ESTABLISHMENT AND CHARACTERISATION OF CELL LINES FROM THE CAERULEAN DAMSEL, *POMACENTRUS CAERULEUS*

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Githa Ann George

Reg. No. 3946

CENTRAL MARINE FISHERIES RESEARCH INSTITUTE

(Indian Council of Agricultural Research)

Post Box No. 1603, Kochi-682 018, Kerala, India

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भारतीय कृषि अनुसंधान परिषद
केन्द्रीय समुद्री मात्स्यिकी अनुसंधान संस्थान
[कृषि अनुसंधान एवं शिक्षा विभाग, कृषि मंत्रालय,भारत सरकार]
Indian Council of Agricultural Research
Central Marine Fisheries Research Institute
[Department of Agricultural Research and Education,
Ministry of Agriculture, Govt. of India]
पोस्ट बोक्स सं.**1603**, एरणाकुलम नोर्त पी.ओ.,कोच्ची–**682018**, केरल, भारत
Post Box No.1603, Ernakulam North P.O., Kochi - 682018, Kerala, India
Phone: 91 484 2394867/2391407 Fax: 91 484 2394909/2396685 Email: director@cmfri.org.in Web: www.cmfri.org.in

Dr. K. S. Sobhana, **27 June 2017**
Principal Scientist
Supervising Guide

Certificate

This is to certify that this thesis entitled, **"Establishment and characterisation of cell lines from the caerulean damsel, *Pomacentrus caeruleus*",** is a bonafide record of research work carried out by **Ms. Githa Ann George (Reg. No. 3946)** under my supervision at ICAR-Central Marine Fisheries Research Institute, from December 2010 to June 2017, in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Kochi. This thesis, as a part or whole has not been presented before, for the award of any degree, diploma or associateship in any University. I further certify that all the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and recommended by the Doctoral Committee of the candidate have been incorporated in the thesis.

Dr. K. S. Sobhana

Declaration

I hereby do declare that the thesis entitled, **"Establishment and characterisation of cell lines from the caerulean damsel, *Pomacentrus caeruleus*",** is a genuine record of research work done by me under the supervision of Dr. K. S. Sobhana, Principal Scientist, ICAR-Central Marine Fisheries Research Institute, Kochi, and that no part of this work, has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title of any University or Institution.

Kochi
27th June 2017

Githa Ann George

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Githa Ann George

Dedicated to my Parents...

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Abbreviations

BOLD	:	Barcode of life database
BSA	:	Bovine serum albumin
CMV	:	Cytomegalovirus
DAPI	:	4', 6-diamidino-2-phenylinodole
DMSO	:	Dimethyl sulphoxide
dNTP	:	Deoxyribonucleotides
DPBS	:	Dulbecco's phosphate buffered saline
EC_{50}	:	Half maximal effective concentration
ECP	:	Extracellular products
EDTA	:	Ethylenediaminetetraacetic acid
EGFP	:	Enhanced green fluorescent proteins
FBS	:	Fetal bovine serum
FITC	:	Fluorescein isothiocyanate
FME	:	Fish muscle extract
FU	:	Fluorescent units
NA	:	Nutrient agar
NCBI	:	National Centre for Biotechnology Informatics
PMS	:	Phenazine methosulfate
PC1CpTr	:	Caudal peduncle cell line from *Pomacentrus caeruleus*
PC1L1Tr	:	Liver cell line from *Pomacentrus caeruleus*

PC1F1Ex	:	Fin cell line from *Pomacentrus caeruleus*
SV40	:	Simian virus 40 promoter
TSA	:	Tryptic soy agar
TSB	:	Tryptic soy broth
XTT	:	(2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H- tetrazolium hydroxide, inner salt, sodium salt

Chapter

1

Introduction

Contents

1.1 Background and scope of the study

As the development of animal cell cultures owed much to the needs of two major branches of medical research *viz*., production of antiviral vaccines and understanding of neoplasia (Freshney, 2010), fish cell cultures were initiated to support the growth of fish viruses (Wolf, 1988) for studies in aquatic animal viral diseases. Tissue culture is considered as the gold standard for identification of diseases associated with intracellular bacterial pathogens, and viruses in fishes and fish cell lines have grown tremendously in number covering a wide variety of species and tissues of origin (Palmeiro and Weber, 2010). Fish cell lines are also used in monitoring aquatic pollution and toxicology and in propagating viruses that are etiologic agents of diseases in economically significant species (Wolf and Mann, 1980; Hightower and Renfro, 1988; Bols and Lee, 1991). Cell lines offer the possibility of performing experiments in a controlled environment, independent of the complexity and variability of experiments *in vivo*. Fish cells, like mammalian cells, can be maintained *in vitro* using primary cultures, which are temporary or cell lines,

which are permanent (Bols and Lee, 1991). Early cultures of primary cells may represent a more appropriate model of tissues *in vivo* (Freshney, 2010). The production of short-term primary cultures however, suffers from a lack of reproducibility in the initiation, and homogeneity of cultures that limit their application (Bols and Lee, 1994). Fish cell lines are advantageous to use because they become fairly homogeneous during early passaging, which makes them reproducible, and they have the ability to be cryopreserved, which provides a convenient source of cells (Bols and Lee, 1991).

Although over 283 fish cell lines have been reported in literature (Fryer and Lannan, 1994; Lakra *et al*., 2010c), only 43 are maintained in public repositories (Lakra *et al*., 2010c). With 53,000 species and subspecies of fish, a dearth in the availability of cell lines can be a limiting factor for isolating live virus, as most viruses are species specific and even tissue specific (Palmeiro *et al*., 2010). Fish cell lines have been developed from a wide variety of edible fish to tackle emerging viral diseases, especially regions in the Asian subcontinent have contributed to over 60% of the total fish cell lines since 1994 (Lakra *et al*., 2010c). However, there are only limited attempts on development of continuous cell lines from marine ornamental fishes, particularly damsel fishes. The rapid pace at which aquariculture activities are developing have come at a cost of poorer fish health and increased prevalence of diseases. There are a number of issues that continue to challenge the ornamental fish industry; one of the more pressing issues is enforcement of management regulations and secondly lack of scientific data. Coral reefs are also subject to a variety of natural and induced impacts; attributing to an observed reduction in collection of commercial aquaria fish from reef fish populations. Collection of in-demand species leading to overexploitation and damage of reef ecosystem remain as challenges to the growing demands in the market (Madhu *et al*., 2009).

