ESTABLISHMENT OF CELL CULTURE SYSTEM FROM *PANGASIANODON HYPOPHTHALMUS* (SAUVAGE, 1878)

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of the requirements
for the degree of

M.F.Sc. (Fish Biotechnology)

By

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Dated: 30th June, 2018

CERTIFICATE

Certified that the dissertation entitled **"ESTABLISHMENT OF CELL CULTURE SYSTEM FROM *PANGASIANODON HYPOPHTHALMUS* (SAUVAGE, 1878)"** is a bonafide record of independent research work carried out by **Mr. A. Sathiyanarayanan** during the period of study from August, 2017 to June, 2018 under our supervision and guidance for the degree of **Master of Fisheries Science (Fish Biotechnology)** and that the dissertation has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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DECLARATION

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सारांश

बायोटेक्नोलॉजी, फिजियोलॉजी, वायरोलॉजी, विषाक्त विज्ञान आदि में अनुसंधान करने के लिए सेल लाइन का उपयोग इन विट्रो उपकरण के रूप में किया गया है। सेल लाइनों को ऊतकों की एक विस्तृत श्रृंखला को कवर करने वाली टेलीस्टो मछली से विकसित किया गया है। वर्तमान अध्ययन का उद्देश्य पेंगासियानोडोन हाइपोफथैमस के चयनित ऊतक नमूने से सेल लाइनों को विकसित करना था जो जलीय कृषि के लिए एक महत्वपूर्ण उम्मीदवार प्रजाति है। पी। हाइपोफथलमस के ऊतकों से तैयार स्पष्टीकरण जैसे। गिल, कौडल फिन, और दिल का इस्तेमाल प्राथमिक संस्कृति के विकास के लिए किया जाता था। गिल, कूडाफिन और दिल से विकसित प्राथमिक संस्कृतियों को क्रमशः 19, 17 और 3 मार्गों तक उपसंस्कृतिबद्ध किया गया था। पीएचजी के रूप में नामित गिल से एक सेल लाइन विकसित की गई थी और आण्विक मार्करों द्वारा विशेषता थी। पीएचजी सेल लाइन को लीबोविट्ज़ -15 माध्यम में 15% एफबीएस (भ्रूण बोवाइन सीरम) के साथ पूरक किया गया था। सेल लाइन में मुख्य रूप से फाइब्रोब्लास्ट-जैसी कोशिकाओं का समावेश होता था। पीएचजी कोशिकाएं 28 से सी के इष्टतम तापमान के साथ 24 से 30° सी तक के तापमान पर बढ़ीं। पीएचजी की वृद्धि दर में वृद्धि हुई क्योंकि एफबीएस एकाग्रता 28% सी पर 10% से 20% तक बढ़ी और इष्टतम वृद्धि 15% एफबीएस पर देखी गई । पीएचजी कोशिकाओं से माइटोकॉन्ड्रियल साइटोक्रोम सी ऑक्सीडेस सब्यूनिट । जीन का अनुक्रम विश्लेषण किया गया था और पीएचपीपोफल्मस के मानक अनुक्रम के साथ एकाधिक अनुक्रम संरेखण ने पीएचपीपोथल्मस से विकसित पीएचजी सेल लाइन की पहचान को प्रमाणित किया था। जीजीपी अभिव्यक्ति के लिए पीजीजी कोशिकाओं का परीक्षण एक पीईजीएफपी प्लास्मिड के साथ किया गया था और 9% संक्रमण क्षमता के साथ सफलतापूर्वक अभिव्यक्त लेनदेन रिपोर्टर जीन 48 घंटे बाद-संक्रमण को व्यक्त किया गया था। विकसित सेल लाइन इन विट्रो जेनेटिक और विषाक्त विज्ञान अनुसंधान के लिए उपयोगी होगी।

ABSTRACT

Cell line has been used as an *in vitro* tool to carry out research in biotechnology, physiology, virology, toxicology etc. Cell lines have been developed from teleost fish covering a wide range of tissues. The present study was aimed to develop cell lines from selected tissue samples of *Pangasianodon hypophthalmus* which is an important candidate species for aquaculture. Explants prepared from tissues of *P. hypophthalmus* viz. gill, caudal fin, and heart were used for development of primary culture. The primary cultures developed from gill, caudal fin,and heart were subcultured up to 19, 17 and 3 passages respectively. A cell line from gill designated as PHG was developed and characterized by molecular markers. PHG cell line was maintained in Leibovitz's- 15 medium supplemented with 15% FBS (Fetal Bovine Serum). The cell line consisted predominantly of fibroblast-like cells. The PHG cells grew at temperatures ranging from 24 to 30 ºC with an optimum temperature ranging of 28 ºC. The growth rate of PHG increased as the FBS concentration increased from 10% to 20% at 28 ºC and optimum growth was observed at 15% FBS. Sequences of mitochondrial cytochrome C oxidase subunit I gene from PHG cells were analyzed and multiple sequence alignment with the standard sequence of *P. hypophthalmus* authenticated the identity of PHG cell line developed from *P. hypophthalmus.* PHG cells were tested for the GFP expression with a pEGFP plasmid and successfully expressed transfection reporter gene 48 hr post-transfection with 9% transfection efficiency. The developed cell lines would be useful for *in vitro* genetic and toxicological research.

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1. INTRODUCTION

Cell line is one of the mostly used *in vitro* tools for carrying out *in vitro* research in virology, toxicology, carcinogenesis, and transgenesis. Fish cell lines have several advantages over mammalian cell lines. Hence, piscine cell lines have been increasingly used as important model systems in embryology, neurobiology, endocrinology and environmental biology (Powers, 1989, Hightower & Renfro, 1988, Braga *et al*. 2006). Fish cell lines have been used as a valuable, rapid, and cost effective tool for toxicological assessment of aquatic pollutants and environmental samples (Goswami *et al.* 2014)

Over the last decade, considerable improvements have been made in the culture of animal cells. Development of antibiotics was the first important step which results in reduction of the contamination problems that plagued earlier cell culture attempts. Later, the development of the techniques, such as the use of trypsin to remove cells from culture vessels and development of a chemically defined culture media like Leibovitz‘ L-15 made it far easier to culture cells necessary to develop continuously growing cell lines. During the 1960‘s and 1970‘s, commercialization of this technology had a further impact on cell culture that continues to this day. One of the main risks faced when isolating tissue is contamination from microbes. Internal organs are considered sterile with the exception of the digestive tract and hence as long as organs are removed aseptically the contamination risk is low. It is prudent, however; to disinfect the area of the incision or even the entire fish before dissection (Wolf and Quimby. 1966). The most common reagents used for decontamination include strong disinfectants (chlorine based), 70% alcohol solution and balanced salt solutions (BSS) containing high levels of antibiotics (Wolf and Quimby. 1966). External organs and larvae or whole fry pose a greater risk of contamination. Treating the fish in a similar manner as for internal organs appears to be the standard method for decontamination. Generally, antibiotic solutions are used to decontaminate

tissue in spite of using strong disinfectant as these can damage cells (Wolf and Ahne. 1982; Chang *et al.* 2001; Lai *et al.* 2003; Chi *et al.* 1999a).

Teleost fish cell lines have been developed from a wide range of tissues such as ovary, fin, swim bladder, heart, spleen, liver, eye muscle, vertebrae, brain, and skin (*Lakra et al.* 2011). In addition, the ovarian tissue also has the high regenerative ability which is a commonly used source (Fryer & Lannan, 1994). The majority of established cell lines have been developed from normal tissue, which transformed spontaneously (Fryer and Lannan, 1994). However, transformation can also be induced through chemical mutagens, infection with a transforming virus or through transfection (Freshney, 2000) Media, growth factors and other supplements will simply optimize the conditions for growth and provide more opportunities to develop a continuous cell line but do not ensure the development of an immortalized cell line. The rainbow trout, *Salmo gairdneri* gonadal cell line, RTG-2 developed by Wolf & Quimby (1962) was the first permanent cell line of fish origin. Since then several short-term and continuous cultures from different organs of different fish species have been developed. At the end of 1994, around 159 cell lines have been reported from fishes of which most cell lines are derived from freshwater teleosts (Fryer & Lannan, 1994, Hong *et al.* 2000). Since then around 124 new fish cell lines from different fish species ranging from grouper to eel have been reported. *In vitro,* culture systems offer several advantages over *in vivo* system as experimental tools. Among the cell lines listed, more than 60% were established from Asian species, which contributes more than 80% of total fish production (Pandey, 2013). Lakra *et al.* (2011) reported over 300 from finfish around the world. Recently Bairoch (2018) enlisted 551 fish cell lines globally in Cellosaurus: *Cell Line Knowledge Resource.*

Sathe *et al.* (1995), and Lakra & Bhonde (1996) have laid the foundation of cell culture work in India. Since the first fish cell line MG-3 derived from gills of *Cirrhinus mrigala*, about 30 fish cell lines have been reported from different groups in India (Goswami *et al.,* 2012). Attempts have also been made in India for development of cell culture systems from crustaceans with limited success

(Kumar *et al.* 2001, Uma *et al.* 2002, Goswami *et al.* 2010). Presently more than 50 cell lines are being maintained and cryopreserved in NRCF (National Repository for Fish Cell line), Lucknow. Some important fish cell lines have been developed from *Tor putitora* (Lakra *et al.* 2006a), *Etroplus suratensis* (Swaminathan *et al.* 2010), *Epinephelus coioides* and *Chanos chanos* (Parameswaran *et al.* 2007), *Lates calcarifer* (Lakra *et al.* 2006b, Parameswaran *et al.* 2006), *Labeo rohita* (Lakra *et al.* 2010a)*, Puntius densonii* (Lakra *et al*. 2010c)*, Puntius sophore* (Lakra & Goswami, 2011).

The integral part of the cell culture system is the characterization of the cell cultures established. Characterization of the cell line is the most important parameter for the species authentication i.e the origin of the cell line. Some other parameters for the species authentication of the cell line are chromosome analysis and isoenzyme electrophoresis. Cell lineage or the type of the tissue origin can be identified by using cell surface markers and antibodies such as vimentin, cytokeratin, and desmin based on the type of the intermediate filament proteins they constitute (Freshney, 2006). Other properties of cell lines includes plating efficiency which indicates the proliferation capacity of the cell line, transfection efficiency for the gene expression studies, viability assay after cryopreservation, and other growth parameters include optimum temperature and FBS concentration required for the cell line maintenance.

The present study, was aimed to develop cell line from *Pangasianodon hypophthalmus* (Sauvage, 1878) catfish which belongs to the Class Actinopterygii of Order Siluriformes and Family Pangasiidae. Catfish occupy an important place in aquaculture owing to their consumer preference and commercial value. It is regarded as the third most important freshwater culture species due to its fast growth and versatile feeding habit. *P. hypophthalmus* is food fish of commercially important in many countries especially in Vietnam (Nguyen *et al*., 2009). Aquaculture of this species introduced to many Asian countries like Bangladesh, China, India, Indonesia, Malaysia, and Myanmar. Because of its significance in the global whitefish supply, the farming of striped catfish is

appearing as a commercial freshwater species (FAO, 2011). *P. hypophthalmus* was introduced into India possibly during 1997 via Bangladesh and adapted for culture in the state of West Bengal. *P. hypophthalmus* commonly called as pangas is a relatively new and fast-growing fish species that has great potential for production and export growth in India Because of its remarkable growth rate (almost one kg in 90 days), there has been much enthusiasm among fish breeders and farmers particularly in West Bengal and Andhra Pradesh for its culture and propagation. It was estimated that over 0.2 million tonnes of *P. hypophthalmus* catfishes are produced in the country per annum (Lakra and Singh, 2010). Recent national production of *P. hypophthalmus* in India accounts for 0.7 million tonnes at present (Lakra and Singh, 2012). The average culture production of *P. hypophthalmus* in tonnes/hectare/year is found to be higher than carp production in the same areas (Lakra and Singh, 2010). Recently channel catfish disease has been reported in *P. hypophthalmus* so developing cell line from this fish could serve as an *in vitro* tool for studying pathogenecity of the virus (Siti-Zahrah *et al.* 2014). No cell line has yet either developed or reported from *P. hypophthalmus* in the National Repository of Fish Cell Line. Hence the present study was carried out to develop and characterize cell line from *P. hypophthalmus*. The present study was carried out to achieve the following objectives.

1. To develop cell lines from the selected tissues of *Pangasianodon hypophthalmus.*
2. To characterize the developed cell lines using molecular markers.

2. REVIEW OF LITERATURE

The number of cell lines developed from different tissues of fish has been increased drastically over the years. In India the projects funded by Department of Biotechnology (DBT), Government of India have boosted fish cell line based research in the country. Development of fish cell lines along with historical background, national, international status, methods used in cell culture, media & supplements used in cell culture, characterization of cell line, application of cell line and conservation of cell lines was reviewed in the chapter.

2.1 Animal Cell Culture- Historical Background

Sydney Ringer in 1882 have laid the foundation of the *in vitro* animal tissue culture. He developed a balanced salt solution which is close to that of the animals body fluids which was first instance of *in vitro* cultivation. Animal cell culture was first successfully undertaken by Ross Harrison in 1907. Harrison cultivated frog nerve cells in a lymph fluid and he observed the growth of nerve fibres *in vitro* for several weeks. This initiative by Harrison paved way for the development of animal cell culture. Montrose T Burrows identified the suitability of the plasma over the lymph fluid for the culture of animal cells. In 1912 Carrel was the first person to cultivate mammalian somatic cells. In 1951 George O Gey and his colleagues established the first mammalian cell line called “Hela cells” from the uterine cervical cancer patient (Yao and Asayama, 2017). The first fish cell line was developed from rainbow trout gonad designated as RTG-2 (Wolf and Quimby, 1962) the cultures were established in cord serum medium maintained at 19ºC for two years.

2.2 International Scenario

At present there are about 551 fish cell lines developed from different tissues covering a wide variety of fishes such as ornamental, fresh water and marine fishes (Bairoch, 2018). Some important cell lines developed over the years were reviewed below.

Chen et al. (1981) established a continuous cell line from the ovary of Japanese eel (*Anguilla japonica*) designated as EO2. The cells were cultured in L-15 medium supplemented with 10% FBS at temperature 32±1°C. They have tested the susceptibility of this cell line to the viruses such as Eel Virus Europe (EVE), Eel Virus Europe X (EVEX), Eel Virus America (EVA) and they found that the cell lines are susceptible to all these viruses.

Li *et al*. (1984) established cell lines from sturgeon *(Acipenser oxyrhyncus)* and guppy *(Poecilia reticulata)* through series of subcultures. The cell lines from heart tissues of sturgeon (SH) and guppy fetel tissues (GFT) have been subcultured over 30 passages. Maximum growth of the cells was observed at 18ºc and require 3-5% salt for optimum growth. The susceptibility of the cell lines was tested with viruses IPNV and amphibian virus LT-IV and found low susceptibility. Three isozymes malate dehydrogenases, lactic acid dehydrogenase, and phosphohexose isomerase were studied by starch gel electrophoresis and found to be suitable markers for distinguishing among different cell lines include SH (sturgeon heart), GFT (guppy fetal tissues) and lumpfish cell lines.

A cell line was developed from gill tissue of rainbow trout (*Oncorhyncus mykiss*) grown in basal medium, L-15 medium supplemented with FBS at 5-10 %. The cell lines were passaged up to 55 times. The morphology of the cells was epithelial-like and the cultures were grown on agar plate stained with DNA staining agent for demonstrating mycoplasma contamination and the contaminant was eradicated by MRA and BM cyclin (Bols *et al*. 1994).

Lio-po *et al.* (1999) established cell lines from brain, gonad, heart, spleen, kidney, fins, skin of catfish and snakeheads (*Clarias batrachus & Channa striatus*) by using four methods of tissue processing The first method involve introduction of explant to the culture flask and allowing it to attach before the addition of the L-15 medium and the second method introduced explant to the flask that already containing culture medium. The third method was forcing the minced explants to the Petridish containing L-15 medium with the aid of syringe plunger and in the fourth method, minced explants were added to EDTA and slowly

agitated using magnetic stirrer for one hour. Among these four processing methods, the introduction of explant tissues to the flask containing L-15 medium supplemented with 15% FBS and antibiotics incubated at 25°C resulted in successful primary cultures and they have studied the susceptibility of these cell lines derived from the primary cultures to the virus isolated from the lesions of EUS affected fishes.

