolayer around the explants were observed after 25 days from the *C. punctata* Muscle tissue (10X)

4.4 Maintenance of the Cultured Muscle and Fin Cells

The primary cell culture system was developed from the muscle and fin of fish. These Primary cell cultures were maintained at 28°C in Leibovitz-15 (L-15) medium supplemented with 20% fetal bovine serum and 500 IU/ml penicillin, 500 µg/ml streptomycin, and 2.5 mg/ml Amphotericin B. The spent medium was partially exchanged with the fresh L-15 medium after 3-5 days. Cultured fin cells line was maintained upto 20 days (Figure 10) and cultured muscle cells line was maintained upto 20 days (Figure 10).

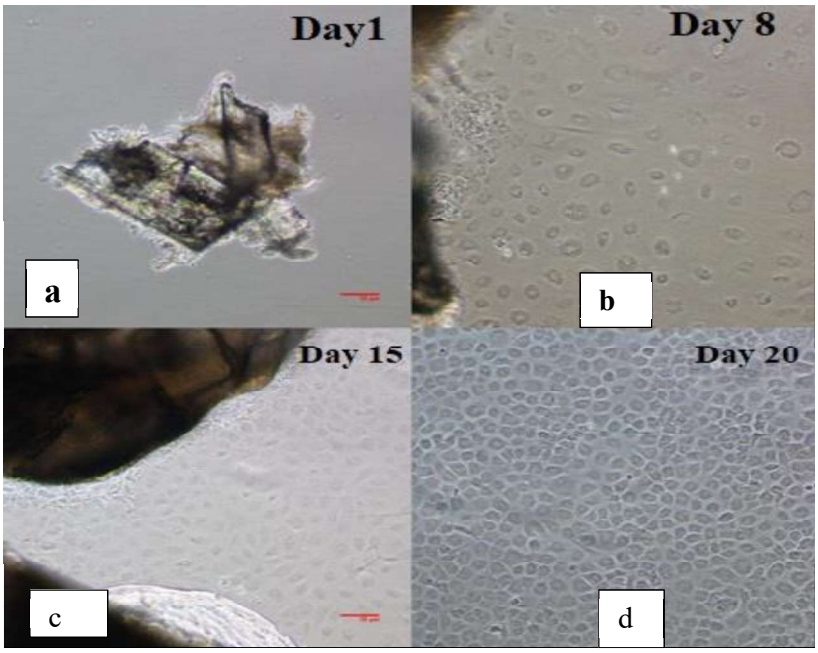


Fig 10. Cultured fin cells line as on Day 1, Day 8, Day 15 and Day 20

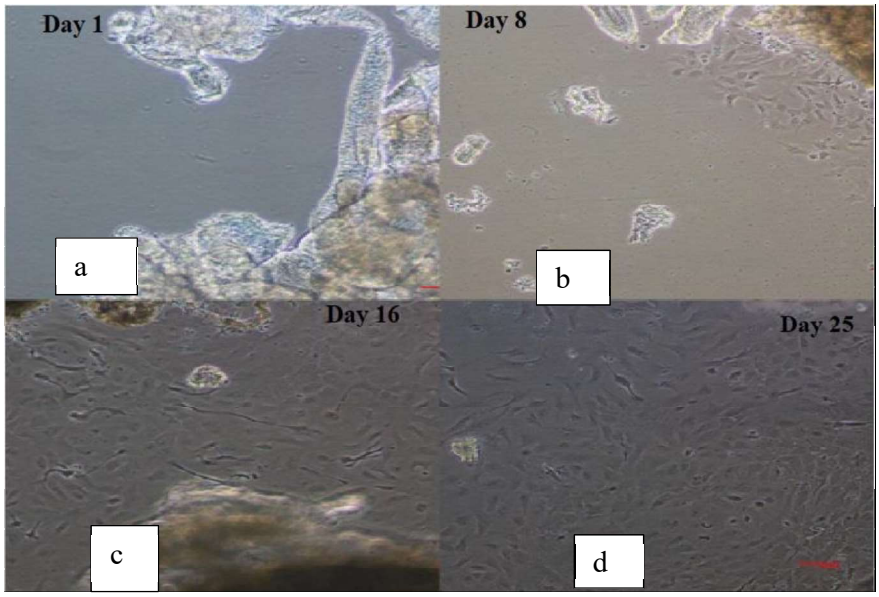


Fig 11. Cultured muscle cells line was as on Day 1, Day 8, Day 16 and Day 25

4.5 Characterization of Cultured Cells