In light of the above, research was undertaken by the ICAR-Central Marine Fisheries Research Institute (ICAR-CMFRI), Kochi, India with an objective to generate scientific knowledge on ornamental fish maintenance, behaviour, influence of social status on sex change, pair formation, breeding, parental care, egg incubation and hatching, captive rearing of larvae and juveniles including disease control and management aspects. These investigations have led to the development of hatchery technology for 14 species of marine ornamental fishes comprising clown fishes *viz., Amphiprion percula, A. frenatus, A. ocellaris, A. sebae, A. sandaracinos, Premnas biaculeatus;* damsels *viz., Dascyllus trimaculatus, D. aruanus; Pomacentrus caeruleus, P. pavo, Chormis viridis, Chrysiptera unimaculata,* dotty back *Pseudochromis dileclus* and *Neopomacentrus nemurus, N. filamentosus,* (Madhu and Madhu, 2006; 2007; Madhu *et al*., 2006a,b; 2008; 2010; Gopakumar *et al.*, 2007, 2009).

1.2 The caerulean damsel as a donor fish for the development of *in vitro* cell lines

Ornamental fish trade comprises a very large and diverse global industry, with trading in over 4500 species of freshwater fish, 1450 species of marine fish, and over 650 species of corals and other marine invertebrates. More than 50% of the ornamental fish supply originates in Asia. Eighty per cent of these are farm-raised freshwater fish, 5% wild-caught freshwater fish and another 15% wild-caught marine species. Since 1985, the value of the industry has grown by an average of 14% per year, and recent estimates place the value of global live ornamental fish trade at USD 900 million (Oliver, 2001, 2003; Wabnitz *et al.*, 2003; Bartley, 2005; UNEP-WCMC, 2007; Whittington and Chong, 2007). Approximately 98% of marine species are collected from coral reef ecosystems of tropical countries, while the remaining

2% of the fish species are captive raised (Miller-Morgan, 2010). A more prevalent market for ornamental fish is based in the United States, Western Europe and Asia. A genuine increase in ornamental fish keeping has also been reported in Australia, Brazil, Israel and South Africa (Miller-Morgan, 2010).

Growth in the global marine ornamental fish trade may be attributed mainly to the scientific advancements of marine aquarium and reef tank technologies as well as the steady increase in the hobby of maintaining marine fishes in closed spaces. In India, about USD 1.17 million worth of ornamental fishes were traded during 2009-10, contributed mainly by indigenous freshwater species collected from rivers. About 85% of ornamental fish exported from India are from the North-Eastern region, a total of 287 native fish species, 92 exotic fish species and 45 ornamental shrimps were found to be exported from India as ornamental fish (Jayalal and Ramachandran, 2012).

Damsel fishes grouped under the family Pomacentridae constitute one of the major groups of marine ornamental fishes inhabiting the tropical coral seas with over 350 species distributed all over the world (Allen, 1991; Green, 2003; Wabnitz *et al.*, 2003). Caerulean damsel (*Pomacentrus caeruleus*, Quoy and Gaimard, 1825) is a popular coral reef associated (Letourneur, 2004) marine aquarium fish and is native to Western Indian Ocean: East Africa (south to Durban) to Maldives (Allen, 1991). They are non-migratory fishes that inhabit lagoons and outer reef slopes usually over rubble near base of reefs (Lieske and Myers, 1994). Males are about 10 cm in total length (Allen, 1986). Their feeding habitats include selective plankton, mainly feeding on animals (Masuda and Allen, 1993).

The caerulean damsel is a hardy aquarium fish which is easy to handle, but loses its colour in captivity, which may be due to the absence of live feeds

(Ajith Kumar *et al.*, 2008). They are monodomous and protogynous hermaphrodites in which the larger fishes establish territories and finally become males (Gopakumar *et al.*, 2002). The mating system is promiscuous in which both males and females spawn with several mates. Species exhibits *dioecism*. They are oviparous, with distinct pairing during breeding. The eggs adhere to demersal substrates which are externally fertilised. Males were found to guard and aerate the eggs. According to the reproductive guild, they are classified as guarders and nesters (Breder and Rosen, 1966).

Caerulean damsels are easily available, their hardy nature and high adaptability to live in captivity make them an excellent candidate for aquariculture. Just as hatchery technology is required for the sustenance of the ornamental fish trade, likewise methods to prevent and control the outbreak of diseases of infectious and non-infectious etiology must be developed. In order to culture infectious pathogens and develop appropriate diagnostics, cell cultures are essential. Besides diagnostics, they may be used to develop vaccines, which are the most promising tool for preventing viral diseases in fish (Palmeiro and Weber, 2010). The pathobiological activities of bacterial extracellular products *in vivo* can be assessed using cell lines (Magarinos *et al.*, 1992).

Transfection is an essential tool for numerous *in vitro* applications including studies of gene expression and intracellular cell signalling. In comparison with many mammalian cell lines, fish cell lines have not been intensively exploited for genetic manipulations (Villalobos *et al.*, 1999). Apart from the Glofish™ zebra danio (*Danio rerio*), the fluorescent medaka (*Oryzias latipes*), the transgenic convict cichlids (*Amatitlania nigrofasciata*) and angelfish (*Pterophyllum scalare*) that fluoresce (Ng, 2010), there is a dearth in information for marine ornamental fish gene manipulations. Hence,

fish cell line transfection protocols need to be standardised which will significantly benefit the growth of the marine ornamental fish industry.

In this backdrop, the present study was undertaken to derive and characterise *in vitro* continuous cell lines from the caerulean damsel, *Pomacentrus caeruleus* (Quoy and Gaimard, 1825).