Chang et al. (2000) developed cell line from fry of Asian seabass (Lates calcarifer) the cells were maintained in EMEM (Eagles minimum essential medium) supplemented with FCS and incubated at 25ºC and the developed cell lines were tested with viruses like birnaviruses, reoviruses, rhabdoviruses and nodaviruses. All these cell lines were tested for CPE after six days of inoculation and these cell lines have good potential for isolation of various fish viruses.

Qin *et al*. (2005) developed and characterized tropical marine fish cell line from the spleen of *E. coioides* which is susceptible to the iridovirus and nodavirus. The cells were grown in L-15 medium supplemented with 10% FBS. The morphology of the cells was found to be fibroblastic by immunofluorescence microscopy. And electron microscopy revealed the CPE after infecting with the virus inoculum which suggests the potential of the cell line as a diagnostic tool for virus isolation and propagation of viruses.

Dong *et al*. (2008) developed a cell line from Mandarian fish fry (*Siniperca chuatsi*) and it has been subcultured over 60 passages. It consists of epithelial-like cells which grow well in DMEM supplemented with 10% FBS. The susceptibility of the cell line was tested with the virus ISKNV (Infectious spleen and kidney necrosis virus) and showed CPE further confirmed by flow cytometry in addition to that apoptosis was observed by FITC staining. From this study, the mandarian cell line forms an efficient tool for studying ISKNV.

Cheng *et al.* (2010) developed cell lines from the brain and fin tissues of cobia *Rachycentron canadum.* The cells were grown in L-15 medium

supplemented with 10% FBS. The origin of the cells was confirmed by the mitochondrial analysis of the COI (Cytochrome C oxidase subunit I) gene from the tissues of the developed cell line and muscle tissues of cobia. The developed cell lines were tested for the susceptibility to the grouper iridovirus. The cell lines were transfected with a green fluorescent reporter gene and it has the ability to express the foreign gene.

Schiotz *et al.* (2011) developed cell lines from head kidney tissues of Atlantic Salmon (*Salmo salar*) to study the transfection efficiency by nucleofection and antibiotic selection with the background knowledge of transfection using calcium phosphate precipitate or lipid reagents. Among all these transfection methods Amaxa electroporation was found efficient with 72-100% viability and low mortality by three combinations of buffers and different electric pulse program such as 20 or 27. Electric pulse 20 and buffer-T combination gave higher viability after transfection.

Choorapoikayil *et al*. (2013) developed a protocol for establishment of cell culture system from embryo and tumor of Wild Zebra fish (*Danio rerio*). The developed protocol can be applicable to establish any cell lines from mutant and transgenic zebrafish. The developed cell line was found fibroblastic for wild-type and endothelial-like for tumor cells. And the cell lines were further used for transfection studies, immunoblotting.

Saint-Jean *et al*. (2014) established a cell line from pronephros tissues of Atlantic Salmon (*Salmo salar*) these cells grow in L-15 medium supplemented with 10% FCS serum and temperature range from 10-15ºC and these cells reached the stage of continuous cell line after 100 subcultures with an epithelial-like morphology and the cells were cryopreserved and revived with 80% viability. The developed cell line was susceptible to the viruses IPNV and IHNV with cytopathic effects (CPE). Transfection of a cell line with a plasmid expressing reporter genes such as MHC (major histocompatibility complex II) and Interleukin 12b, as a result, established cell line was useful to study gene manipulation and virus isolation.

Fu *et al.* (2014) developed a continuous cell line from the brain of Mandarian fish (*Siniperca chuatsi*) which grows well in L-15 media supplemented with 10% FBS at temperature 28ºC. The origin of the species was confirmed by amplification of the nuclear marker 18s rRNA. The developed cell line was cryopreserved at different passages and revived at 80-90% viability. The cell line was tested for the susceptibility of the ISKNV (Infectious spleen and kidney necrosis virus) and the confirmation of the infection was done by reverse transcriptase PCR and this study forms an efficient tool for viral propagation and gene expression studies.

Wang *et al*. (2014) established a cell line from the snout tissue of the common carp which grows well at a temperature ranging from 22-27º C in L-15 media supplemented with 10% FBS the developed cell line has been subcultured more than 115 times. The developed cell line was tested for the susceptibility of the KHV (Koi Herpes virus) and confirmed by the CPE and the replication of the virus was confirmed by immunofluorescence and western blot assays. From this study, the developed cell line provides a useful tool for KHV isolation and detection.

Wei *et al.* (2014) established the ovarian cell line from the southern catfish (*Silurus meridionalis*) which helps in studying endocrine disruptors and the interactions of somatic and germ cells. And they analyzed the mRNA expression of the developed cell line by RT-PCR against the control using β-actin gene.

Gökçe & Üçüncü, (2017) established a fibroblastic cell line PSF from the caudal fin tissue of Guppy (*Poecilia reticulata*) grown in L-15 media supplemented FBS. The proliferation of the cell line was studied by different concentrations of FBS and temperature and the optimum conditions were found to be 28ºC and 10% FBS concentration. And the developed cell line was cryopreserved and revived with 85-90% successfully.

A fibroblastic cell line named ACBA from the Bulbus arteriosus Arctic charr (*Salvelinus alpinus*) and they passaged the cells over 40 times. The remarkable future of the cell line was the survival of the cells even at 1ºC. The cells were tested for the susceptibility with viruses like VHSV, IPNV, CSV which allows the replication of all the tested viruses. And the developed cell line produced MH- class I proteins which indicated the possibility of the cell line to study immune function (Semple *et al*. 2017)

Minghetti *et al*. (2017) established a first fish intestinal cell line (RTgutGC) from Rainbow trout (*Oncorhynchus mykiss*) which grown in L-15 medium supplemented with 5% FBS serum. The developed cell line was useful for toxicology and physiology.

A new brain cell line EMB was developed from the kelp grouper (*Epinephelus moara*) they subcultured the cells over 60 passages. The cells grew well in L-15 media supplemented with 20% FBS, 2- mercaptoethanol, and bFGF (basic fibroblastic growth factor). The developed cell line was susceptible to the SGIV (Singapore grouper iridovirus) and RGNNV (Redspotted grouper necrosis nervous virus) and replication of the virus was confirmed by q-PCR. And the cell line was transfected with the pEGFP plasmid for the gene expression study. (Liu *et al*. 2018)

2.3 National Scenario

In India, at present there are about 51 fish cell lines from 24 different fish species being maintained in the National Repository of fish Cell Line (NRFC), NBFGR, Lucknow. All these cell lines were deposited by various researchers. Fish cell lines have been developed from a broad range of tissues such as ovary, fin, swim bladder, heart, spleen, liver, eye muscle, brain, and skin. Development of new cell lines from *Etroplus suratensis* (Babu *et al. 2012*), *Clarias batrachus* (Babu *et al.* 2013), *Schizothorax richardsoni* (*Goswami et al.* 2013), *Catla catla* (Chaudhuri *et al*. 2014), *Channa striatus* (Majeed *et al*. 2014), *Puntius chelynoides* (Goswami *et*

al., 2014) and *Wallago attu (*Dubey *et al.,* 2014) have promoted research programme in fish biotechnology and conservation of fish germplasm in India.

Kumar & Singh (2000) reported the first cell culture from the ovarian tissue of African catfish (*Clarias gariepinus*). They have studied the attachment of explant and cell proliferation by supplementing the Leibovitz (L-15) culture media with fish muscle extract (FME) and further enriching with prawn muscle extract (PME), lectin-2, and LPS (lipopolysaccharides) and addition of ovary extract (OE), prawn haemolymph (PHL) and glucose in place of any of the above said growth factors resulted in strong attachment of explant. For the subculture of the confluent monolayer, cell dissociation solution TPVG (containing 0.0125% trypsin) resulted in complete extraction of cells from the culture flask.

Hameed *et al.* (2006), developed a new epithelial-like cell line, SISK, from the kidney of Asian sea bass, *Lates calcarifer,* in Leibovitz L-15 medium supplemented with 15% FBS. The cells were maintained to 100 passages at an optimum growth temperature of 28^{o}C. Karyotype of the cells was 48. Cells were successfully revived after cryopreservation with a survival rate of 80-90%. Virological studies showed that the cell line was susceptible to MABV, NC1, and nodavirus.

Parameswaran *et al.* (2006a), developed two new epithelial-like cell line, SISE, and SBES, from blastula of Asian sea bass, *Lates calcarifer,* in Leibovitz L-15 medium supplemented with 15% FBS. Both the cell lines were maintained to 70 passage at an optimum growth temperature of 28^{o}C. Karyotype of the cells was 48. SISE Cell line was characterized by CFLSM and was successfully transfected with pEGFP-N1.SBES cell line was shown to differentiate into neuron-like cells, muscle cells and beating cardiomyocyte on treatment with all-trans retinoic acid. SISE Cells were successfully revived after cryopreservation with a survival rate of 70-80%. Virological studies showed that both the cell lines were susceptible to IPNV, VR-299, and nodavirus.

Parameswaran *et al.* (2006b) developed a new epithelial and fibroblast-like cell line, SISS, from the spleen of Asian sea bass, *Lates calcarifer,* in Leibovitz L-15 medium supplemented with 15% FBS. The cells were maintained to 70 passage at an optimum growth temperature of 28^{o}C. The cell line was characterized by CFLSM, immunocytochemistry, and was successfully transfected with the pEGFP-N1 vector. Cells were successfully revived after cryopreservation with a survival rate of 70-80%.Virological studies showed that the cell line was susceptible to IPNV, VR-299, and nodavirus.

Parameswaran *et al.* (2007) developed a new epithelial cell line, SIGE, from Eye of Orange-spotted grouper, *Epinephelus coioides,* in Leibovitz L-15 medium with 15% FBS. The cells were maintained to 100 passage at an optimum growth temperature of 28 ^{o}C. The cell line was authenticated with a karyotype of 48 chromosomes. Cells were successfully revived with a survival rate of 90% after cryopreservation. Virological studies revealed that the cell line was susceptible to Nodavirus, MABV, NC-1 and Y6 virus.

Ahmed *et al.* (2009) studied the morphology of the cells derived from the tissues of eye and brain of *Labeo rohita* (rohu) and *Catla catla* (catla). At initial subcultures, the cell lines consist of epithelial and fibroblastic-like cells. After 10 subcultures, only epithelial-like cells were observed in rohu eye cells and only fibroblastic-like cells in catla brain cells. They characterized cell lines by using the proliferation markers such as pan Cytokeratin, Ki-67 for eye and fibronectin, desmin for the brain. These cell lines showed maximum growth at an optimum temperature of 28°C. The growth rate of the cells increased as the FBS concentration increased from 2% to 20% at 28 °C.

Sobhana *et al*. (2009) developed a gill cell line from the explants of the grouper (*Epinephelus malabaricus*). The developed cell line was maintained in L-15 medium supplemented with 20% FBS and the primary culture was observed with heterogeneous population of cells. The epitheliod cells dominated over the fibroblast after subsequent passages.

Lakra *et al.* (2010) developed two cell lines PDF and PDH from the caudal fin and heart tissue of *P. denisonii*, respectively. These cell lines were maintained at 26 °C in Leibovitz-15 medium supplemented with 10% fetal bovine serum. Diploid count of 50 chromosomes at passage 50 was observed in both the cell lines, the cell doubling time was found to be 28 and 30 h for PDF and PDH cell lines respectively. The viability of the PDF and PDH cell lines was 70% and 76%, respectively, after 4 months of storage in liquid nitrogen (−196 °C). The origin of the cell lines was confirmed by the amplification of 653 bp fragments of cytochrome c oxidase subunit I of mitochondrial DNA genes.

Swaminathan *et al.* (2010) developed an epithelial-like cell line, PSF, from caudal fin of Pearl spot, *Etroplus suratensis,* in Leibovitz L-15 supplemented with 15% FBS. The cell lines were passaged 35 times at an optimum growth temperature of 28^{o}C. 2n=48 chromosomes were revealed through karyotyping. The authenticity of the cell line was also confirmed through molecular biology tools of 16S rRNA and COI mitochondrial gene sequence analysis. Cells were successfully cryopreserved and revived with the survival rate of 70%. MTCC 3904, an extracellular product of *Vibrio cholerae* was found to be toxic to the cell line hence can be used for studying the mechanism of bacterial ECP toxicity.

Lakra and Goswami (2011) developed a continuous cell line from fin of Pool barb *Puntius sophore* in L15 media supplemented with 10% FBS at 28 ^{O}C. The authenticity of the cell line was also confirmed through molecular biology tools of 16S rRNA and COI mitochondrial gene sequence analysis. Cells were successfully cryopreserved and revived with the survival rate of 75%. The PSCF cells were successfully transfected with green fluorescent protein (GFP).

Babu *et al*. (2011) established and characterized cell line from the fin tissues of *Clarias batrachus* in a culture medium L-15 supplemented with 15% FBS. The growth rate of fin cells increased as the FBS concentration increased from 2% to 20% at 28 °C with optimum growth at a concentration of 15% or 20%. The cells were found to be susceptible to fish nodavirus and IPNV and infections

were confirmed by cytopathic effect and reverse transcriptase–polymerase chain reaction.

Goswami *et al.* (2012) developed PCE cell line from the eye of *Puntius chelynoides* in Leibovitz L-15 media supplemented with 20% fetal bovine serum (FBS) at 24 °C. The authenticity of the cell line was also confirmed through amplification of mitochondrial COI & 16S rRNA and sequence analysis. The cell line was transfected with pEGFP-C1 plasmid, the applicability of cell line for the genotoxic study was confirmed by comet assay and further cell line was successfully cryopreserved and revived at an interval of six months. Cell culture systems were also successfully developed from other tissues including fin, heart and swim bladder by using explant method.

Majeed *et al.* (2012) established the permanent cell line from gill tissue of (*L. rohita*) for the study of gene expression and toxicology. They studied the toxicity of the cell line when exposed to the Malathion at the concentration level of 30% mg/l (w/v) to fishes which caused complete mortality to the cell line.

Goswami *et al*. (2013) developed a new cell line from the caudal fin tissue of snow trout (*Schizothorax richardsonii*) named as SRCF. The cells were grown in Leibovitz L-15 media supplemented with 10 % fetal bovine serum (FBS) at 24 °C. The authenticity of the cell line was also confirmed through amplification of COI & 16S rRNA and sequence analysis. The cell line was transfected with a pEGFP-C1 plasmid which suggested that the developed cell line was useful in the gene expression study.

2.4 Cell Culture Methods

The procedure for cell culture involves preparation and sterilization of glassware, equipment, reagents, and media. As the cell culture is being carried out under highly aseptic conditions, all media and solutions are required to be sterilized thoroughly. Two methods namely explant culture and enzymatic dissociation methods are available 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 *et al*., 1994).

2.5 Commonly used Tissues for Cell Culture

The first consideration in the development of cell culture is the choice of the organ to be used. Different organs of fish can be used for developing primary culture and subsequently to permanent cell lines. Most commonly used organs are kidney, gill, liver, heart, spleen, fin, eye, muscle, brain, and other tissues can also be used. Minghetti *et al*. (2017) developed first fish intestinal cell line from rainbow trout designated as RTgutGC. Fish bone derived cell lines are used for the study of bone related disease which emerged as a major *in vitro* fish model system and another advantage of the bone derived cell line is the ability to differentiate into mesenchymal stem cells (Rafael *et al*. 2010; Marques *et al*. 2007)

2.6 Contaminants & Test for Detection of Contamination

Contaminants in cell culture are not easily eliminated. Microbial contamination is the major issue in cell culture technique. Contamination can arise from the operator or from the laboratory equipment. Bacteria, Fungus, Moulds, yeast, and Mycoplasma are the common contaminants in cell culture. Utmost care should be taken to get rid of contamination in cell culture. For decontamination routine sterilization methods such as swabbing, flaming, dry heat, wet heat, radiation, filter sterilization should be practiced for the aseptic condition of the working environment. Myoplasmas are the most common contaminants in the cell culture (chen *et al*, 1977) fluorescent staining of DNA by Hoechst 33258 is one of the easiest and most raliable technique and reveals mucoplasma infections as a fine particulate or filamentous staining over the cytoplasm. And other techniques for the screening of mycoplasma are PCR, ELISA assay, immunostaining, autoradiogarphy (Freshney, 2010). Incidence of mycoplasma in primary cultures and continuous cell lines were reported earlier (Ludovici *et al*. 1973).