The cell culture system from the fin & muscle tissues of *C. puntata* was characterized for species authentication.

4.5.1 Molecular Characterization for Species Authentication

The origin of the cultured cells was confirmed by the amplification of mitochondrial genes such as the COI sequence. Amplificon size of COI of cultured cells yields 569 bp (Fig. 13) Sequence alignment of COI derived from cultured cells demonstrated 99.8% similarity with the known mitochondrial DNA sequence derived from *C. punctata* voucher specimens confirming species of origin (Table No.5).

Table No. 5: GenBank accession numbers of the COI of *C. punctata*

Mitochondrial gene COI	GenBank matching accession no. LN995842.1

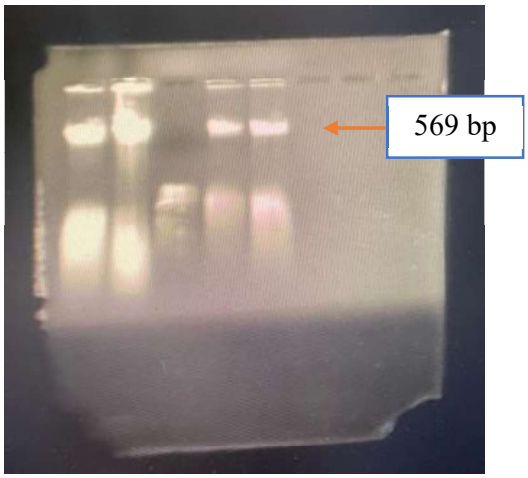


Fig.12 PCR amplification of 569 bp of *C. punctata* genome using oligonucleotide primers from conserved portions of COI region. Lane 5 denotes 10bp DNA ladder, Lane 5 denotes *C. punctata* culture cell COI