1.3 Objectives of the study

- Evaluation of tissues from *Pomacentrus caeruleus* for developing primary cell cultures
- Development of cell lines from primary cultures
- Determination of the optimal growth kinetics of the derived cell lines
- Standardisation of cryopreservation techniques for long term storage of the cell lines
- Characterisation of cell lines by chromosome studies, immunotyping for cell type markers and confirmation of the species of origin using molecular tools
- Transmission electron microscopy (TEM) analysis to check for the presence of contaminating viruses or mycoplasmas
- Evaluation of gene transfection efficiency
- *In vitro* cytotoxicity assays using bacterial extracellular products

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Chapter

2

Review of Literature

Contents

2.1 Animal cell culture

Animal cell culture dates back to the early twentieth century and since that time a multitude of techniques, each designed for the solution of a particular problem, had been devised. The pioneering work of Ross Harrison in 1907 demonstrated that culturing tissues *in vitro* not only kept cells live, but

enabled them to grow as they would *in vivo* by growing frog nerve cells using the 'hanging drop' technique. Alexis Carrel demonstrated the effectiveness of strict aseptic control in enabling the prolonged subculture of cells and used chick embryo extracts mixed with blood plasma to support cell growth (Carrel, 1912). Carrel flasks were designed by Carrel and Ebeling in 1923 which promoted aseptic conditions and paved the way for modern routine cell culture flasks. The discovery of antibiotics by Fleming for which he was awarded the Nobel prize in Physiology or Medicine in the year 1945 was another major milestone that facilitated prolonged cell culture by reducing contamination issues, which facilitated long-term cell line propagation, although many people were already warning against continuous use and the associated risk of harbouring cryptic, or antibiotic-resistant, contaminations (Parker, 1961). Jonas Salk succeeded in developing polio vaccine using polio virus cultured in human embryo cells, the first virus to be grown without using solid animal tissue or eggs (Enders *et al.*, 1949). The development of defined culture media by Morgan *et al*., 1950; Parker *et al*., 1954; Eagle, 1955, 1959; Waymouth, 1959 and also serum-free media (Ham, 1963, 1965) became the turning point where tissue culture was elevated to the status of a routine laboratory tool.

Biochemical and molecular analysis gained impetus with the standardisation of modern tissue culture technology, as the conditions for the maintenance and further applicability was clearly defined. Kohler and Milstein in 1975 produced hybridoma cells capable of the continuous production of a single type of antibody (Kohler and Milstein, 1975). These monoclonal antibodies have been particularly valued as diagnostic and therapeutic agents because of their ability to selectively bind specific compounds. By 2007, the centenary year of tissue culture, such monoclonal antibodies were being commercially produced in multi-kilogram quantities (Freshney, 2010).

2.1.1 Recent advances in animal cell culture

Animal cell culture is an exciting area of research that generates a pronounced avenue to unravel and exploit novel strategies having profound applications (Freshney, 2010). Standardisation and commercialisation of cell culture techniques drew the benefit of reliable media and sera and control of contamination with antibiotics as well as clean-air equipment which have broadened the scope of this field from a laboratory tool to a large scale industry. Large- scale culture applications have led to the manufacture of automated equipment, and today's high-end cell culture robots are able to harvest, determine cell viability and perform all liquid handling. Hence there has been a growing importance for cell-based assays, particularly in the pharmaceutical industry. Tissue culture has also been used for diagnosis and toxicology. The toxic effects of pharmaceutical compounds and potential environmental pollutants can be assayed *in vitro*. Toxicological investigations using cell culture have been extremely useful for clarification of action mechanisms of toxic substances with specialised cell systems; clarification of the effects on basic cell functions mainly with fibroblasts and epithelioid cells and in metabolism investigations (Paganuzzi- Stammati *et al.*, 1981). Developments with human embryonal stem cell cultures (Thomson *et al.*, 1998; Webber and Minger, 2004) has made possible the study of brain or nervous tissue, that will differentiate into neurons and may provide useful and specific models for neuronal diseases (Ebert *et al.*, 2009). The prospect of transplantation of cultured cells has generated a whole new branch of culture, that of tissue engineering (Atala and Lanza, 2002; Vunjak-Novakovic and Freshney, 2006), encompassing the generation of tissue equivalents by organotypic culture, isolation and differentiation of human embryonal stem (ES) cells and adult totipotent stem cells such as mesenchymal stem cells

(MSCs), gene transfer, material sciences, construction and utilisation of bioreactors, and transplantation technology. With the success in human induced pluripotent stem cells (iPSCs), cell culture has far reaching applications as in the production of new disease models and in drug development as well as application in transplantation medicine once technical limitations (for example, mutation through viral integration) are eliminated. In biomedical field, cultured cells are already used routinely for a variety of applications, for example Genzyme's Epicel® (cultured epidermal keratinocyte autografts) for burn patients and Carticel® (cultured autologous chondrocytes) for cartilage repair, as well as at *in vitro* fertilisation (IVF) clinics where the zygote is cultured usually for a few days prior to implantation in the mother's uterus (Clarke and Dillon, 2011). Mario Capecchi, Martin Evans and Oliver Smithies won the Nobel Prize for Physiology and Medicine in 1997 for their work using embryonic stem cells from mice for introducing specific gene modifications (knockout mice). The Nobel Peace prize for physiology and medicine was awarded in 2012 for ground breaking discoveries in stem cells to John B. Gurdon and Shinya Yamanaka. While Gurdon in 1962 introduced the concept of cloning, it was Yamanaka (Takahashi and Yamanaka, 2006, 2007) who discovered that reprogramming can be accomplished by just four specific transcription factors in the egg, the cell could return to its primitive or stem cell form. They demonstrated induction of pluripotent stem cells from mouse embryonic or adult fibroblasts by introducing just four factors, Oct3/4, Sox2, c-myc, and Klf4, under ES cell culture conditions. These iPS cells are capable of differentiating into various cell types, such as beating cardiac muscle cells, neurons and pancreatic cells (Zhou *et al.*, 2009). Cell culture is becoming the next big thing in pharma research, as laboratories rush to meet demand for

stem cell and biologics. The global cell culture market was worth USD 3.4 billion in 2011, and it is expected to grow at a compound annual growth rate (CAGR) of 9.3% between 2011 and 2018 to reach USD 6.3 billion in 2018.

2.2 Fish cell culture

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

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

A major breakthrough in application of fish tissue culture to virology was probably when Schlumberger (1949), used roller tubes and mammalian-type medium in a tissue culture study of a neoplasm from adult goldfish, *Carassius auratus*. The etiology of the tumour could not be concluded with the then available techniques. Soon afterwards, Sanders and Soret (1954) as well

as Soret and Sanders (1954) reported growth of an arthropod-borne virus in intact *Gambusia* embryo grown in mammalian-type medium. Their work marked the first report of a synthetic medium designed for mammalian cells (Medium 199) which was used for teleost tissues.