2.7 Cell Culture Media and Reagents

. In general, most fish cultures use media developed for mammalian cell culture. Eagle‘s Minimal Essential Medium (EMEM) supplemented with fetal bovine serum (FBS) is commonly used culture medium for the cells of mammals, birds, reptiles, amphibians and of course fish (Wolf and Quimby, 1966). Other media routinely used in fish culture are Glasgow MEM, Hank‘s MEM (HMEM) and Leibovitz L-15 medium (L-15). Leibovitz- 15, an amino acid-rich nutrient medium that does not require CO_2 buffering has been successfully used with fish cell lines (Leibovitz, 1963). Fetal bovine serum (FBS) is used as supplements with the tissue culture media with a varying concentration range of 5% to 20%. Fish serum was used (1%) in combination with FBS in developing fish cell lines (Chen *et al.,* 2004; Lakra *et al.,* 2006a). Some other additives such as fish muscle extract, sucrose, prawn shell extract can also be used for better results. Various growth factors such as mammalian epidermal growth factor (mEGF) (Watanabe *et al.,* 1987), basic fibroblast growth factor (bFGF) (Chen *et al.,* 2004) had been used to stimulate the growth of the fish cell line.

2.8 Characterization of the Cell Line

The ease of handling and simpler growth requirements make cross-contamination of cell line a more likely possibility and hence proper characterization and identification of the cell lines are required. Other properties of cell lines which are characterized includes plating efficiency, transfection efficiency, viability assay after cryopreservation, cell cycle analysis, comet assay and other growth characteristics required for maintenance of cell line

2.8.1 Species Authentication

Characterization of a cell line is necessary to authenticate the origin of the cell line, absence or presence of cross-contamination and its applicability for various fields of research. Cell lines are characterized by using molecular markers such as 16S rRNA, 18S rRNA and Cytochrome Oxidase subunit I (COI) region of mitochondrial genes for authentication of fish cell lines (Ahmed *et al.,* 2009a; Lakra

et al., 2011). In addition, isoenzyme analysis (Steube *et al.*, 1995), chromosome markers (Stulberg *et al.*, 1976), DNA fingerprinting (Stacey *et al.*, 1992; Matsuo *et al.*, 1999) and mini and micro-satellite analysis (Matsuo *et al.*, 1999) have also been used in conjunction with PCR methods to authenticate origin of cell line.

2.8.2 Chromosome Analysis

Cytogenetic analysis is used to establish the common chromosome complement or karyotype of a species or cell lines. The relative stability of chromosome morphology with regard to environmental factors makes this kind of study useful in research on phylogeny of species groups, their taxonomic status, and possible patterns of speciation (White 1973). Cell culture offer several advantages for chromosome studies such as large number of metaphases can be obtained, chromosome morphology is generally better than obtained from direct tissue preparation,

2.8.3 Growth Studies

Growth studies of cell lines are also carried out to determine the optimum temperature and FBS concentrations for growth of cell lines, type of media to be used and requirement of other growth factors. Optimum temperature for growth of most cell line was found to be 28 ^{o}C. Basically, the temperature ranged from 24 to 32 ^{o}C supported the growth of cell lines with an optimum temperature of 28 ^{o}C has been described by many researchers (Tong *et al.* 1997; Lakra *et al.* 2006a). A temperature of 35-37 ^{o}C has been reported to be lethal to many fish cells (Tong *et al.* 1997). Alvarez *et al,* (1991) reported the optimum in *vitro* growth temperature to be a few degrees above the preferred environmental level for live fish.

2.8.4 Transfection Efficiency

Transfection efficiency of cell line is another important parameter that must be determined as exogenous DNA delivery to cultured cell is very useful for both basic research and applied research related to gene expression studies. When the

cell lines were transfected with pEGFP vector DNA, significant fluorescent signals were observed by many researchers indicating their potential utility for transgenic and genetic manipulation studies (Qin *et al.* 2006; Parameswaran *et al.* 2007; Zhou *et al.* 2007; Ahmed *et al.* 2008; Ku *et al.* 2009; Lakra and Goswami 2010).

2.8.5 Immunocytochemistry

Epithiloid or fibroblastic morphology of cells can be confirmed by immunohistochemistry (IHC) using cell-specific marker. Basically monoclonal antibodies directed against Vimentin and Cytokeratin are used for conforming fibroblastic and epthiloid morphology respectively. (Lakra and Goswami 2010)

2.8.6 Cryopreservation

Cell lines are cryopreserved in order to prevent loss of cell line by contamination, to minimize the genetic change in continuous cell lines and to avoid aging and transformation in cell lines. Cryopreserved cell lines are instrumental in germplasm conservation of important and endangered fish species. In order to prevent the loss of cell lines due to contamination and to reduce the possibility of cross contamination cell lines are cryopreserved and revived after regular interval of time. Additionally cryopreservation of cell line also prevent mutations that may alter the original functional characteristics of cell line due to over passaging .These cryopreserved cell line will be instrumental in conserving biodiversity of this important species. Hence cell lines are cryopreserved in liquid nitrogen at -196 ^{o}C and viability is accessed at regular interval by many researchers. Viability of cells after cryopreservation vary from cell line to cell line, it was found to be around 73% for GF-1 (*E. coioides*) (Chi *et al.*, 1999) and 80–85% for SF (*L. calcarifer*) (Chang *et al.*, 2001), 75% PSCF (*Puntius sophore*) (Lakra *&* Goswami, 2011).

2.9 Application of Cell Lines

Fish cell cultures can be used to address a diversity of questions related to basic fish research including toxicology, virology, immunology,

reproductive biology, carcinogenesis, regulation and expression of genes, DNA replication and repair.

2.9.1 Virology

The most widely employed application of fish cell cultures is in the field of isolation of fish viruses that are agents of epizootics of commercially important aquaculture fish species. Many fish cell lines have been established from fish tissues for detection and isolation of fish viruses. The cell lines from different tissues of different species are valuable for studying species-specific responses to viral infection at the cellular level. With the help of *in vitro* culture techniques now it is possible to investigate unique viruses that do not replicate in standard fish cell lines but require highly differentiated cells. Purified viruses can be used as vaccines are likely to be the first health product to be obtained from piscine cell cultures, while the Fish viral vaccines are still in the developmental phase (Sindermann, 1990).

2.9.2 Immunology

Cell culture systems developed from fish also plays important role in understanding fundamental aspects of fish immunology (Faisal and Ahne, 1990). There are possible considerations for developing vaccines for the important fish pathogens and in comparative immunology and the immune system. In vitro systems have been used to study the effects of various substances such as antibiotics on the modulation of the cells of the immune system.

2.9.3 Toxicology

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

2.9.4 Biomedical Research

Fish cell culture can also be employed for the production of an important protein or biomarker involved in vital functioning as they would be expected to perform the appropriate post-translation modifications correctly. The two important sources from which they can be purified are tissues and cell culture systems. In the field of reproductive biology of fishes, cell culture systems are being used to understand the hormonal regulation of fish reproductive cycles.

Cell cultures offer several advantages as modern research tools. For research in general, cultures allow cellular phenomena to be studied in a controlled and defined environment, independent of the complexities and variability of systemic or larger physiological controls. Cell lines are the only regularly available source of biological material for an experiment on particular species (Bols *et al.,* 2005). Only a little work has been done on the development of fish cell lines from Indian subcontinents. Published information from India is also available on the standardization of explant method to develop cell lines from the various tissues of various Indian fishes. There is need to initiate cell lines of the cultivable species, particularly for viral disease diagnosis, toxicological studies, screening and certification of fish for import and export. Development of fish cell lines has several applications in aquaculture and in aquatic toxicology. Cultured fish cells have more advantages than live fish as the experimental material; the materials are cheap and easy to obtain and the experimental condition could be controlled accurately. Toxicology is concerned with mechanisms of toxicity at all organizational levels within an organism.

2.9.5 Vaccine Development

Further, cell culture systems can be used to study and identify new biomarkers and also in the drug development process. With the availability of the whole genome sequences of most economically important fishes now it is also possible to reconstruct the established cell line by genetic engineering. Such genetically altered cell lines can be used as a biomarker or a cell-based reporter

systems and a platform for the expression of endogenous immune or pathogen gene (Collet *et al*. 2017).

2.9.6 Fish Stem Cell Culture

Long-term undifferentiated cell culture systems derived from inner cell mass of developing embryos are called as Embryonic stem (ES) cell culture systems (Evans and Kaufman, 1981). These cells are pluripotent in nature and can be induced to differentiate into cells of different lineage. Previously ES cell lines were limited to mice (Evans and Kaufman, 1981, Bradley *et al.* 1984) and rat (Iannaccone *et al.*1994, Liao *et al.* 2009) only.

The procedure involved in the development of ES cell culture have been basically based on the methods established for development of ES cell lines from mice (Evans and Kaufman,1981; Martin, 1981). In order to prevent further differentiation of ES cells they have been maintained on feeder layers and in conditioned media (Martin, 1981; Handyside *et al.,* 1989) or in a medium supplemented with leukemia inhibitory factor (LIF) (Pease *et al.,* 1990; Hasty *et al.,* 1991) or with LIF-related cytokines (Conover *et al.,* 1993; Nichols *et al.,* 1998; Piquet-Pellorce *et al.,* 1994; Yoshida *et al.,* 1994). Extensive studies have been done in small model fishes, such as zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*), to develop embryonic stem (ES) cell lines and gene targeting technique in fish, as they offer the possibility of combining embryological, genetic and molecular analysis of vertebrate development.

Earlier it was difficult to obtain eggs or fry of some fish species because they are pelagic spawners (Wolf and Quimby 1966). However, due to recent advances in aquaculture embryonic form of many species are readily available, as a result of which, number of embryonic stem-like cell lines were established by various workers from fish species.

ES-like cell lines have been established in medaka (Wakamatsu *et al.,* 1994; Hong *et al.,* 1996, 2000) and zebrafish (Sun *et al.,* 1995). Hong *et al.,* 1998b reported about a medaka ES-like cell line, MES1, which retained a diploid

karyotype and ability to form viable chimeras. Attempts were made to derive ES cell lines by using feeder layer techniques from the zebrafish (Collodi *et al.,* 1992; Sun *et al.,* 1995) and medaka fish (Wakamatsu *et al.,* 1994) have achieved only partial success. A pluripotent cell line, LJESI, had been established from blastula-stage embryos of *Lateolabrax japonicus,* which differentiated into cells of different lineage after treatment with retinoic acid (Chen *et al.,* 2003a). A long-term embryonic cell culture from *Sparus aurata* have been derived by Bejar *et al.* (2002) which were characterized for totipotency and transfected with a GFP plasmid. A team led by Hong Yunhan of the National University of Singapore successfully produced the world's first haploid embryonic stem cells and semi-cloned fish. In India, Parameswaran *et al.* (2006b) developed a continuous embryonic SISE cell line and a pluripotent embryonic stem cell line SBES from blastula-stage embryos of sea bass (*Lates calcarifer*) (Parameswaran *et al.* 2006c), Lakra *et al.* (2010d) reported about embryonic cell culture system derived from catfish (Heteropneustes fossilis) and Dash *et al.* (2010) developed ES-like cell CCES from mid blastula stage embryos of a freshwater fish catla (*Catla catla*). Recently a spermatogonial stem cell culture system (SSCs) from the testis of commercially important carp, *Labeo rohita* was developed and cells were characterized by various markers like Vasa, Pou5f1/pou5f1, Sea-1, Tra-1-81, plzf, Gfr1/gfr1 and c-Kit/c-kit by immunocytochemical and/or quantitative real-time polymerase chain reaction (RT-PCR) analyses. (Panda, 2011).

Holen *et al*. (2003) developed embryonic stem-like cell from the midblastula stage embryo of marine flatfish (*Scophthalmus maximus*) in DMEM medium supplemented with 20% FCS, TEE (turbot embryo extract), bFGF (basic fibroblastic growth factors) and LIF (Leukaemia inhibiting factor). The developed cell line was characterized by immunocytochemistry for the cell morphology.

Sha *et al*. (2010) developed an embryonic cell line CSEC from the half-smooth tongue sole (*Cynoglossus semilaevis*) the cells grew well in DMEM supplemented with 15% FBS, bFGF (Basic fibroblastic growth factors), LIF (Leukaemia inhibitory factor) and mercaptoethanol (ME). The cells were passaged

over 50 times and the developed cell lines were transfected with GFP reporter gene and the cell line was found susceptible to the virus LCDV (Lymphosystis Disease virus).

Dash *et al*. (2010) developed embryonic stem-like cell (CCES) culture from the mid-blastula stage embryo of Catla (*Catla catla*) in L-15 media supplemented with 10% FBS, FEE (fish embryo extract), bFGF (basic fibroblastic growth factors). The developed cell line was characterized by immunocytochemistry for the cell morphology and reverse transcriptase PCR for the marker genes such as oct4 and Vasa.

Kaufman (2016) established two methods of cell culture systems from zebrafish blastomeres for the directed differentiation toward myogenic lineage and the neural crest cell culture with the applicability of chemical screening. Chemical screening of zebrafish blastomeres has been used to identify chemicals that promote vascular endothelial cell differentiation.

Jin *et al*. (2017) developed a continuous cell line ZBE3 from the blastomeres of Zebrafish (*Danio rerio*). The cells were grown at a temperature ranging from 20- 32º C with an optimum temperature of 28º C in ESM 2 or ESM 4 medium supplemented with 15% FBS. The cell line was transfected with pEGFP N-3 plasmid and the cell line was found susceptible to the viruses Red-spotted Grouper necrosis virus, Singapore grouper iridovirus, and the grass carp reovirus and the viral replication was confirmed by RT-PCR

2.10 Cell Line Repositories.

Bairoch *et al*, (2018) has enlisted 551 cell lines in the International cell line authentication committee (ICLAC) and he also reported the misidentification of cell lines with cross contamination. The sources for the cell line at the global level was reviewed which includes American Type Culture Collection

(ATCC, Riken cell Bank (RIKEN), European collection of Cell Cultures (ECACC), Coriell Cell Repositories (CCR) funded by National Institutes of Health (NIH).

In India, a National Repository of Fish Cell Lines (NRFC) has been established at ICAR-NBFGR, Lucknow with the financial assistance from DBT, New Delhi during which is mandated to receive, authenticate, characterize, store and maintain fish cell lines developed in the country. NRFC has a facility for development, characterization, and maintenance of cell lines. The objective is to receive cell lines developed at various research institutes of India. It acts as a National Referral Centre of Indian and exotic fish cell lines in the country. At present, 50 fish cell lines are being maintained in the repository (Goswami *et al*, 2014)

National Centre for Cell Science (NCCS) functions as a national repository for cell lines or hybridomas. The repository at National Centre for Cell Science is the only repository that houses human and animal cells in India. The NCCS repository serves to receive, identify, maintain, store, cultivate and supply animal and human cell lines and hybridomas. The repository has procured cultures from various sources within the country and abroad from 35 animal species. A major bulk of the cell lines stocked in the repository has been procured from the American Type Culture Collection (ATCC) and the European Collection of Animal Cell Cultures (ECACC). At present, the total number of culture strains is 1127, of which about 300 are available for distribution to users on registration. National Centre for Cell Science (NCCS) that provides biological products, technical services and educational programs to private industry, government and academic organizations. Their objective is to acquire, authenticate, preserve, develop and distribute biological materials, information, technology, intellectual property and standards for the advancement and application of scientific knowledge.

3.MATERIAL & METHODS

3.1 Location

The present work was carried out in the Cell Culture Facility, Central laboratory, and Fish Genetics and Biotechnology laboratory, ICAR- CIFE, Mumbai.

3.2. Experimental Animals

Healthy fingerlings of *P. hypophthalmus* were maintained in the Central Wet laboratory, ICAR- CIFE. Fingerlings (15 to 20g) were transported live to the FGB laboratory and maintained in sterile, aerated water before explant preparation.