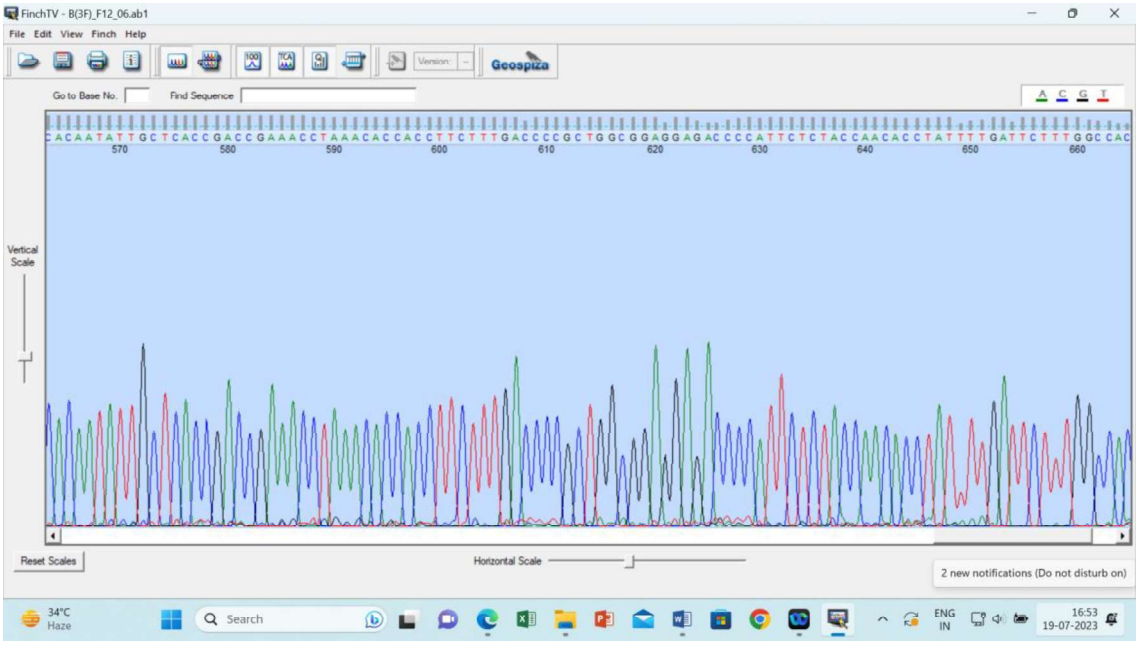


Fig.13 Sequence peaks of 569 bp of *C. punctata* genome using oligonucleotide primers from conserved portions of COI region.

CHAPTER 5

Discussion

5. Discussion

Cell line derived from fish has been established as a promising tool for studying many key issues of aquaculture covering fish growth, disease, reproduction, genetics, and biotechnology. In addition, fish cell lines are very useful in vitro models for toxicological, pathological, and immunological studies (Goswami M *et al*., 2022). The establishment of permissive cell lines from host animals is essential for the isolation, identification and study of pathogenic viruses (Swaminathan TR *et al*., 2012). Fish cell culture offers several advantages over mammalian cell culture in terms of adaptation to a broad range of temperatures, higher tolerance to hypoxia, and easier maintenance of cell culture for longer periods (Goswami M *et al*., 2022). Rastogi A *et al*., (2022) developed three cell lines, namely PFB, PFS and PFH have been established from the brain, spleen, and heart of the blood parrot cichlid which would be useful for surveillance of diseases suspected to be of viral origin in this candidate species and also other ornamental cichlids. However, the number of cell lines developed from fish fin and muscle tissue was very much limited. Fish Fin and Muscle cell lines have the potential to be used in invitro studies, germplasm conservation, and Biotechnological studies. The present study aimed to develop and growth optimization of primary cell culture systems from the fin muscle tissue of *C. punctata*

5.1 Development of *Channa punctata* Culture System

In the present study, primary cultures were initiated from the fin and muscle tissues of *C. punctata* using the explant technique. The attachment of explants to the cell culture flasks is the primary requirement for successful cell culture systems. Two methods, namely explant and enzymatic dissociation were predominantly used to develop primary cell culture. The explant technique has many advantages over the trypsinization method in terms of speed, ease and maintenance of cell interactions, and the avoidance of enzymatic digestion which can damage the cell surface (Avella M *et al*., 1994). In the present study, the primary cell culture developed from *C. punctata* was heterogeneous in nature. Many Primary Cell Cultures have been developed by using explant techniques from different tissues of fishes and shellfishes such as *P. indicus* and *Penaeus vannamei* (Toullec JY *et al.,* 1996), *Schizothorax esocinus* (Khurshid K *et al*., 2022), *Labeo rohita* (Rathore G *et al.,* 2007), *Oryzias latipes* (Wick E *et al*., 2018) etc. Yadav MK *et al .,* (2022 have established a continuous cell line, designated as *L. calbasu* fin (LCF), from caudal fin of *L. calbasu* using the explant method developed.