Grützner (1958) made a major contribution by trypsinisation of fish tissues yielding cultivable cells which grew in to a monolayer. Digestion was done at 20°C or less and solutions were diluted to correspond to the reported freezing point of tench (*Tinca tinca*) serum. Grutzner's monolayers were successfully subcultured, thus contributing to the establishment of fish cell culture. Clem *et al*. (1961) were the first to establish monolayer cell cultures from marine teleosts and obtained best results in commercial medium modified with 0.07M additional NaCl.

RTG-2 cell line of rainbow trout, *Salmo gairdneri* gonad origin initiated in 1960 was the first permanent fish cell line to be developed (Wolf and Quimby, 1962). Clem *et al*. (1961) initiated trypsinised blue-striped grunt, *Haemulon flavolineatum* fin cell cultures which provided the GF-1 cell line, the first line of marine fish origin. The next established line, FHM was initiated in 1962; which was an epithelial-like cell from the fathead minnow, *Pimephales promelas* (Gravell and Malsberger, 1965). Fryer (1964) made a quantitative study of requirements for dispersing tissues and culturing cells from embryonic Pacific salmon and rainbow trout hepatoma and established five different lines of cells from these tissues.

Several cell cultures and cell lines from a variety of fishes have been developed since the first cell line from rainbow trout. A comprehensive list of most fish cell lines developed before 1980 has been published (Wolf and Ahne, 1982). Most of the established cell lines had been derived from cold water fish of European origin (Nicholson, 1988).

Fryer and Lannan (1994) reported on 159 cell lines originating from 74 fish species representing 34 families of fishes. A majority of these cell lines have been established from freshwater or anadromous fish species. The rapid expansion of aquaculture and associated viral diseases in North America, Europe and Japan led to the subsequent development of several fish cell lines for health management purposes (Lannan *et al*., 1984). Hightower and Renfro (1988) reviewed current applications of fish cell lines in the study of intracellular movement of organelles, xenobiotic metabolism, thermal stress, ion transport, endocrinology, immunology and cancer biology. They are, however, mostly used in monitoring aquatic pollution and toxicology, and in propagating viruses that are etiologic agents of disease in economically significant species (Wolf and Mann, 1980; Hightower and Renfro, 1988; Bols and Lee, 1991).

Zhou *et al.* (2008) developed a tail fin cell line from the Chinese sturgeon, *Acipenser sinensis* a cartilaginous ganoid. The species is considered to be a living fossil. The developed cell lines could serve as a model 'living fossil' and used to support research on cellular genetics and cell biology. Yadav *et al.* (2012) developed a caudal fin cell line from the endangered mahseer, *Tor tor* which would open new vistas of *in vitro* research in genetics and conservation of endangered mahseer species.

Five single-cell clone lines were developed from normal primary cultures of rainbow trout pituitary glands and passaged for over 150 times. Karyotype analysis showed that each of these five single-cell clones contain from 59$\pm$1 to 60$\pm$2 chromosome complements. Immunocytochemical analysis with monospecific antisera of Gh and Prl showed that cells of all the five single-cell clones produced both Gh and Prl, pituitary hormones. This observation was further supported by results of RT-PCR analysis that mRNAs

of gh, prl, tsh, gth-1, gth-2, sl, pomcb, cr and rtE2Ra genes were detected in each of these five single-cell clones. The expression of gh and prl genes in the single-cell clone lines is responsive to induction by E2, dexamethasone, and o, p'–dichlorodiphenyltrichloroethane (Chen *et al.*, 2010).

Three ES cell lines MES1 to MES3 from medaka, *Oryzias latipes* were established in 1996 using bFGF, a potent mitogen for embryonic stem cells using feeder-free culture conditions (Hong *et al*., 1996). Since ES cells have been derived from both mouse (advanced) and medaka (primitive), it has provided the possibility of deriving ES cells from the broad vertebrate species. MES1 was further characterised and revealed that they include stable growth, high alkaline phosphatase activity, a typical ES cell phenotype (a small size, a round/polygonal shape, large nuclei and prominent nucleoli), a normal karyotype and the ability for spontaneous differentiation into various cell types including pigment cells, muscle cells, nerve cells and fibroblasts (Hong *et al.*, 1996). ES cell clones have been formed from compacted cell colonies of undifferentiated ES cells in MES1 (Hong *et al.*, 2011). Wang *et al.* (2011) identified seven medaka pluripotency genes by homology search and expression *in vivo* and *in vitro*. The genes were categorised based on their expression pattern by carrying out RT-PCR. Gonad specific expression was elicited by *nanog* and *oct4* genes, gonad preferential expression by *sall4* and *zfp281* and the genes *klf4, ronin* and *tcf3* expressed in several somatic tissues apart from the gonads. The genes are maternally inherited, with high expression during early embryogenesis. Pluripotent markers, *nanog* and *oct4* are highly expressed in pluripotent blastomeres of 16-cell embryos. In the adult testis, *nanog* expression was specific to spermatogonia, the germ stem cells, whereas *tcf3* expression occurred in spermatogonia and differentiated cells. These genes have been uniquely identified as pluripotency markers *in vitro* due to their high levels of

expression in undifferentiated ES cells but dramatic down-regulation upon differentiation (Wang *et al.,* 2011). Pluripotent embryonic stem cells (ES) provide an efficient approach for genome manipulation with many applications in marine biotechnology and developmental studies. The application of feeder-free culture system has led to the establishment of ES-like cells in several marine fish species, including the zebrafish, *Danio rerio* (Sun *et al.*, 1995), gilthead seabream, *Sparus aurata* (Béjar *et al.*, 2002), red seabream, *Pagrus major* (Chen *et al.*, 2003), seaperch, *Lateolabrax japonicas* (Chen *et al.*, 2007), Asian seabass, *Lates calcarifer* (Parameswaran *et al.*, 2007), Atlantic cod, *Gadus morhua* (Holen *et al.*, 2010) and carp, *Labeo rohita* (Goswami *et al*., 2012a). Embryonic stem (ES) cells can differentiate to become any tissue in the body (Hong *et al*., 2000). Successful protocols for grafting of embryonic cells to host embryos, for germline transmission of desired genome, can be instrumental in evolving effective programmes for production of transgenics and rehabilitation of endangered species (Diwan *et al.*, 2010).