3.3 Explant Preparation

3.3.1. Preparation of Donor Fish

As contamination is the major problem in cell culture experiments, adequate care was taken to minimize the possible routes of contamination. The donor fish i.e *P. hypophthalmus* was starved for a day or two to reduce the possibility of contamination from feces and regurgitated feed. During this period, fish was allowed to swim in well aerated, autoclaved water for reducing the microbial load adhered on to the skin and gills. Donor fish (*P. hypophthalmus*) was then sacrificed by euthanization using ice for 5-10 min.

3.3.2. Decontamination

External decontamination is necessary prior to dissection. The decontaminating solutions for this purpose included chlorine solution (500 ppm available chlorine), 70% ethanol, iodophore solution (0.5 w/v iodine). External organs like gills, skin or fin were washed with KmNO4 solution before dissection. The commonly used antibiotics in the present experiment were penicillin (400 IU/ml) and streptomycin (400 µg / ml) with an anti-fungal amphotericin B (10 µg/ml). The tissue of interest was aseptically picked up and washed three to five times with the antibiotic solution.

3.3.3. Dissection

Eye, fin, heart, gill and swim bladder tissues were taken out aseptically and washed with PBS containing 500 IU/ml penicillin, 500 µg/ml streptomycin and 2.5 mg/ml Fungizone. The tissues were minced into small pieces; explants of 1mm^3 sizes were prepared and washed thrice with PBS containing antibiotics. These explants were then seeded into 25 cm^2 cell culture flasks. The adherence of explants was accomplished by addition of 0.2 ml of FBS, then the flasks were incubated at 28^oC and allowed to attach to the surface of the flask overnight. After 18-24 hrs L-15 (Leibovitz*)* medium supplemented with 10% FBS was added gently. The medium was changed after an interval of 3-5 days.

3.4 Morphological Observation

The flasks were observed daily for attachment of explants, spreading and proliferation of cells, contamination using an inverted microscope (Nikon, Japan).

3.4.1. Subculture and Maintenance

. The dispersed cells adhered to the culture substrate proliferate whereas dead cells cannot adhere to the flask and hence floats. Dead cells were removed during the subsequent medium exchange. The optimum pH and incubation temperature maintained were 7.4 and 28^0C respectively for the culture of fish cells. Upon reaching 90%-95% confluency, the cells were trypsinized using TPVG solution (0.1% trypsin, 0.2% ethylenediaminetetraacetic acid, EDTA, and 2% glucose in 1× PBS).

At the time of the first subculture, when the confluent monolayer had formed in the primary culture, the old medium was removed and cells were dislodged by treatment with TPVG solution twice for 30 seconds each. The detached cells were resuspended in 5mL of fresh growth medium (L-15 plus 15% FBS) and seeded in 25 cm^2 plastic culture flasks. From second passage onwards, a split ratio of 1:2 was maintained for subsequent passages. In the initial subcultures, 50% of the culture medium was replaced with the fresh medium.

3.5. Characterization of Cell Line

3.5.1. Quantitation of Cells

Cells from T flasks were harvested using Trypsin-EDTA, and viable cells were counted using hemocytometer.

3.5.2. Giemsa Staining for Cell Morphology Confirmation

Monolayers with 80% confluency were rinsed with PBS, fixed in methanol for 10 min, thereafter stained with undiluted Giemsa for 2 min following the protocol of Freshney (2010). Photomicrographs were taken using a camera attached to the inverted microscope.

3.5.3. Measurement of Cell Doubling Time

The interval required for a cell population to double in the middle of the logarithmic phase of growth. Cells/Population Doubling time (DT). DT= T ln (Xe/Xb) where T= is the incubation time in any unit, Xe=cell number at the end of the incubation time, Xb= cell number at the beginning of the incubation time.

3.5.4. Plating Efficiency

Plating efficiency is a measure of the number of colonies originating from single cells. Plating efficiency was determined at seeding concentrations of 200, 500 and 1000 cells per 25cm^2 flasks in duplicates. The cells were incubated at 28 ^{0}C in L-15 medium with 20% FBS. After two weeks, the medium was discarded, and the cells were fixed with 5 ml of crystal violet (1%)-formalin (25%) stain fixative for 15 min, then rinsed with tap water and air-dried. The colonies were then counted (X) under the microscope, and plating efficiency (Y) was calculated using the formula (Freshney, 1994) $Y=100X/Z^{-1}$ where the Z= number of cells seeded.

Plating efficiency = Number of colonies formed/ Number of cells plated x 100

3.5.5. Growth Studies

To check the optimum conditions required for growth and maintenance of cells, cells were grown at different temperatures and FBS concentrations in L-15 media. To determine the optimum temperature, the flask was incubated at 20, 24, 28 and 32ºC over 7 days at seeding concentration of $1x10^5$ cells in 25 cm^2 tissue culture flask. On alternate days, two flasks from different temperatures at which they were incubated, were trypsinized and cell counting was performed using a hemocytometer. Analogous procedures were performed for the effects of various concentrations of FBS (10, 15, and 20 %) on cell growth at 28ºC over 7 days.

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3.5.6. Molecular Characterization

3.5.6.1 DNA Extraction

Genomic DNA from the PHG cell line and the gill tissue of *P. hypophthalmus* was isolated following Sambrook *et al*. (2001). First old media from the culture flasks were removed and then the cells were washed with 2ml PBS and the cells were detached by trypsinization. Then the detached cells were suspended with 2ml PBS. Then it was transferred to a centrifuge tube and centrifuged at 8000 rpm for 8 minutes. For the lysis of the cells, 500µl of TEN buffer, 50 µl of 10% SDS and 5 µl of proteinase K were added and incubated at 55 ºC for 1 hour. After cell lysis, an equal volume of Tris-saturated phenol was added to the lysate and mixed well until emulsion forms. The tubes were centrifuged at 8000 rpm for 8 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 emulsion forms and the centrifuged at 8000 rpm for 8 min at 4ºC.Aqueous phase was transferred to a separate tube and added an equal volume of chloroform-isoamyl alcohol (24:1), mixed well and then centrifuged at 8000 rpm for 8 min at 4ºc. 0.6- 0.7 volume of isopropanol was added to the tubes for precipitation and again centrifuged at 8000 for 8 min and the supernatant was removed and then the precipitate was washed twice with 500 µl of 70%

alcohol. Finally, air dried and suspended in 30 µl of TE buffer. DNA was then incubated with 1 µl of RNase A at 37°C for 1 h to remove RNA and stored at -20°C for further use.

3.5.6.2. Amplification of COI gene

The universal pair of primers FishF1 and FishR1 (Ward *et al*., 2005) were used to amplify the mitochondrial cytochrome C oxidase I (COI) gene. Details of the primer used in this study is given in Table 1.The mtDNA gene COI was amplified in a 12.5µl reaction volume with 1.25 µl of 10X Taq polymerase buffer, 0.25µl of each dNTP (0.05 mM), 0.5µl of each primer (0.01 mM), 0.125 µl of Taq polymerase and 0.5µl of genomic DNA (50-100ng /µl). Details of a composition provided in Table 2. The thermal cycler Agilent 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 gel documentation system (OmegaLum G, Aplegen, USA)

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	FishF2	TCGACTAATCATAAAGATATCGGCAC	26	Ward *et al*. 2005
2	COI	FishR2	ACTTCAGGGTGACCGAAGAATCAGAA	26	

Table 2: Composition of PCR master mix.

Components	Volume/Reaction (µl)
DD Autoclaved Water	9.375
PCR buffer (10x with $MgCL_2$)	1.25
dNTPs (2.5mM each)	0.25
Primer Forward (10mM)	0.50
Primer Reverse (10mM)	0.50
Taq. Polymerase (0.75U)	0.125
Template DNA (100ng/µl)	0.50
Total volume	12.5

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

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

3.5.6.3 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 on the basis of molecular weight by moving negatively charged nucleic acid molecules

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

3.5.6.4 Preparation of 1 % Agarose gel solution and gel casting

0.5g of agarose and 50 ml of TAE were mixed to form a solution. The solution was boiled to dissolve the agarose, preferably in a microwave oven for 1 minute. Then 2.0 µl ethidium bromide (10 mg/ml) was added to solution carefully Solution was cooled down to about 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 gets solidified, the comb was carefully removed. The holes that remained in the gel were the wells or slots. The gel was put, together with the casting tray, into a tank with 0.5 X TAE 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 (DNA ladder is also highly recommended). The lid of the electrophoresis chamber was closed and the current was set to 300mA and voltage 80V for 40 minutes was applied. 20 ml of Cold 1.0 X TAE was poured into the tank as gel running buffer. DNA ladder (molecular weight markers) was loaded into the first slot After markers were added, samples were mixed with bromophenol blue (dye are added to visualize DNA movement) and glycerol and later loaded into 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 Small DNA strands moved faster, large DNA strands moved slowly through the gel matrix.

3.5.6.5 Sequence Analysis

The amplified products were eluted using gel extraction kit (Qiagen, USA) and sequencing was performed with the same set of primers by the external sequencing facility. Both the forward and reverse sequences were obtained. The forward and reverse sequences were aligned using multiple sequence alignment.

The aligned sequences were ran for BLAST using BLASTn i.e, BLAST nucleotide for the match with the known sequence of *P. hypophthalmus.*

3.5.7. Transfection

Transfection of PHG with pEGFP-C1 vector was carried out as follows

A 70-90% confluent cells at 10^{th} passage was trypsinized from Flask and then seeded at a density of 1 x 10^5 in a 6 well plate individually at the 28ºC normal atmospheric incubator. The cells were grown at 28ºC in the incubator until they were 70% confluent. The culture medium was aspirated from each well, and the cells were gently rinsed twice with PBS and supplemented with 400µl of fresh L-15 medium devoid of serum and antibiotics. The plasmid DNA (200ng of pEGFP-C1) was dissolved in 100µl of Opti- MEM and 0.5µl of plus reagent was added, and the mixture was incubated for 5 minutes at room temperature. Lipofectamine LTX was mixed gently, and this was added to a mixture containing plasmid DNA and incubated for 30 min at room temperature. The mixture was added dropwise to each well-containing cells and mixed gently by rocking the plate back and forth. The cells were incubated at 28ºC in BOD incubator for 20 to 24 hours prior to testing for transgene expression. Then the old medium was changed with fresh medium after 4-6 hours. The cells were examined under a fluorescence microscope for expression of green fluorescence signals. Transfection efficiency was determined by percentage of the fluorescence protein-positive cells to the no.of viable cells 24~48 h after the start of transfection.

4. RESULTS

Cell culture systems were developed from different tissues of *P. hypophthalmus* viz. caudal fin, gill, and heart. Cell culture systems developed from caudal fin and gill were successfully subcultured to establish permanent cell lines designated as PHCF and PHG respectively .The PHG cell line developed from the gill was characterized for species authentication, growth parameters at different temperature & FBS concentrations and transfection efficiency.

4.1 Primary Culture

Primary cultures were established by using explants technique. A total of 116 explants were prepared from different tissues viz. heart, gills, and caudal fin tissues. The details are given in Table 4

Table 4: Details of cell culture system developed from the differenet tissues of *P. hypophthalmus*

S.No	Tissue samples	Number of Expalnts prepared	Radiation time	Number.of successful primary cultures	Number of passages
1.	Caudal Fin	36	5 days	9	17
2.	Gill	65	3 days	12	19
3	Heart	15	6 days	3	3
Total	**3**	**116**	**-**	**24**	-

4.1.1 Development of Cell Culture System from Caudal Fin Explant

A total of 36 explants were prepared and among them, 9 were used to develop cell culture. Morphological observation under the inverted microscope revealed that all the explants prepared from fin explants were found to be attached properly after 18-24 hrs of explant preparation. The radiation of cells started after 5 days of explant preparation from fin and a confluent monolayer around the explants was observed within 10-15 days. Majority of the cells proliferating from fin explant were fairly heterogeneous in nature i.e. consist of both epithelial and fibroblastic cells (Figure 1).

Fibroblast- like cells morphology was observed after 8 passages by Giemsa staining. The consistent subculture of caudal fin cells resulted in the development of a permanent cell line designated as PHCF. The PHCF cell line was maintained up to 17 passages.

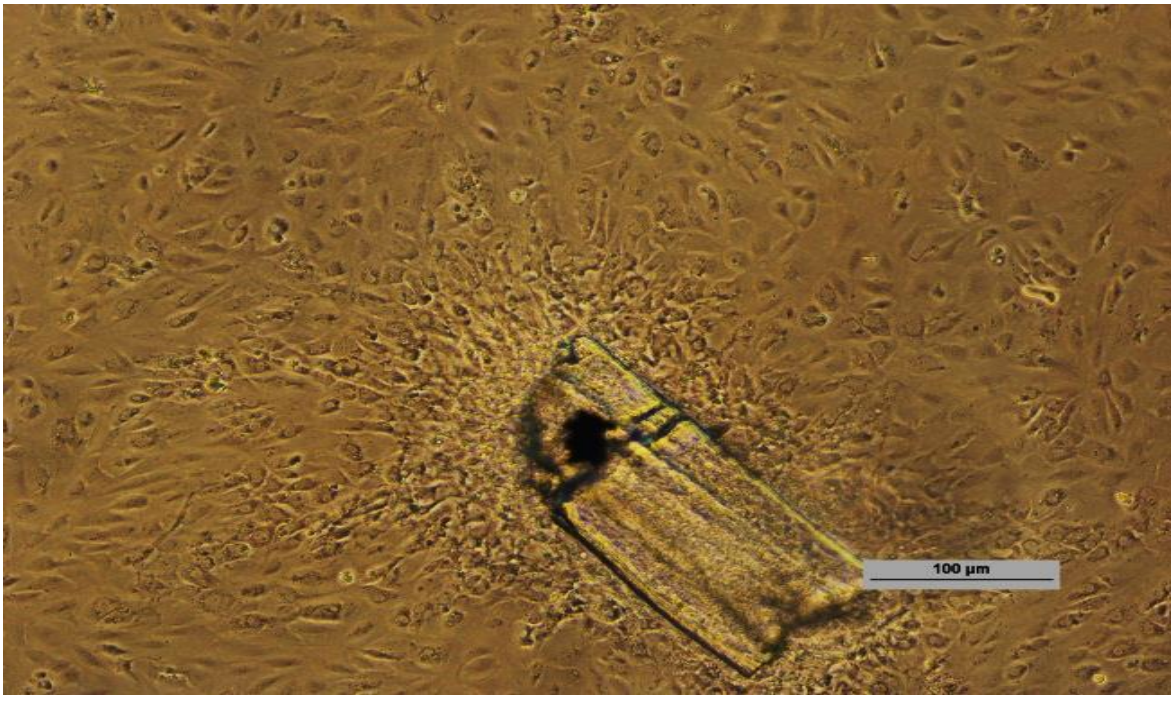

Plate.1 Phase contrast photomicrographs of PHCF cells along with the caudal fin explant of *P. hypophthalmus* (100X)