During the primary muscle culture, *C. punctata* was maintained in L-15 media and supplemented with 20% FBS along with antibiotic-antimycotic Solution. The predominance of fibroblast cells over epithelial cells was reported in cell cultures from fish in previous studies (Bejar J *et al*., 1997; Lai YS *et al*., 2003;Lakra WS *et al*., 2006;Babu VS *et al*., 2011;Lakra WS and Goswami M, 2011;Goswami M *et al*., 2012). Currently, more than 80% of the cell lines established since 1994 have used the L-15 medium (Lakra WS *et al*., 2010). Several researchers have tested different media for the suitability of cell growth and among them, L-15 was found to be most suitable for attachment and proliferation of cells (Kumar GS and Singh IS, 2001).

5.2 Molecular Characterization

In the present study, the cultured cells were confirmed by the amplification of mitochondrial genes such as the COI sequence. Ampliicon size of COI of cultured cells yielded 569 bp. Sequence alignment of COI derived from cultured cells demonstrated 100% similarity with the known mitochondrial DNA sequence derived from *C. punctata* voucher specimens and that confirms the species of origin. Hebert PD *et al*., (2003) have demonstrated the utility of the COI gene as a universal barcode, referred to as DNA barcoding for the genetic identification of animal life. 569 bp region, often described as the “barcode region” by using a universal primer mix targeted at conserved sequences of the cytochrome C oxidase I gene (COI). Cooper JK *et al*., (2007) used the COI region for the identification of 67 cell lines. Similarly, the cell line from *Amphiprion ocellaris* was also confirmed by the COI gene (Yashwanth BS *et al*., 2020).

Barcoding has also been employed to validate the identity of animal cell lines (Lorenz JG *et al*., 2005) (Cooper JK *et al*., 2007) and is a recommended characterisation step for materials in biodiversity repositories (Hanner R and Gregory TR , 2007). DNA barcoding through the Barcode of Life Database (BOLD), with universal primers for mitochondrial CO1 gene is used for fish species identification (Ivanova NV *et al*., 2007). It has been applied for confirmation of the species of origin for several fish cell lines viz., brain (CB) and fin (CF) cell lines from *Rachycentron canadum* (Cheng T *et al.,* 2010) fingerlings cell line (SBT-E1) from *Thunnus maccoyii* (Bain PA *et al*., 2013) caudal fin (PDF) and heart (PDH) cell lines from *Puntius denisonii* (Lakra WS *et al.,* 2011) caudal fin (PSCF) cell line from *Puntius sophore* (Lakra WS and Goswami M, 2011) fin (RTF) cell line from *Puntius denisonii* (Swaminathan TR *et al*., 2012) and intestine (RTgutGC) cell line from *Oncorhynchus mykiss* (Kawano A *et al*., 2011).

CHAPTER 6

Conclusion

6. Conclusion

Cell culture is a reliable and essential biological in vitro tool for numerous disciplines, including physiology, toxicology, immunology, cancer, genetics, virology, biomedical sciences, conservation genetics, and pharmacology.

Fingerlings of *C. punctata* (6.31-12 gg in weight) were maintained in an FRP tank with sterile and well-aerated water in ICAR-NBFGR before explant preparation. An explant technique was followed to develop primary cultures. Primary cultures were developed from fin and muscle tissues of *C. punctata* the growth of muscle tissue was slower than fin tissue .The developed Cell culture system was maintained in an L-15 medium supplemented with 20% FBS and penicillin (400 IU/ml) and streptomycin (400 μg/ml) with an anti-fungal amphotericin B (10 μg/ml) at the temperature of 28°C . The cultured cells were characterized by molecular characterization.

DNA barcoding-based authentication of cell lines is one of the most important criteria for confirming the species' origin of the cell line. The cell culture system was authenticated using the Cytochrome C Oxidase subunit I (COI) gene. The gene sequence of COI derived from the cultured cells revealed 100% similarity with the known sequences of *C. punctata* in NCBI. This primary culture of different tissues would play an important tool in studying in vitro research for conservation genetics, biotechnological studies, virology, and germplasm conservation and also help in establishing cell lines from these primary cultures.

CHAPTER 7

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7. Bibliography

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