Aquaculture production presently is heavily dominated by China and other developing countries in the Asia-Pacific region which accounts for 89% by volume of global production and 77% by value (FAO, 2008), which substantiates the findings reported by Lakra *et al*. (2010c) that about 125 teleost fish cell lines have been developed since 1994 with more than 60% of the cell lines being established from the Asian region. The largest number of marine fish cell lines was derived from groupers (Osteichthyes: Serranidae). The species from the genus *Epinephelus* account for roughly 30% of the marine fish cell lines developed since 1994 (Parameswaran *et al.*, 2007; Sobhana *et al.*, 2008, 2009, 2011b; Ku *et al*., 2009; Wei *et al*., 2009; Lakra *et al.*, 2010 c; Ou-yang *et al.*, 2010; Gong *et al.*, 2011; Huang *et al*., 2011; Lei *et al*., 2012; Swaminathan *et al*., 2012a), the highest taxonomic representation overall.

2.3 Fish cell culture in India

Fish tissue culture has advanced in a very short span of time from an esoteric art to a workaday tool of many uses. A few pioneering works in India include, a cell line (MG-3) from gills of mrigal, *Cirrhinus mrigala* and a cell line (RG-1) from gills of rohu, *L. rohita* (Sathe *et al*., 1995; 1997), both cell lines were characterised with respect to isoenzyme pattern and chromosome number. The profile of three isoenzymes were tested (*viz.*, lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and glucose 6 phosphate dehydrogenase (G6PDH) and RG-1 cell line only differed from MG-3 cell line in mobility pattern of G6PDH. Both cell lines crossed 20 passages and had a chromosome number of 50.

Primary cultures from the kidney of *Heteropneustes fossilis* (Singh *et al.*, 1995), larvae of *Poecilia reticulata* (Kumar *et al*., 1998), caudal fin of *Tor putitora* (Prasanna *et al*., 2000), caudal fin of rohu (Lakra and Bhonde, 1996), heart tissues of catla, rohu and mrigal (Rao *et al.*, 1997), ovary of *Clarias gariepinus* (Kumar *et al*., 2001), kidney of *Siganus canaliculatus* (Ravi, 2007), fin tissues of *L. rohita* (Rathore *et al.*, 2007) and gill of *Epinephelus malabaricus* (Sobhana *et al.*, 2009) have also been reported.

A growth medium with Leibovitz's L-15 as the base, supplemented with fetal bovine serum (FBS) (10% v / v) , fish muscle extract (10% v/ v) , prawn muscle extract (10% v/v), lectin (concanavalin A, 0.02 μg mL^{-1}), lipopolysaccharide (0.02 μg mL^{-1}), glucose D (0.2 μg mL^{-1}), ovary extract (0.5% v/v) and prawn haemolymph 0.5% has been formulated with 354±10 mOsm for the development and maintenance of a cell culture system from the ovarian tissue of African catfish, *C. gariepinu*s (Kumar *et al*., 2001). The cell culture was passaged 15 times after which they ceased to multiply and

consequently perished. The cell cultures were maintained for 12–15 days without fluid change between the passages.

Successful development of fish cell lines or cell culture systems have been achieved from a few freshwater teleosts. Lakra *et al.* (2006a) developed a new fibroblast like cell line from the fry of golden mahseer, *T. putitora* (Ham). A few other cell lines established include those derived from eye muscle, brain, heart, macrophage and thymus tissues of catla, *Catla catla* (Ishaq Ahmed *et al.*, 2008, 2009a, b; Chaudhary *et al.*, 2012 a, b; Chaudhary *et al*., 2013), liver, kidney and eye tissues of rohu, *L. rohita* (Lakra *et al.*, 2005; Ishaq Ahmed *et al.*, 2009b), fin and heart tissues of common carp, *Cyprinus carpio* (Lakra *et al*., 2010a), caudal fin, eye, gill, kidney and brain tissues of the green chromide, *Etroplus suratensis* (Swaminathan *et al.*, 2010; Babu *et al*., 2012) and fin tissue of the Indian walking catfish, *Clarias batrachus* (Babu *et al.*, 2011).

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

passage numbers 5, 8, 15 and 18. The cells were characterised for chromosome number (2n=100) at 10 and 20 passages. The cell cycle analysis by FACS (Fluorescence Activated Cell Sorter) revealed that most of the cells on the first and fourth day of culture were in S-phase, indicating a high growth rate.

C. catla cell lines have been developed from eye muscle (SICE) (Ishaq Ahmed *et al.*, 2008) which showed strong positive reaction to epithelial markers *viz.*, pancytokeratin, cytokeratin 19 and the proliferative marker Ki67. The cell cycle analysis by fluorescence-activated cell sorter revealed that most of the cells on the second day of culture were in S-phase, indicating a high growth rate. Similarly, catla cell lines have also been developed from brain (Ishaq Ahmed *et al*., 2009a), heart muscle (Ishaq Ahmed *et al*., 2009b), adherent peripheral blood mononuclear cells (macrophage cell line) Chaudhary *et al*., 2012a), thymus (Chaudhary *et al*., 2012b) and an epithelial cell line (Chaudhary *et al.*, 2013).

2.4 Cell lines from marine fishes

Fish are cultured throughout the world for conservation, restoration, harvest, recreation, food, ornamental markets and scientific research. Fish cell cultures gained impetus due to the rapidly developing mariculture activities world over which led to outbreak of viral diseases that has become a serious issue causing heavy economic losses (Seng and Colorni, 2002). Permissive cell cultures are progressing as viral diagnostic tools, to isolate, propagate and assay infectious viruses, and to prepare and clone attenuated virus strains for antiviral vaccination. Moreover, many new continuous cell cultures are constantly being developed as a result of intensive efforts in several parts of the world, to provide cell cultures from local species utilised in aquaculture (Fernandez *et al.*, 1993 a, b; Chang *et al*., 2001; Lai *et al*., 2003). Since cell

cultures derived from the same species or a species closely related to that in which the disease occurs would be the most sensitive for virus isolation, cell lines derived from local species are given high priority. The host and tissue specificity of virus underlines the need for developing cell lines from different species in different regions (Cheng *et al*., 1993). Cell lines also offer the advantage of allowing problems to be investigated quickly and inexpensively at the molecular and cellular levels in order to gain information that can be used to design the most efficient *in vivo* studies of the problems (Kawano *et al.*, 2011).