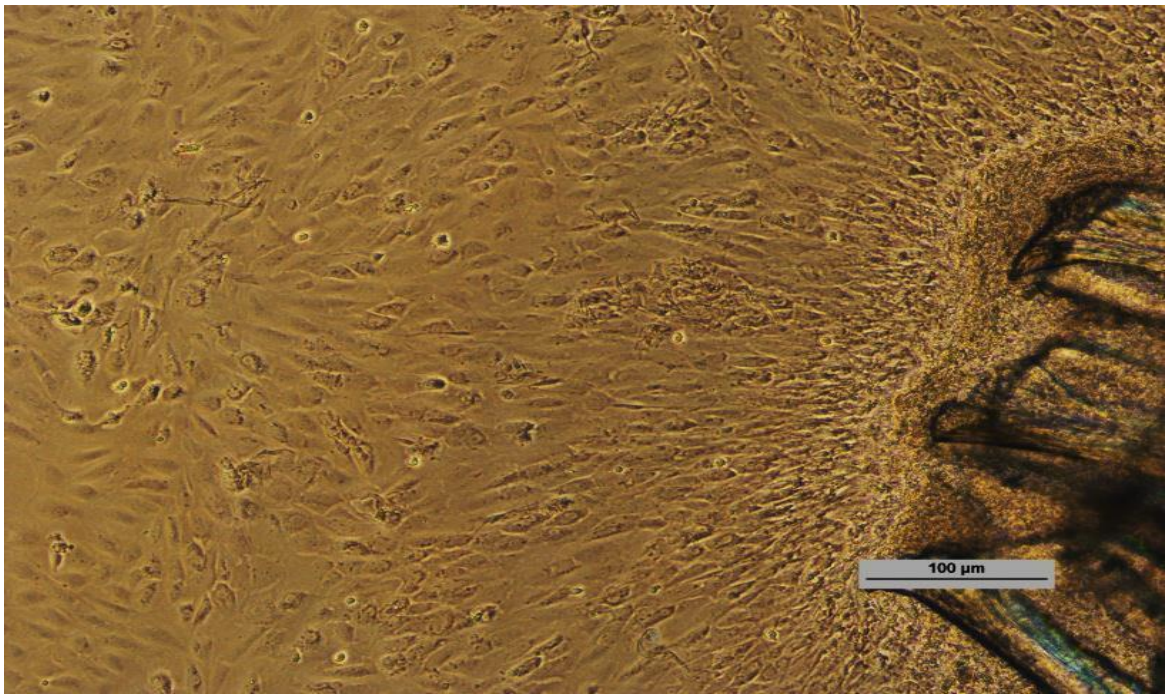

Plate.2 Phase contrast photomicrographs of PHCF cells derived from the caudal fin of *P. hypophthalmus* showing proliferated cells (100X)

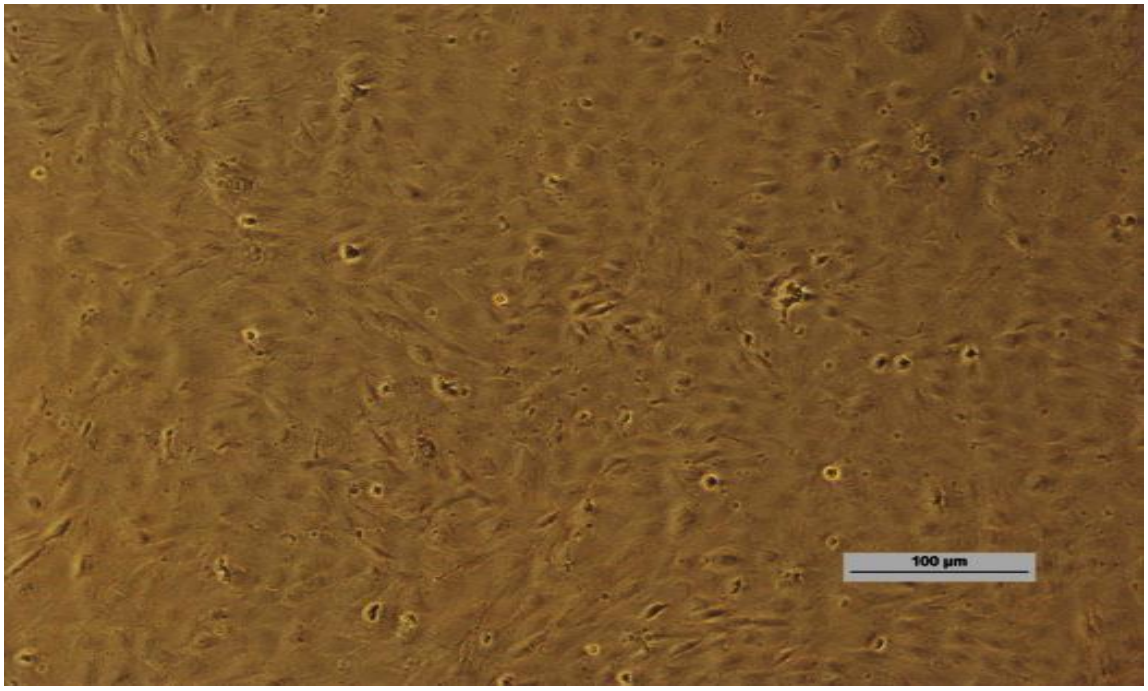

Plate.3 Phase contrast photomicrographs of PHCF cells at passage 12th (100X)

4.1.2 Development of Cell Culture System from Gill Explant

A total of 65 explants were prepared and among them, 12 were used to develop cell culture. Morphological observation under the inverted microscope revealed that all the explants prepared from gill explants were found to be attached properly after 18-24 hrs of explant preparation. The radiation of cells started after 3 days of explant preparation from gill and a confluent monolayer around the explants was observed within 7-10 Days. Majority of the cells proliferating from gill explant were fairly heterogeneous in nature i.e. consist of both epithelial and fibroblastic cells. The morphology of the cells were heterogeneous in nature to the passage 8^{th}. The spent medium was exchanged with the new medium 4-5 days once. For the subculture of cells L-15 media and 15% FBS were used.

Fibroblast- like cells morphology was observed after 15^{th} passages by Giemsa staining. The consistent subculture of caudal fin cells resulted in the development of a permanent cell line designated as PHG. The PHG cell line was maintained up to 19 passages.

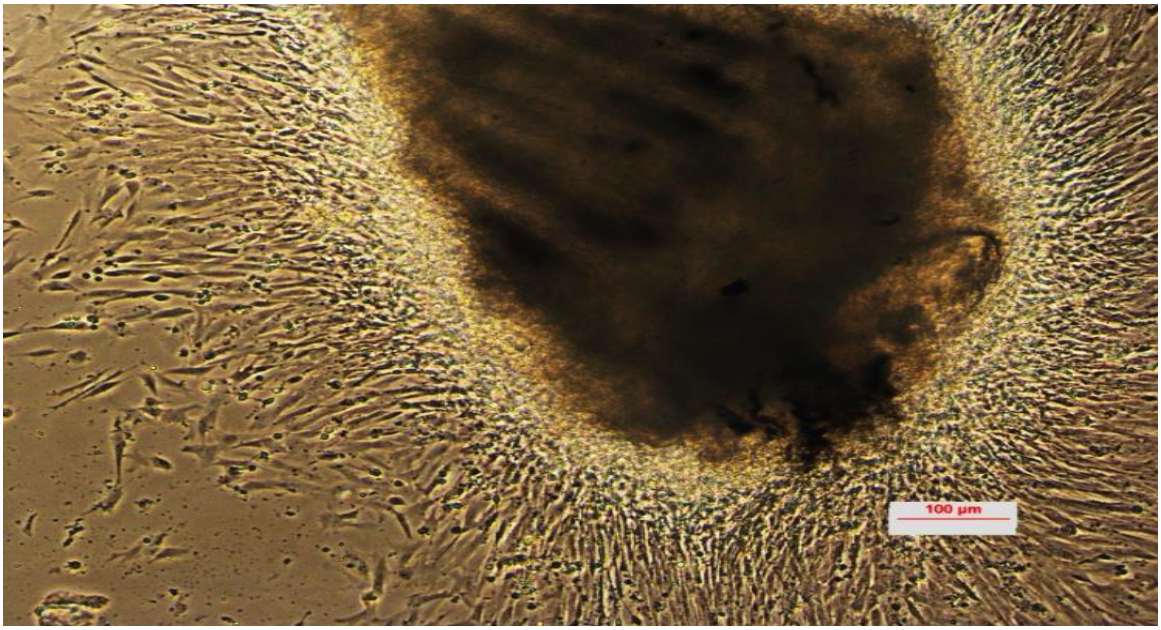

Plate.4 Phase contrast photomicrographs of PHG cells derived after 5 days of explant preparation from the gill explant of *P. hypophthalmus* (100X)

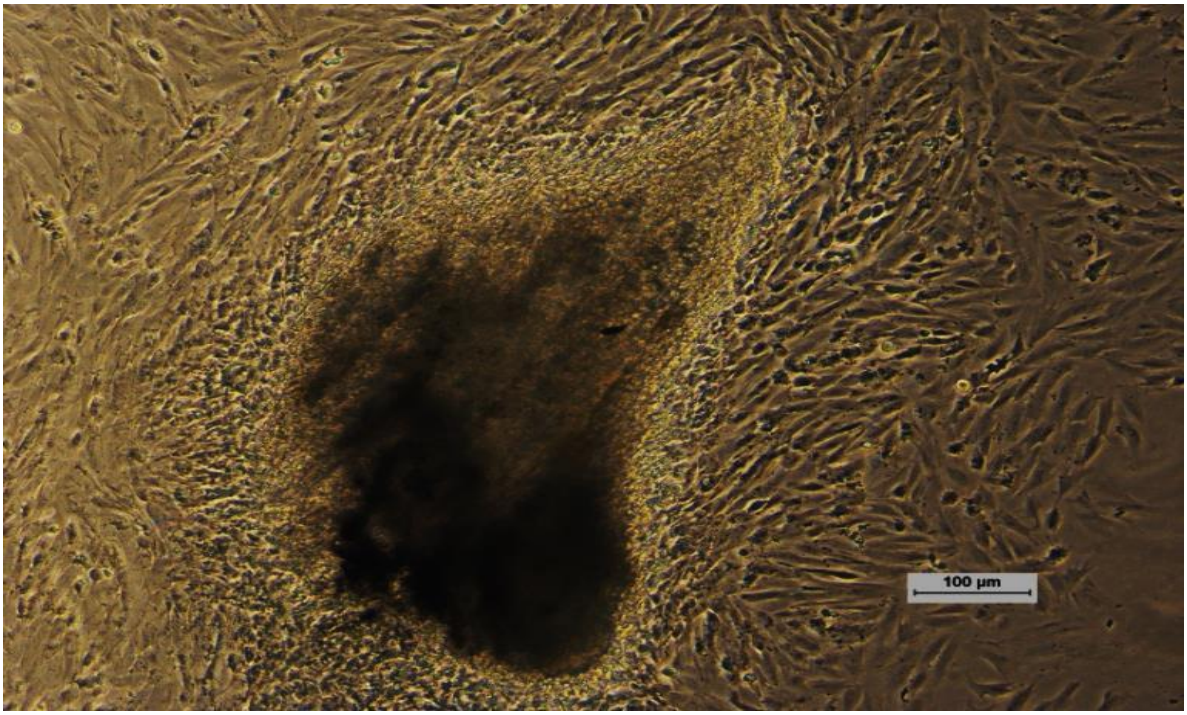

Plate. 5 Phase contrast photomicrographs of PHG cells Confluent monolayer around the explant observed after 7 days. (100X)

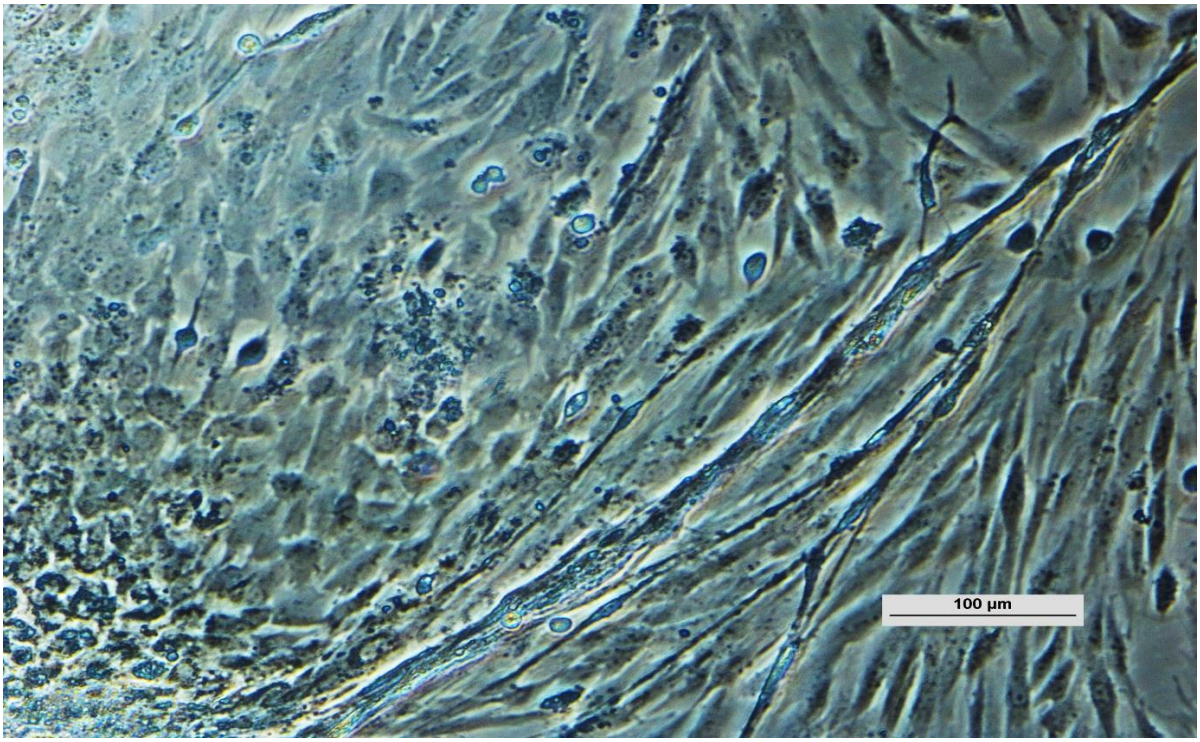

Plate. 6 Phase contrast photomicrographs of PHG cells Confluent monolayer around the explant observed after 7 days. (200X)

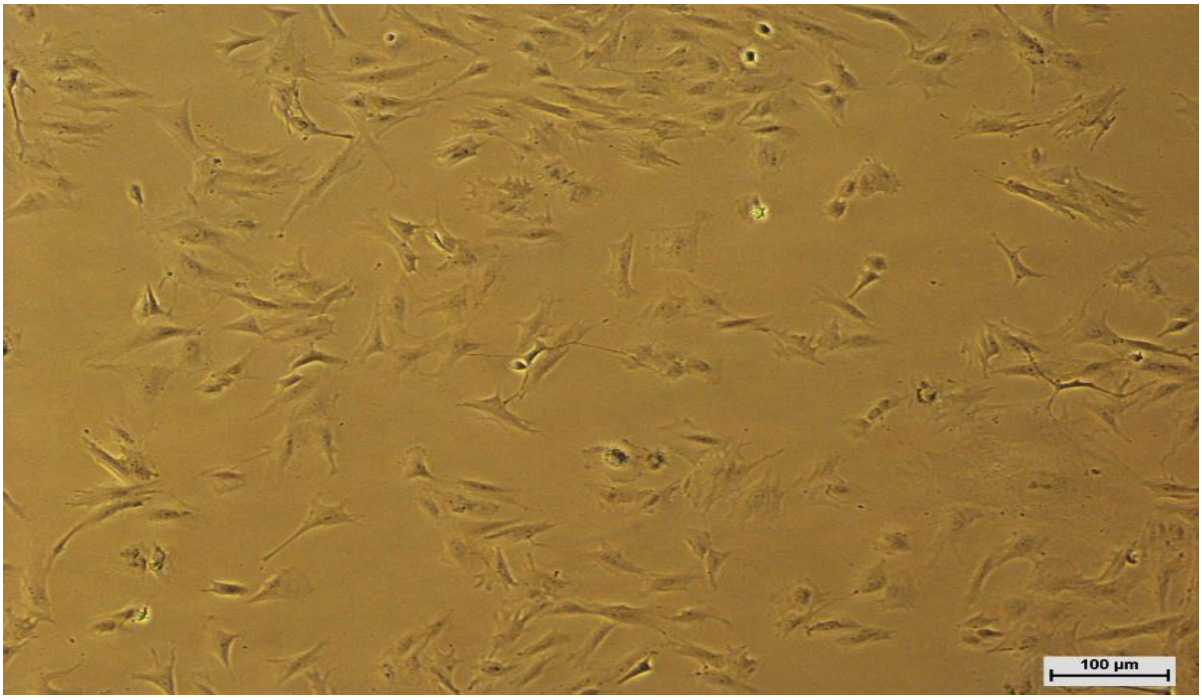

Plate.7 Phase contrast photomicrographs of PHG cells at 1st passage (100X)