Three continuous cell lines have been established from gonads of Japanese striped knife jaw, *Oplegnathus fasciatus* (JSKG cell line), embryos of a hybrid of kelp grouper (*Epinephelus moara*), red spotted grouper, *E. akaara* (KRE cell line) and skin of greater amberjack (also called purplish amberjack) *Seriola dumerili* (PAS cell line) (Fernandez *et al.*, 1993 b). JSKG and PAS cell lines exhibited optimal growth response in Eagle's minimum essential medium (Eagle's MEM) buffered with a combination of tris and sodium bicarbonate. These cell lines were initiated at a higher NaCl concentration of 0.206 M but gradually adapted to a low NaCl concentration of 0.116 M after several subcultures. Optimum growth temperatures were 25°C for JSKG and PAS cells and 30°C for KRE cells. The modal chromosome number is 83 for the JSKG cell line, 92 for the KRE cell line and 96 for the PAS cell line. Results of plating efficiency indicated that all three cell lines were composed of transformed cells. JSKG and PAS are susceptible to nine fish viruses including channel catfish virus (CCV) and chum salmon virus (CSV). The KRE cell line is susceptible to CCV and rhabdoviruses of the vesiculovirus group.

Faisal (1995) designed a study to identify the optimal conditions for spot (*Leiostomus xanthurus*) liver cell survival and propagation *in vitro.* Liver tissues were digested by trypsin disaggregation. The isolated liver cells attached to the substrate within 2-6 h and formed confluent monolayers by 6^{th}-8^{th} day of culture. Intercellular junctions were frequently observed between neighbouring hepatocytes. The best results were obtained when the cells were seeded at a density of 1 x $l0^6$cells per 25 cm^2 flask. RPMI-1640 and L-15 media supported cellular growth in the presence or absence of 5% CO_2. Cultured spot liver cells formed a monolayer over a wide range of osmolalities (*i.e*., 295 to 355 mOsm kg^{-1}). The cells were able to synthesise DNA, RNA, and proteins. Coral tree extract, concanavalin A, and bacterial lipopolysaccharides augmented DNA synthesis in cultured liver cells. Subculturing was possible and three continuous cultures (designated as SLW, SLN, and SLF) with the potential to become immortal cell lines were developed.

A continuous cell line (SAF-1) was developed from fin tissues of an adult gilt head seabream, *Sparus aurata* (Béjar *et al*., 1997). The cells grew in DMEM-F12 basal medium supplemented with 5% FBS at the optimal temperature of 25°C. They exhibited fibroblast-like morphology, high plating efficiency, doubling time of 2 days and high survival after storage in liquid nitrogen. Karyotype and DNA content of the cell line revealed a mostly euploid and rather stable cell population. This cell line has been shown to be susceptible to several fish viruses and to bacterial extra cellular products.

The tropical marine food fish, the Asian seabass, *Lates calcarifer*, has been used extensively to develop cell cultures. Chang *et al.* (2001) developed a cell line from seabass fry (SF cell line) which was maintained in Earle's minimum essential medium (EMEM) supplemented with FCS and incubated at 25°C. To assess viral replication efficiency, SF cell line was inoculated with

iridoviruses, birnaviruses, reoviruses, a rhabdovirus, and a nodavirus; were found to be susceptible with high titres of GRV, IPNV, LDV and ThRV and smaller titres were observed of GIV and GNV. Cytopathic effect (CPE) was also observed daily after virus inoculation. SF cell cultures infected with an iridovirus, a nodavirus and a reovirus were further elucidated by electron microscopy. All the viruses tested were shown to induce CPE on SF cells. The SF cell line consists predominantly of epithelial-like cells. Sahul Hameed *et al*. (2006) established and characterised India's first marine fish cell line (SISK) from kidney of seabass, *L. calcarifer.* The cell line was subcultured more than 100 times over a period of 2 years. The epithelial-like cell line was found to be strongly positive for the epithelial markers: cytokeratin 19 and pancytokeratin. The cells were able to grow at temperatures between 25°C and 32°C with an optimum temperature of 28°C. Microsatellite markers with loci LCAM03, LCAM38 and LCAM21 were used to confirm the species of origin. The cell line was found to be susceptible to fish nodavirus (VNN) and marine birnavirus – Nc1 (Mabv Nc-1). A continuous cell line was obtained from the blastula stage embryo of the seabass, *L. calcarifer* and grown in Leibovitz's L-15 with 15% FBS. The average viability of the cells after recovery from cryostorage was found to be 70- 80%. Cells were transfected with pEGFP-N1 plasmid using lipofectamine 2000 and a transfection efficiency of about 1/5000 cells was obtained within 12h post-incubation. The cells were found to be susceptible to infectious pancreatic necrosis virus (IPNV VR-299) and nodavirus, and the infection was confirmed by CPE, RT-PCR and electron microscopy. The cells of the embryonic cell line were found strongly positive for cytokeratin 19 (Parameswaran *et al*., 2006a).

Two cell culture systems of epithelioid cells (LCE) and fibroblastic cells (LCF) were also developed from fry and fingerlings of *L. calcarifer*

(Lakra *et al.*, 2006b). A brain cell line designated ASBB was developed from the Asian seabass (Hasoon *et al.*, 2011). The average viability of the cells after cryopreservation was estimated to be about 90%. The modal chromosome number was 48. Cells of ASBB were found to be susceptible to marine fish nodavirus. Swim bladder and muscle cell lines were developed from the tissues of *L. calcarifer* (Lai *et al.*, 2008). The cell lines were subcultured over 100 times. The cells showed 95% viability on recovery from cryogenic storage. Apoptosis was clearly induced in both cell lines when treated with grouper iridovirus (GIV), as demonstrated by DNA fragmentation and chromatin condensation. Apoptosis was confirmed by DNA ladder and TUNEL (terminal uridine deoxy-nucleotidyl transferase dUTP nick end labelling) assays, the formation of chromatin condensation and apoptotic bodies was observed by Hoechst nuclear staining using the fluorescent dye Hoechst 33258 and the presence of phosphotidyl-serine (PS) on the plasma membrane of apoptotic cells was assayed by staining with annexin V. A brain cell line, SBB-W1, was also developed from the adult seabass, *Dicentrarchus labrax* (Servili *et. al.*, 2009). These long term cell cultures were grown in suspension as neurospheres that were immunopositive for nestin, a marker for neural stem cells, or grown as adherent monolayers displaying both glial and neural morphologies. Immunostaining with anti-glial fibrillary acidic protein (a glial marker) and anti-neurofilament (a neuronal marker), yielded positive staining in most cells. Sox 2, a marker for neural stem cells, could be detected from these cell extracts as well as proliferating cell nuclear antigen, a marker for proliferating cells. SBB-W1 could be transfected using pEGFP-N1 indicating their viability and suitability as convenient models for neurophysiological or neurotoxicological studies.