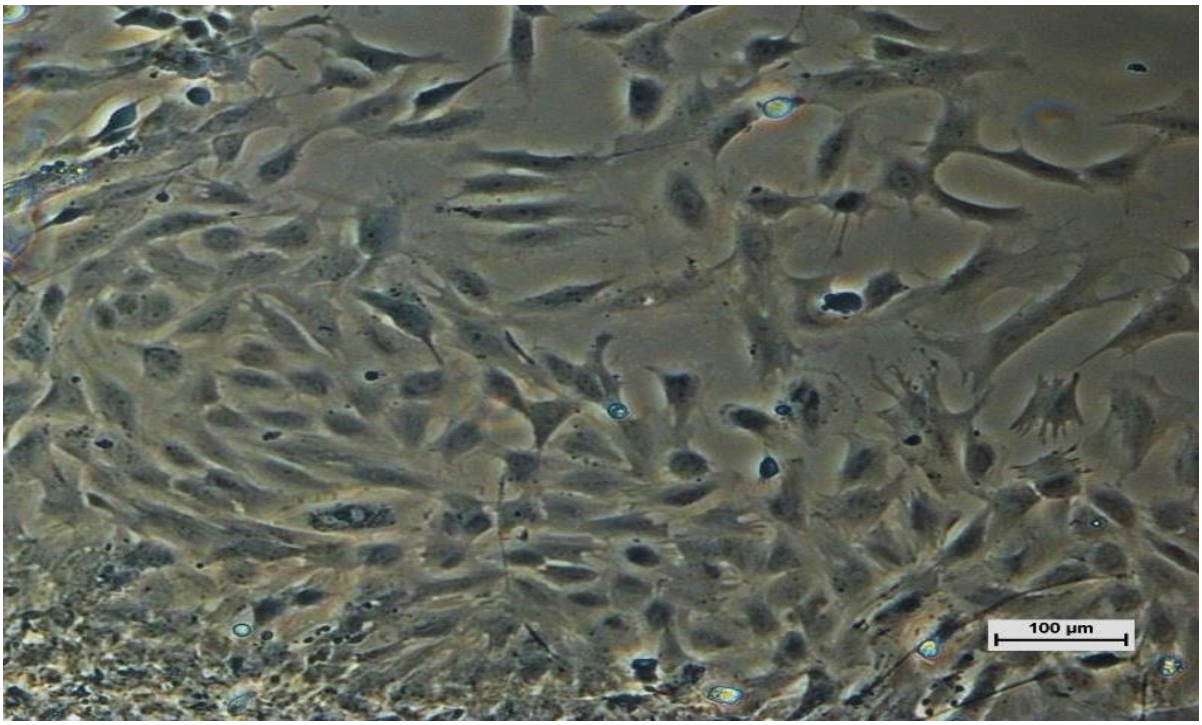

Plate. 8 Phase contrast photomicrographs of PHG cells at 1st passage (200X)

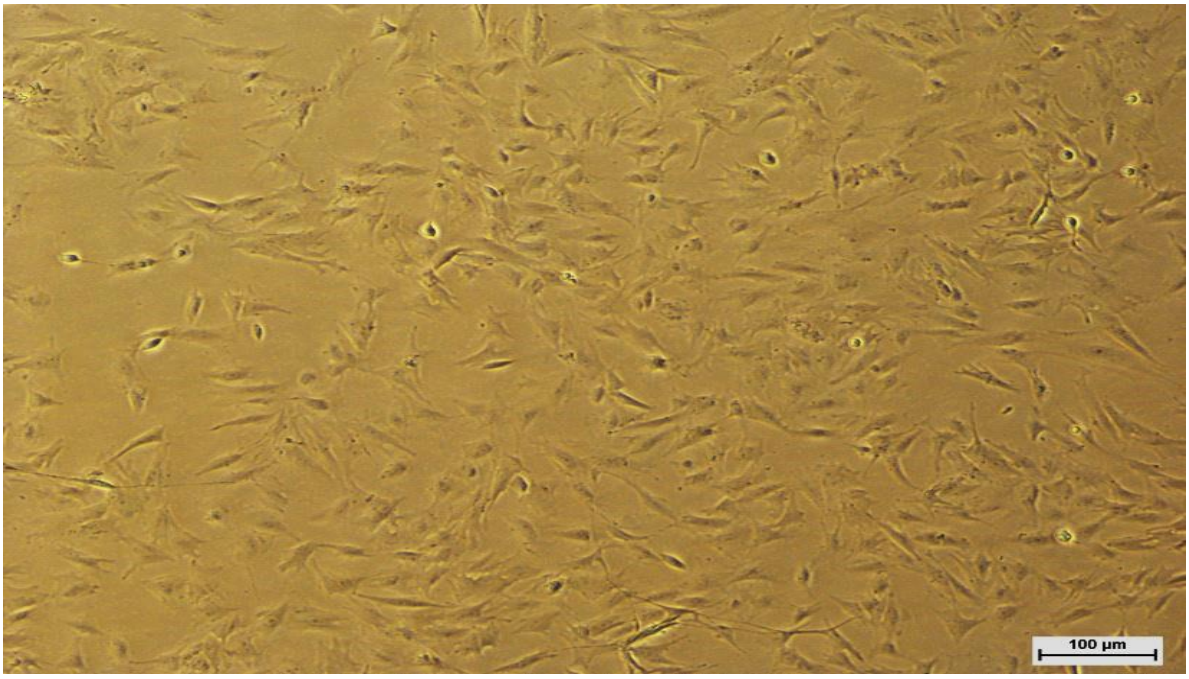

Plate. 9 Phase contrast photomicrographs of PHG cells at 5th passage (100X)

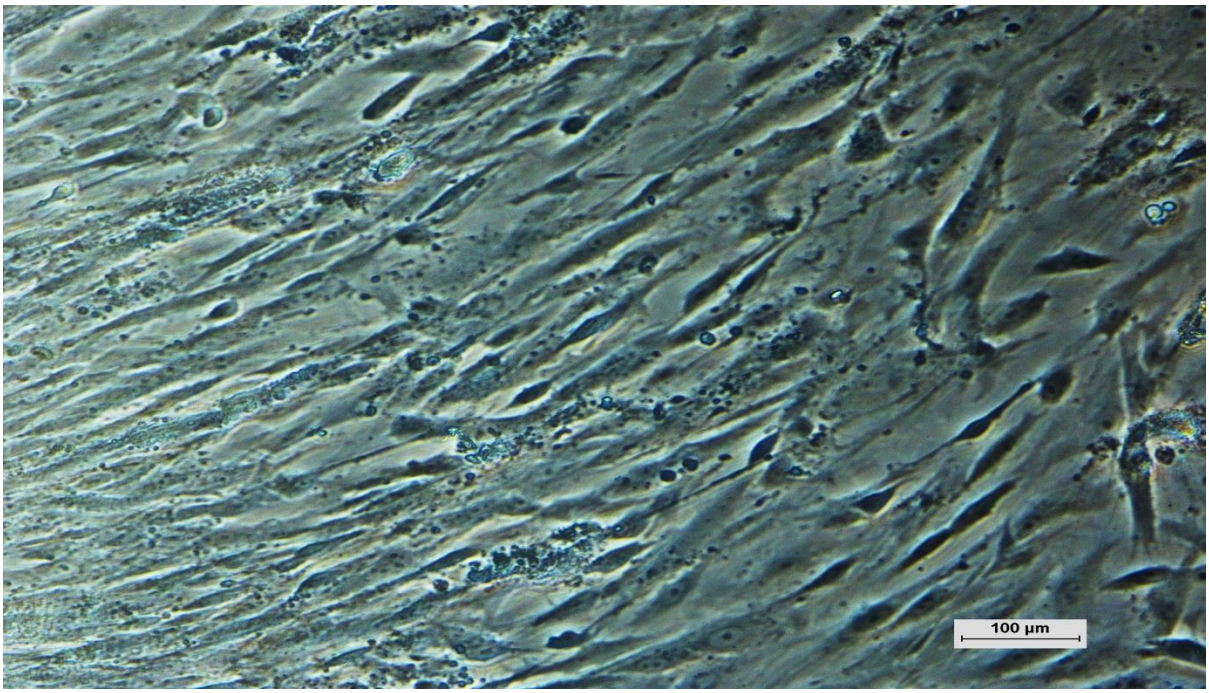

Plate. 10 Phase contrast photomicrographs of PHG cells at 5th passage (200X)

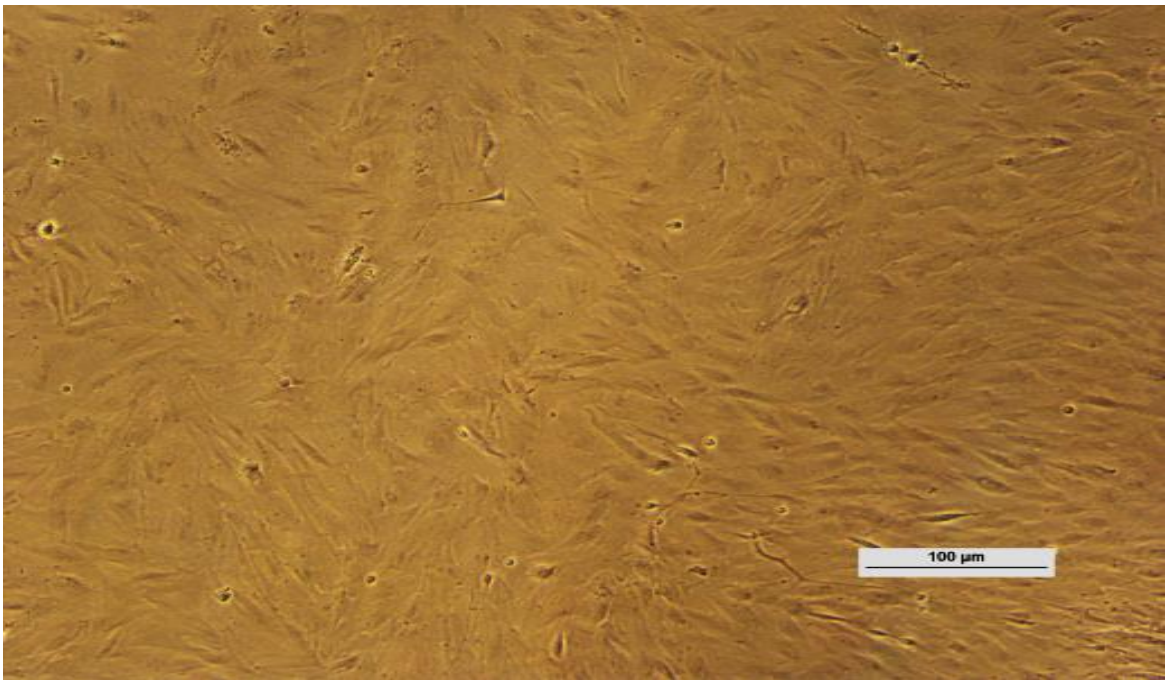

Plate. 11 Phase contrast photomicrographs of PHG cells at 19th passage (100X)

4.1.3 Development of Cell Culture System from Heart Explant

A total of 15 explants were prepared and among them, 3 were used to develop cell culture. Morphological observation under the inverted microscope revealed that all the explants prepared from heart explants were found to be attached properly after 18-24 hrs of explant preparation. The radiation of cells started after 6 days of explant preparation from the heart and a confluent monolayer around the explants was observed after 15-20 Days. Attempts made to initiate primary cultures from the heart tissues were subcultured to 3 passages. Majority of the cells proliferating from heart explant were epithelial in nature.

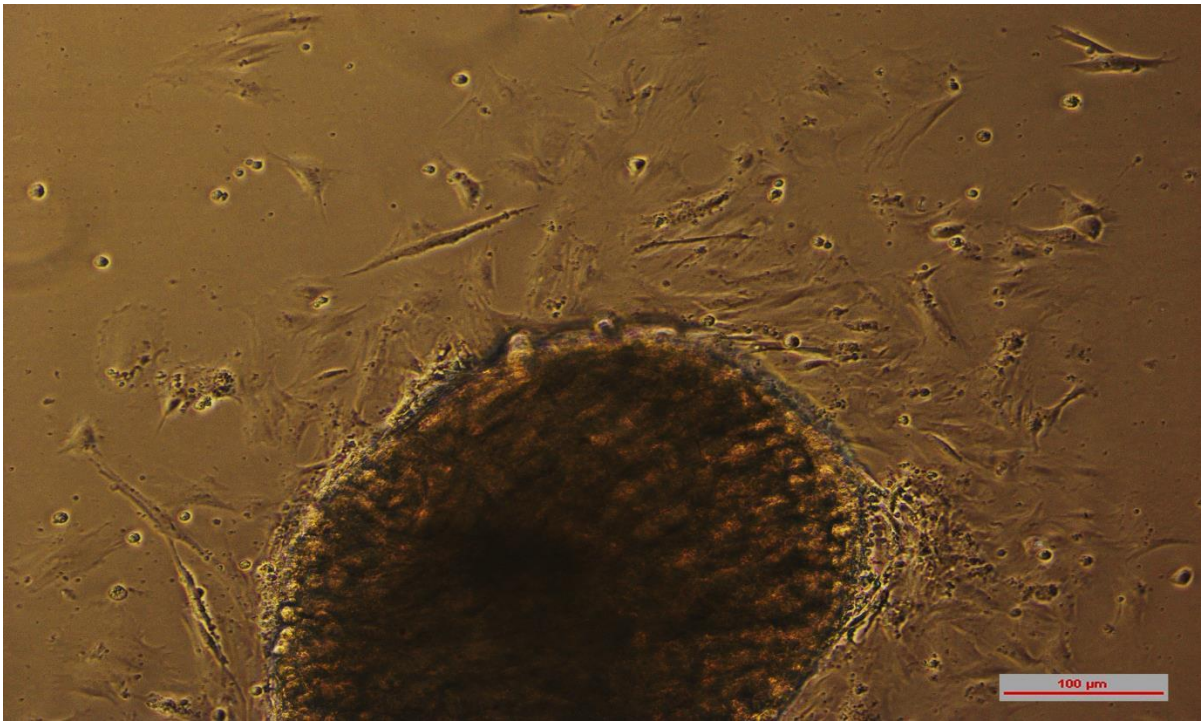

Plate. 12 Phase contrast photomicrographs of PHH cells derived from the heart tissue of *P. hypophthalmus* after 7 days of explant preparation (100X)

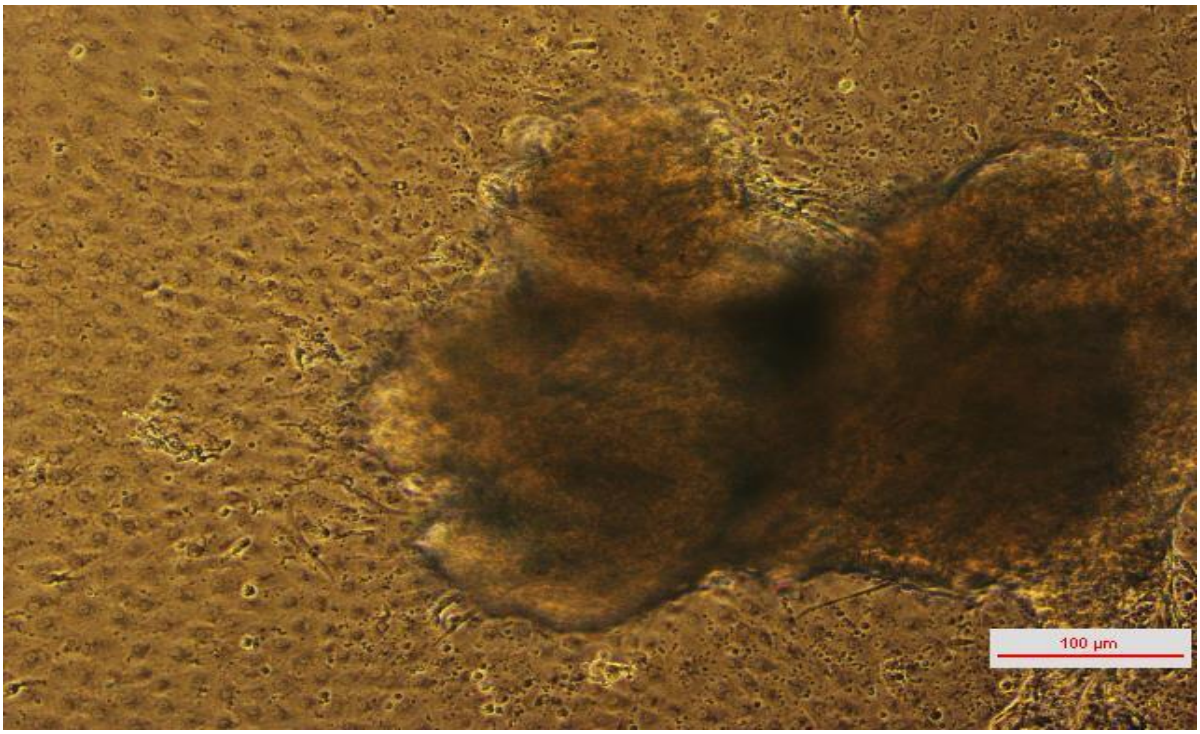

Plate. 13 Phase contrast photomicrographs of PHH cells confluent monolayer observed around the explant after 15 days(100X)