Kang *et al*. (2003) established and characterised two cell lines, FFN cells from the fin tissue and FSP from the spleen tissue of the flounder, *Paralichthys olivaceus*. The cells multiplied well in Eagle's MEM supplemented with 10% FBS at an optimal growth temperature of 25°C, and consisted of epithelioid cells. Heteroploid chromosome number of 64 and 62 was observed in FFN and FSP cells respectively. Both the cell lines were found susceptible to a wide range of fish viruses such as IPNV, marine birna virus, chum salmon virus, infectious haematopoietic necrosis virus (IHNV), spring viraemia of carp virus (SVCV) and hirame rhabdovirus.

In 2004, Pombinho *et al.* developed and characterised two new cell lines, designated VSa13 and VSa16, derived from the vertebra of the gilthead seabream which were capable of mineralising *in vitro* and of expressing genes found in chondrocyte and osteoblast cell lineages. Scanning electron microscopy and von Kossa staining confirmed that VSa13 and VSa16 mineralised their extracellular matrix forming calcium-phosphate crystals when cultured in DMEM. Cell lines expressed alkaline phosphatase and Gla proteins (osteocalcin and matrix Gla protein, MGP) in the process of mineralisation. VSa13 and VSa16 cell lines also expressed osteocalcin and MGP in a mutually exclusive manner.

A continuous cell culture was derived from the tail fin of red seabream, *Pagrus major*, mandarin fish fry (MFF-1) and had been subcultured over 60 passages during a period of 18 months (Imajoh *et al*., 2007). MFF-1 consisted of predominantly epithelial-like cells and was grown in DMEM supplemented with 10% FBS. MFF-1 could produce high titres of Infectious spleen and kidney necrosis virus (ISKNV) by continuous viral passages which were further confirmed by MTT, indirect immunofluorescence assay, QC-RT-PCR analysis. Flow cytometry analysis showed that approximately 80% cells could

be infected by ISKNV at 3 days post-infection. Abundant ISKNV particles were observed in the cytoplasm of the ISKNV infected MFF-1 cells by transmission electron microscopy. In addition, apoptosis was observed in the MFF-1 cells upon ISKNV infection by FITC- annexin V staining.

2.5 Ornamental fish cell lines

Clem *et al.* (1961) initiated cell cultures from trypsinised fin tissues of the blue-striped grunt, *Haemulon flavolineatum* which provided GF-1 cells, the first line of marine fish origin which is also coincidentally the first ornamental fish cell line. Hu and Chavin (1960) confirmed the positive impact of adrenocorticotropic hormone (ACTH) in hormonal stimulation of melanogenesis by employing goldfish fin explant cultures (Wolf and Quimby, 1969).

Xing *et* al. (2009) developed a zebrafish blastula stage embryo cell line using RTS34st as a feeder layer to develop and maintain the cell cultures through initial passages. ZEB2 was then grown for 2 years without feeders in L-15 containing 15% FBS for 120 population doublings. ZEB2J was heteroploid, had detectable telomerase activity, and was adherent. After growing into monolayers, some cells continued to grow into mounds. Cultures expressed *Pou-2* mRNA and contained many alkaline phosphatase and a few stage-specific embryonic antigen-1-positive cells. In dishes coated with a phospholipid polymer (2-methacryloxyloxyethyl phosphorylcholine, MPC), ZEB2J formed spherical aggregates (Xing *et al.*, 2008). Xing *et al.* (2009) developed a spleen stromal cell line, ZSSJ with the aim to act as feeder layer for zebrafish embryonic cell line, ZEB2J. Ghosh *et al.* (1994) developed a zebrafish liver cell line (ZF-L) which was hypodiploid and exhibited epithelial morphology. ZF-L cell homogenates exhibited alanine and aspartate

aminotransferase, glucose-6- phosphatase and alkaline phosphatase enzyme activities.

Goldfish (*Carassius auratus*) is a eurythermal temperate fish that survives at higher temperatures and a popular ornamental fish. Goldfish cell lines were developed from fin (Wolf and Mann, 1980, ATCC No. CCL-71, Mauger *et al*., 2006), skin (Lee *et al*., 1997), scales (Akimoto *et al*., 2000), muscle and swim bladder (Rougee *et al.*, 2007) as well as caudal fin (Ippei *et al*., 1979; Kondo *et al*., 2004). Cell lines were transfected with pEGFP-N1 vector DNA using TransIT-LT1 transfection reagent and a cytomegalovirus promoter and fluorescent signals were observed within 24h of incubation, suggesting that GFTF cells could be a useful tool for transgenic and genetic manipulation studies (Yan *et al*., 2011). Permanent cell lines, GEM-81 and GEM-218, from spontaneous erythrophoromas (tumours derived from red pigment cells or erythrophores) of goldfish (Matsumoto *et al*., 1980) were found to have stem cells, melanoblastomas, which are capable of melanogenesis. These cells were induced to melanise by cultivation in autologous serum (Matsumoto *et al*., 1984).

Wang *et al*. (1995) and Belosevic *et al*. (2006) developed goldfish macrophage cell lines, GMCL and PKM from goldfish kidney hematopoietic tissues respectively. GMCL cell lines were stored by cryopreservation at 80˚C in medium containing 10% DMSO, or at 4˚C for more than 12 months. Virtually all (>99% of macrophages) stained positive for non-specific esterase. Long-term cultured macrophages engulfed sheep red blood cells and both amastigotes and pro-mastigotes of *Leishmania major*. After stimulation with phorbol esters (PMA) and/or lipopolysaccharide (LPS), cultured fish macrophages produced reactive oxygen and nitrogen intermediates. Macrophage hematopoiesis of primary kidney macrophage (PKM) cultures

from goldfish kidney tissues indicated that three distinct subpopulations developed in response to endogenous macrophage growth factors. These macrophage subpopulations expressed several differentiation markers, including the hematopoietic stem cell antigen AC133, c-kit, granulin, CD63, macrosialin, c/EBPb, legumain, and the colony-stimulating factor receptor-1 (CSF-1R). Macrophage developmental pathways were elucidated using PKM cell lines, that showed distinct differentiation pathways: one consistent with the ‘‘classical’’ pathway (MPS) of macrophage development (progenitors/ monocytes/mature macrophages), and an ‘‘alternate’’ pathway (AP-macrophages) where mature macrophages appeared to rapidly develop from early progenitors in the absence of an intermediate monocyte stage.