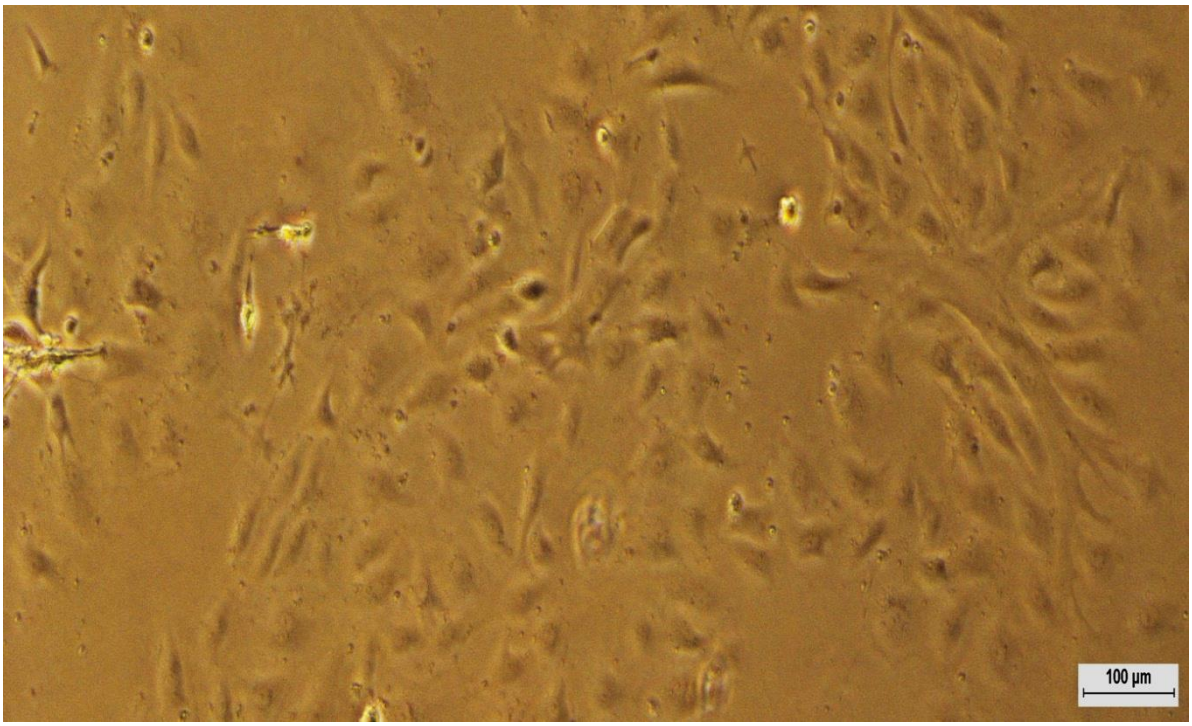

Plate. 14 Phase contrast photomicrographs of PHH cells at 3rd passages.

4.2. Subculture and Maintenance

Primary cultures developed fom the Caudal fin and gill explants were subcultured to 17 and 19 passages respectively. The cell line has been maintained in L-15 medium supplemented with 15% FBS. Primary cultures that proliferated to confluency were subcultured, split into two flasks and passaged cells were developing in patches having heterogenous morphology, comprising thin bipolar cells, fibroblast-like cells and epithelial-like cells.

4.3 Characterization of PHG Cell Line

Cell line PHG developed from the gill of *P. hypophthalmus* was characterized by the following parameters.

4.3.1 Detection of Contamination

Most of the explants setup at the initial stages of cell culture systems were contaminated with fungus and bacteria. Then handling of cell culture was improvised, afterwards cell culture systems were developed without any contamination. Flasks were routinely observed for the morphological changes as well as contamination.

4.3.2 Morphological Observation

Morphology of the cells observed were bipolar elongated appearance with a narrow diameter indicated the fibroblast morphology.

4.3.3 Cell Doubling Time

The estimated doubling time of PHG cell line at 17^{th} passage was 38.02 hrs.

4.3.4 Cell Plating Efficiency

Plating efficiency for each cell line was determined at different seeding concentrations of 200, 500 and 1000 cells. PHG cells showed plating efficiency of 45% when seeded in a density of 1000 cells /ml.

4.3.5. Growth Studies

4.3.5.1 Temperature

To determine the optimum temperature the cells were seeded at a concentration of 1×10^5 cells /ml in 25 cm^2 culture flask at different temperatures such as 20, 24, 28, 30, and 32ºC. The observed optimum temperature for the growth of the cells was 28 ºC. The cells were capable to proliferate in temperature ranging from 24ºC to 32ºC. But the maximum growth was observed at 28ºC.

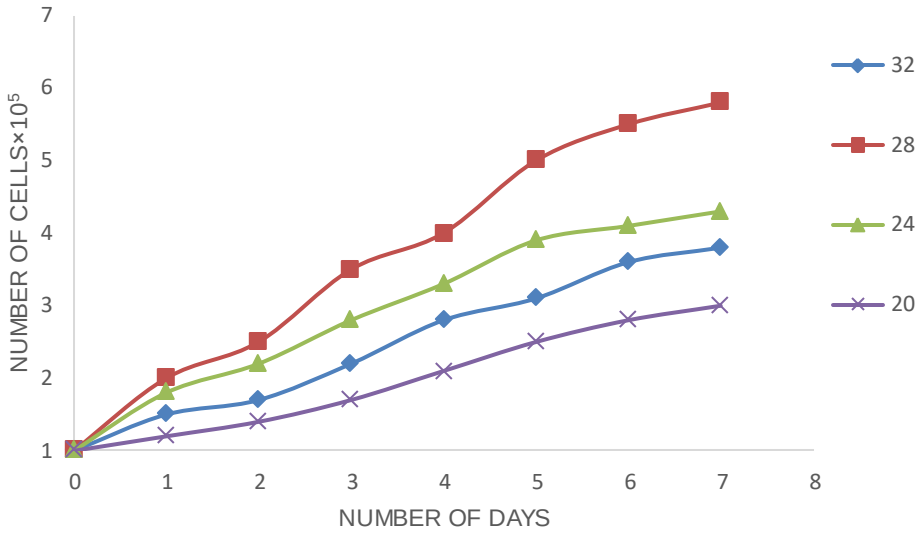

Fig.1 Growth of PHG cell line at different temperatures (ºC).

4.3.5.2 FBS Concentration

For the initial subcultures 10 % FBS was supplemented with L-15 medium. The growth of the cells increased as the concentration of the FBS increased from 10% to 20%. Cells grew reasonably well in the optimum concentration of FBS at 15%.

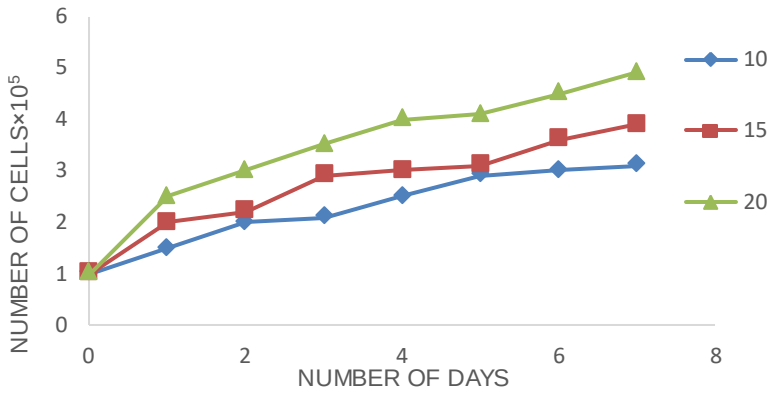

Fig.2 Growth of PHG cell line at different concentrations of FBS (%)

4.4 Authentication using Molecular Markers

4.4.1 PCR amplification of COI gene

PCR (Polymerase Chain Reaction) was carried out to authenticate the origin of the cell line. The mitochondrial region COI gene from the PHG cell line and the gill tissue was amplified using universal primers yielded amplicons size of 655 bp. Sequence alignment of COI gene derived from the PHG cells revealed 99% similarity with the known mitochondrial sequence of *P. hypophthalmus* (Genbank accession number: KR0802631.1)

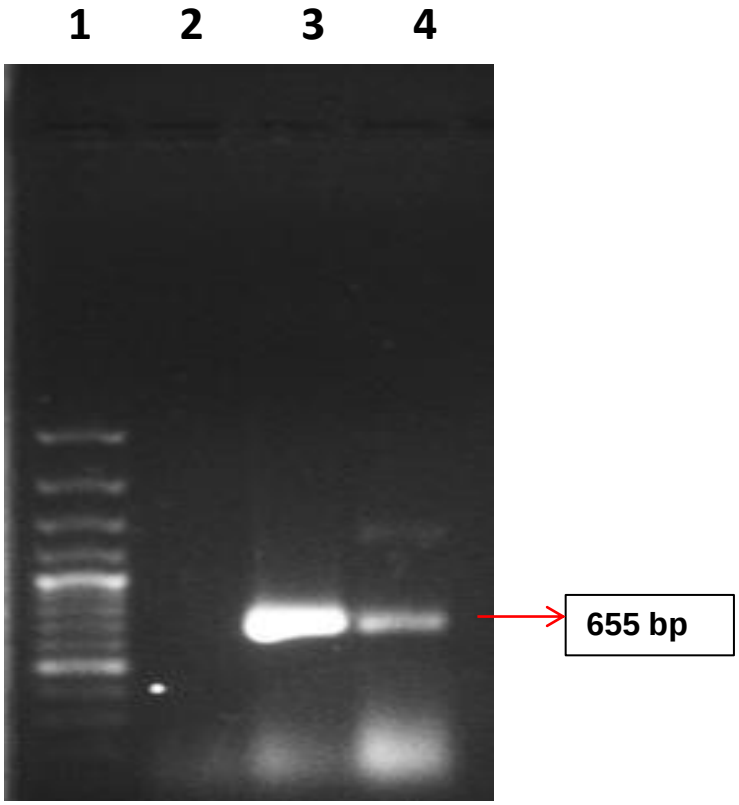

Plate. 15 PCR Amplification of 655 bp fragment of *P. hypophthalmus* genome using oligonucleotide primers from the conserved portions of COI region. Lane 1- Generuler express 100 bp DNA ladder (Fermentas) Lane 2- Negative control; Lane 3- PHG Positive control, Lane 4- PHG COI.

4.4.2 Transfection

The PHG cell line at 10 passage was successfully transfected with pEGFP-C1 plasmid using Lipofectamine and plus reagent (Invitrogen). The expression of the pEGFP in the PHG was detected 18hrs post transfection. The estimated transfection efficiency was 9%.

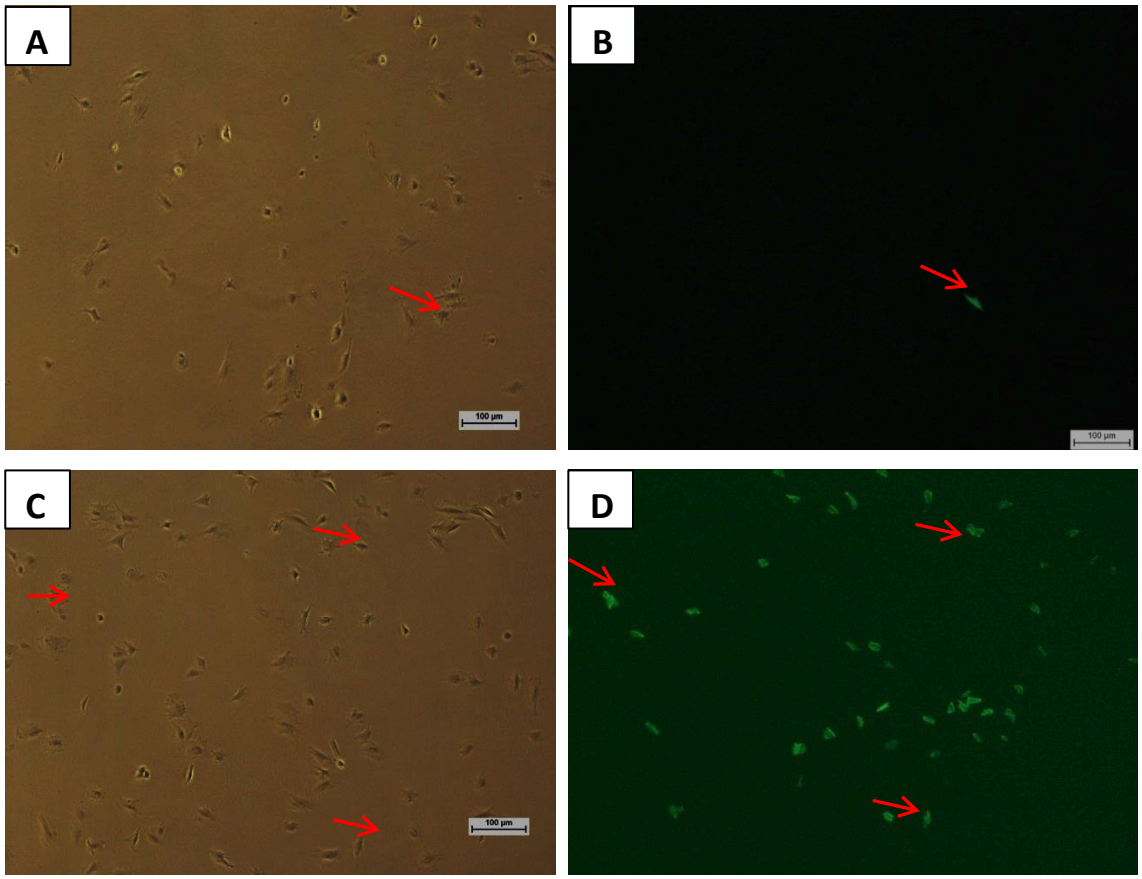

Plate.16 Green fluorescent protein expression of PHG cell line transfected with pEGFP-C1 (100X)

5. DISCUSSION

Cell lines are very important biological *in vitro* tools for carrying out investigations into physiology, virology, toxicology, carcinogenesis, biomedical science and transgenesis. Teleost fish cell lines have been developed from a wide range of tissues such as ovary, fin, swim bladder, heart, spleen, liver, eye muscle, vertebrae, brain, and skin *(* Lakra *et al.* 2011) The rainbow trout gonadal cell line, RTG-2 developed by Wolf & Quimby (1962) was the first permanent cell line of fish origin. Developing cell lines from different tissues of fish could be a valuable tool for *in vitro* with particular reference to fish physiology and other related studies. The present study, was aimed to develop cell culture system from *P. hypophthalmus* (Sauvage, 1878) which is a major candidate species for aquaculture in India. In the present study two permanent cell lines i.e. PHG and PHCF were developed and cell line from gill i.e. PHG was characterized.

5.1 Development of Primary Culture

In the present study primary cultures were developed from the gill, heart and caudal fin tissues using explant technique. The explant technique has many advantages over trypsinization methods which enable quantitative analysis, ease of culture and has no chemical attack (Avella *et al*. 1994). Many continuous cell lines have been developed by using explant technique from different fishes such as *Epinephelus malabaricus* (Sobhana *et al*. 2009), *Clarias gariepinus* (Kumar & Singh, 2000), *Lates calcarifer* (Parameswaran *et al*. 2006), *Puntius denisonii* (Lakra *et al*. 2010) and etc.

Nanda *et al.* (2014) studied comparison of explant method and trypsinization method and reported better attachment of cells by using explant technique over trypsinization method in *Cirrhinus mrigala.* The cells radiating from the primary cultures were consisted of heterogeneous population i.e., epithelial and fibroblast cells. Such heterogeneous population of cells at the primary cultures were reported by many researchers (Lakra *et al*. 2010, Kamalendra *et al*. 2010),

Ahmed *et al*. 2008, Hameed *et al*. 2006). After 12th passages of PHG, fibroblast cells gradually increased in numbers. Such predomination of fibroblast cells over the epithelial cells was also reported by many researchers (Bejar *et al*. 1997, Lakra *et al*. 2006, Goswami *et al*. 2012). Chi *et al.* (1999) reported presence of both epithelial cells and fibroblast-like cells in primary culture of grouper fin (GF-1) cells. However, they reported that in subsequent subcultures, the fibroblast-like cells proliferated more rapidly than the epithelial cells and ultimately predominated.

5.2 Development of Cell Line

For the development of cell lines, primary cultures initiated from caudal fin, gill and heart were used. The primary cultures were initiated by the explant technique. The explant technique was found to be more efficient to develop confluent monolayer cultures from the targeted tissues. Attachment of the cells is the major requirement for successful cell culture system. The cells require an intact cell membrane integrin protein which aids in the attachment (Bols & Lee, 2005). The degree of attachment of the explants to the flask on incubation varies with the type of tissues. Attachment of caudal fin, gill, and heart explants exhibited appreciable attachment to the flask surface, attributed to the several cell attachment factors released by these tissues. In the present study PHCF and PHG cell lines were developed and subcultured up to 17 and 19 passages respectively.

5.3 Characterization of Cell Line

5.3.1 Growth Studies

The optimum physico-chemical environment like culture medium, FBS concentration, growth supplements, incubation temperature, etc. varies considerably across fish species. Most commonly used parameters for growth study of any cell line are temperature and FBS concentration. Fish cell line has the ability to grow in temperature ranging from 24ºC to 32ºC (Tong *et al*. 1997, Lakra *et al*. 2006, Lakra & Goswami, 2011, Goswami *et al.* 2012). In the present study the optimum temperature for the developed cell line PHG was found to be 28ºC. The

major advantage of the piscine cell line is the growth. Piscine cell lines can be grown over a wide range of temperature whereas in the case of mammalian cells require 34 -37ºC.

Another parameter to be characterized for the growth study is the suitability of the cells to grow in L-15 medium with optimum FBS concentration. The optimum growth of the cell line PHG was observed in the L-15 medium with 15% FBS which indicated the confirmation with Lakra *et al,* 2006. Many other media had been tested by previous workers for the suitability of the cells growth medium. Among the several tested media, L-15 was found to be most suitable for attachment and proliferation of cells (Kumar & Singh 2001). The growth rate of cells increased when the FBS concentration increased from 10 % to 20%. Although better relative growth can be attained by 10-15% FBS concentration (Wolf & Quimby,1969) in the economic point of consideration. (Bols *et al*. 1994) concluded that the optimal level of serum supplementation in the cell culture is 10%. Some fish cell lines have been observed to grow in 2% serum (Jensen *et al*. 2012) in contrast to others which required 20% or even 30 (Bryson *et al.* 2006).

5.3.2 Plating Efficiency

The estimated plating efficiency of the PHG cell line was 45% with 20% FBS. Previous reports were 64% at 1000 cells per flask by Goswami *et al*. (2012); and Bejar *et al*. (1997) recorded 65.3% for the continuous cell line SAF-1 at 10% FBS. Chi *et al.* (1999) recorded the plating efficiency of 21% of the GF-1 cells seeded at a density of 100 cells per flask at subculture 50 and this increased to 80% at subculture 80.

5.3.3. Molecular Characterization

Species authentication of cell lines is one of the most important parameters to authenticate the origin of the cell line. In the present study, the PHG cell line was authenticated using COI gene. The gene sequence of COI derived from the PHG cells revealed 100% similarity with the known sequences submitted

in NCBI, Genbank database.

Hebert *et al.* (2003) have demonstrated the utility of COI gene as a universal barcode, referred as - DNA barcoding for the genetic identification of animal life. It has been also used to identify species and to study relationships among organisms (Ward *et al.,* 2005). COI region has been consistently used by many researchers for identification of cell lines (Lakra *et al.* 2010b; Lakra & Goswami, 2011) and Cooper *et al.* (2007) used COI region for identification of 67 cell lines used for barcode analysis. Other alternatives such as 16S ribosomal RNA gene sequence can also been used to confirm the origin of muscle and fin cell lines (Zhao & Lu, 2006; Kochzius *et al.* 2008). Lakra & Goswami, (2011) confirmed the identity of the PSCF cell line from *Puntius sophore* by amplifying the 655 bp fragments of COI (cytochrome oxidase Subunit I) of mitochondrial DNA . Similarly cell line from the ornamental fish *Puntius denisonii* was also confirmed by the same sequence Swaminathan *et al.* (2012).

5.3.4. Transfection Efficiency

In the present study the PHG cell line was successfully transfected with pEGFP-C1 plasmid using lipofectamine LTX and Plus Reagents (Invitrogen). However, the estimated transfection efficiency was 9% which is comparable to PSCF cell line with 10% efficency (Lakra & Goswami, 2011). Zhou *et al.* (2008) reported 2% transfection efficiency in a CSTF cell line developed from Chinese sturgeon *Acipenser sinensis*. Ku *et al*. (2009) transfected continuous cell lines derived from the *Epinephelus quoyanus* brain, heart, and gill tissues with the vector pEGFP-C3 under the control of CMV (Cytomegalovirus) promoter using lipofectamine 2000. The expression of GFP in these cell lines could be detected as early as 30hrpost transfection. Similarly Qin *et al.* (2006) transfected *Epinephelus coioides* spleen cell line with pEGFP-N3 plasmid vector this cell line expressed the reporter gene 3 hrs post transfection. This reveals the importance of PHG cell line in gene expression studies and for the production of recombinant proteins.

5.4 Application of Cell Line

Piscine cell lines are used for the toxicological assessment of aquatic pollutants at the cellular and molecular levels. Bols & Lee (2005) enlisted the number of cell lines that have been used over the years for the ecotoxicology assessment. Tanneberger *et al*. (2012) used the piscine cell line RT gill-W1 to predict the acute toxicity of 35 chemicals and found the EC_{50} value for those tested chemicals. Tan & Schirmer, (2017) developed a cell culture based biosensing technique for the detection of toxicity in water.

For the assessment of toxicants number of assays employed in the cell culture system are MTT assay, AB assay, and NR uptake assay. Babu *et al*. (2012) studied the cytotoxicity caused to the three cell lines developed from the *Etroplus suratensis* to tannery effluents and three end points were studied such as MTT 3-(4,5 – dimethylthizol-2-yl)-2,5- diphenyl tetrazolium bromide, NR (Neutral red) and AB (Alamar Blue). Cell lines are also used for testing the in vitro cytotoxicity caused by the bacterial extracellular products (ECPs). Hameed *et al*. (2006) reported that ECP from *Vibrio* strains produced shrinking, detaching, and eventual monolayer destruction. Zhao *et al*. (2010) tested the ECP from *V. alginolyticus* which caused apoptosis, cell rounding, and osmotic lysis in EPC (Epithelioma papulosum cyprini) cells which led to the eventual death of the fish cells within hours of infection

In the present study, the developed cell line PHG will be useful in evaluating the acute toxicity of the various chemicals and assessment of effluents. In addition, the PHG cell line faciliates the study of gill related diseases in *P. hypophthalmus* and also useful in carrying out *in vitro* cytotoxicological studies. PHG cell line also aids in the conservation of the germplasm of the important species *P. hypophthalmus.*

6. SUMMARY & CONCLUSION

Fish cell line has been used as a major *in vitro* tool for in various fields such as biotechnology, biomedical sciences, conservation genetics, cellular physiology, virology and toxicology etc. Hence developing cell lines from different tissues could be highly beneficial for studying the physiology of the fishes. *Pangasianodon hypophthalmus* is a major candidate species for aquaculture in India which emerged as a third most culture species next to the carps. At the moment, in Indian freshwater cage farming, *P. hypophthalmus* is being cultured economically (NFDB, 2016). It is evident that the fish production from the reservoirs can be greatly improved by undertaking cage culture. Channel catfish virus disease is an acute hemorrhagic disease reported in cage cultured. *P. hypophthalmus* (Zahrah *et al.* 2014).Hence developing cell lines from the economically important fish could serve as an *in vitro* tool for studying the pathogenicity. Considering all these facts, the present study was aimed to develop cell line from the selected tissues of *P. hypophthalmus* and to characterize the developed cell line by using molecular markers.

Fingerlings of *P. hypophthalmus* were maintained in the Central Wet lab, ICAR-CIFE and sacrificed to initiate primary cultures from different tissues such as caudal fin, gill and heart. Explant technique was followed to develop primary cultures. Primary cultures developed from the caudal fin, gill and heart were subcultured to 17, 19 and 3 passages. Established cell lines were designated as PHCF, PHG, and PHH respectively. The developed cell lines were maintained in L-15 medium supplemented with 15% FBS at an optimum temperature of 28ºC. The optimum temperature for the cell lines which was in conformity with the earlier reported cell lines (Lakra *et al*. 2006, Goswami *et al*. 2012). Among the three developed cell lines PHG cell line from the gill tissue was characterized by various parameters such as growth study, cell plating efficiency, cell doubling time, molecular analysis, and transfection.

Growth parameters such as temperature and FBS concentration were estimated by seeding the cells at varying concentration of FBS and varying temperature and the maximum growth of the cells were observed at temperature 28 ºC and 20% FBS concentration. The optimum FBS concentration for the growth of the cell line was 15% which was in conformity with the earlier reported cell lines (Majeed *et al*. 2012; Hameed *et al.* 2006). Plating efficiency of the PHG cell line was determined 45% by seeding the cells at 500, 1000 cells per flask which indicated the proliferation and colony formation ability of the cells. The origin of the cell line was authenticated by amplification of the COI gene.

The applicability of the cell line to express the foreign gene was studied by transfecting the cells with pEGFP-C1 plasmid using lipofectamine and plus reagent (Invitrogen) The estimated transfection efficiency was 9% which also in conformity with the previous reports (Goswami *et al*. 2012; Lakra and Goswami, 2011). This would be a useful model for gene expression and transgenic studies.

The developed cell lines from different tissues of *P. hypophthalmus* would be instrumental in carrying out *in vitro* genetic and toxicological research. In addition, the developed cell line will play an important role in conservation of germplasm of this important fish species.

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ABBREVIATIONS

µ	micron
µg	Micrograms
^{o}C	Degree Centigrade
bFGF	Basic Fibroblast Growth Factor
bp	base pair
BSS	Balanced Salt Solutions
BTF	Bluefin Trevally Fins
BTMS	Bluefin Trevally Muscles
CCV	Channel Catfish Virus
CSV	Chum Salmon Virus
COI	Cytochrome Oxidase I
DDW	Double Distilled Water
DNA	Deoxyribonucleic Acid
dNTP	dideoxynucleotide tri phosphate
ECP	Extra Cellular Products
EDTA	Ethylene Diamine Tetra Acetic Acid
EMEM	Eagle‘s Minimal Essential Medium
EtBr	Ethidium Bromide
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
GNNV	Grouper Nervous Necrosis Virus
HMEM	Hank‘s Minimal Essential Medium

ICC	Immunocytochemistry
IHNV	Infectious Hematopoietic Necrosis Virus
IPNV	Infectious Pancreatic Necrosis Virus
ISAV	Infectious Salmon Anaemia Virus
KCl	Potassium Chloride
kD	Kilo Dalton
L-15	Leibovitz- 15
mg	Milli gram
mM	Milli Molar
nM	Nano Moles
PBS	Phasphate Buffer Saline
PCR	Polymerase Chain Reaction
pM	Pico Moles
rRNA	ribosomal Ribonucleic Acid
SDS	Sodium Dodecyl Sulphate
SGIV	Singapore Grouper Iridovirus
SHRV	Snakehead Rhabdo Virus
SVCV	Spring Viremia Carp Virus
TAE	Tris - Acetate - EDTA buffer
TE	Tris - EDTA buffer
VHSV	Viral Hemorrhagic septicemia Virus

APPENDIX

Equipments

- Phase contrast inverted green fluorescent microscope (Nikon, Japan).
- Laminar Flow (Thermo scientific USA)
- BOD incubator (Labtro, India)
- Autoclave (Expo Hi-Tech, India),
- Deep Freezers (-20°C)
- Electronic Balance (APX-100, Denver Instruments, USA; Shimadzu, Japan);
- Electrophoresis assembly (Bangalore Genei Pvt. Ltd., India; Technosource, India);
- Gel Documentation system (Omega Lum G, Aplegen, USA);
- High speed cooling centrifuge (Eppendorf, Thermo Scientific);
- Spectrophotometer Nanodrop 2000/2000c (Thermo Scientific, USA);
- PCR thermal Cycler (LabIndia, Applied Biosystems; BioRad, USA, Takara, Japan
- Water bath (Julabo, Germany; LablineBiosystems,USA).

Chemicals and Reagents

- Fetal Bovine Serum (FBS) was from Gibco
- Leibovitz (L-15) Media for cell culture work and Antibiotics (penicillin, streptomycin, amphotericin B) were from HiMedia Laboratories
- Trypsin with EDTA
- Dulbecco's Phosphate-Buffered Saline (Thermo Fisher scientific).
- Trypan blue dye
- Lipofectamine 3000 and plus Reagents were from Invitrogen.
- DNA primers were from Xcelris, India;
- PCR reagents were from SRL, India and Thermo Scientific, USA;
- Gel Extraction kit from Qiagen,USA
- Plasmid Extraction kit from Qiagen,USA.

Cell Culture Vessels, Plasticwares and Glasswares

- Glasswares for the work was acquired from Borosil,India
- Plasticwares from Tarsons Pvt. Ltd., India.
- Cell culture flasks and Petridish were from Thermo Fisher Scientific.

All solutions, plasticwares and glasswares except heat labile components were sterilized by autoclaving at 15lb for 20 min. Heat-labile solutions were filter sterilized using 0.22-micron cellulose acetate disposable syringe filters (Osmonics, USA).

Reagents for Genomic DNA Isolation

DNA Extraction Buffer

Tris-HCl	7.88g
NaCl	23.37g
EDTA	1.46g

dH2O to 1000 ml

pH : adjust to 8.0

SDS (10%)

SDS	10 g
dH2O	90 ml

Proteinase K (20 mg/ml)

Proteinase K	20 mg
dH2O	1 ml

Stored at -20°C.

Chloroform: Isoamyl alcohol (24:1)

Chloroform	24 vol.
Isoamylalcohol	1 vol.

Stored at room temperature.

TE Buffer 1X

Tris	10mM

EDTA	1mM
MW	200 ml

pH adjusted 7-8 by HCL, filtered through 0.2µm filter and sterilized by autoclaving.

Reagents for Agarose Gel Electrophoresis

TAE (50X)

Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA (0.5 M; pH 8.0)	100 ml

Final volume made to 1000 ml.

Gel loading dye (6X)

Bromophenol blue	0.25 g
Xylene cyanol	0.25 g
Glycerol	3 ml

Make the volume to 10ml using distilled water and stored at 4°C.

Reagents for Plating Efficiency

Formalin (10%)

Formaldehyde	1 ml
DDW	9 ml

Mix 1 ml formaldehyde in 9 DDW.

Reagents for Staining

Giemsa stain

Giemsa powder	0.5g
Glycerol	33ml
Methanol	33ml

Dissolve giemsa powder in 33ml glycerol & incubate overnight at 60˚C in water bath. After cooling at room temperature, add 33ml methanol to the above solution. Filter the solution with filter paper.