Grist *et al.* (1986) had surveyed DNA repair activity in a number of lower vertebrate tissues and cell lines including fat head minnow, *Pimephales promelas* cell lines, GEM 218 and GEM 81 from goldfish erythrophoromas, and the goldfish fibroblastic lines RBCF-1. They found that fish cell cultures retained O^6-methylguanine acceptor activity and the ability to remove pyrimidine dimers caused by exposure to ultraviolet radiation. They concluded that cells from lower vertebrates are good *in vitro* models for studies of DNA repair function.

Cell lines have also been developed from embryo of mosquitofish *Gambusia affinis,* whole embryo of guppy *Poecilia reticulata,* green swordtail *Xiphophorus helleri,* southern platyfish *Xiphophorus maculatus* and from peduncle of golden gourami *Trichogaster trichopterus* (Wolf and Mann, 1980). SGP cell line was established from the muscle tissue of snakehead gourami *Trichogaster pectoralis.* Similarly from the golden gourami *Trichogaster trichopterus* muscle tissue, GP cell line was obtained (Fryer and Lannan, 1994).

A few cell lines were obtained from the tropical freshwater ornamental fishes. Karunasagar *et al.* (1995) developed a cell line that was sensitive to the snakehead fish cell line C-type retrovirus. In 2011, Lakra *et al*. developed cell lines from the caudal peduncle (PDF cell line) and heart (PDH cell line) of *Puntius denisonii*, which showed optimal growth at 26˚C in Leibovitz's L-15 medium, supplemented with 10% FBS and had a diploid chromosome number of 50. The cell lines PDF and PDH showed a population doubling time of 28 and 30h at the 50^{th} passage. After cryogenic storage for a period of 4 months, 70 and 76% viability was obtained respectively for PDF and PDH cell lines respectively. Confirmation of the origin of the cell line was carried out by amplification of cytochrome c oxidase subunit I of mitochondrial DNA genes (Lakra *et al*., 2011). A continuous caudal fin cell line was developed from *Puntius sophore* (Lakra *et al*., 2011) which was successfully transfected with green fluorescent protein (GFP) reporter plasmid (pEGFP-C1) driven by cytomegalovirus promoter gene and expression was detected as early as 16h and estimated transfection efficiency was 10%. Swaminathan *et al*. (2012b) assessed the potential of *P. denisonii* tissues (fin, heart and swim bladder) for development into cell lines. Of these a fibroblast-like fin cell line was found to be sensitive to bacterial extracellular products from *Vibrio cholera* MTCC3904 and *Aeromonas hydrophila*. Similarly, Goswami *et al.* (2012b) developed and characterised PCE cell line from the eye tissues of *Puntius* (*Tor*) *chelynoides* and the cell lines were found to have high plating efficiency, and species of origin was confirmed using both 16S rRNA as well as CO1 gene.

Cell lines have also been established from marine ornamental fish species. Two clonal cell lines each from four different tissues of *Amphiprion sebae viz*., spleen (CFSP and CFSP2), brain (CFBR and CFBR4), caudal peduncle (CFCP and CFCP2) and fin (CFFN and CFFN2). The eight cell lines

have crossed 70 passages. The optimum temperature for growth of the cells was found to be 28°C. The cells were found to express GFP indicating that the cell lines could be used for transfection studies. Sequence homology of the cell lines were carried out using mitochondrial 16S rRNA (Mulloorpeedikayil *et al.*, 2011). Tissues from *Amphiprion percula* were evaluated for their potential to develop cell lines by explant as well as trypsinisation method (Sreedevi *et al.*, 2011). Gill, fin and caudal peduncle tissues were found to have better potential for attachment, growth, multiplication, formation of confluent monolayers and successful subculturing. All the three cell culture systems derived were successfully cryopreserved and revived after 6 months of storage in liquid nitrogen with 70% recovery of cells (Sreedevi *et al.*, 2011). Sobhana *et al.* (2011a) developed cell lines from fin and caudal peduncle tissues *viz*., DT1F4Ex, DT1CpEx and DT1CpTr from the three-spot damsel, *Dascyllus trimaculatus*. The cultures were fast growing lines that required only 2% FBS in Leibovitz's L-15 medium. Chromosome analysis was carried out and the modal chromosome number was 48. The cultures were cryopreserved and successfully revived after two years in storage.

2.6 Methods in cell culture

Stages of cell culture comprises: (1) acquisition of the sample, (2) isolation of the tissue, (3) dissection and/or disaggregation, and (4) culture after seeding into the tissue culture vessel. After isolation, a primary cell culture may be obtained either by allowing cells to migrate out from fragments of tissue adhering to a suitable substrate or by disaggregating the tissue mechanically or enzymatically to produce a suspension of cells, some of which will ultimately attach to the substrate. Mechanical and enzymatic disaggregation of the tissue avoids problems of selection by migration and yields a higher number of cells that are more representative of the whole tissue

in a shorter time. However, just as the primary explant technique selects on the basis of cell migration, dissociation techniques will select protease- and mechanical stress-resistant cells (Freshney, 2010).

2.6.1 Culture vessels and substrates

Most normal cells need to spread out on a substrate to proliferate (Folkman and Moscona, 1978; Ireland *et al.*, 1989; Danen and Yamada, 2001; Frame and Norman, 2008; Zhang *et al.*, 2008), and inadequate spreading due to poor adhesion or overcrowding will inhibit proliferation. Cells shown to require attachment for growth are said to be anchorage dependent; cells that have undergone transformation frequently become anchorage independent and can grow in suspension (Freshney, 2010). For the routine growth of piscine cell lines, non-vented flasks are favoured because the loss of medium through evaporation is less. Most plastic vessels are treated to promote animal cell attachment and proliferation, but some have hydrophobic surfaces to keep the cells in suspension. Fish cells also attach to glass Petri dishes. However, salmonid cell lines have been found to spread poorly in Lab Tek chamber slides (Nunc) with a glass surface, but do spread well when these slides have a plastic Permanox surface (Bols *et al*., 2011).

Laminin has been used for the attachment and spreading of carp myosatellite cells (Koumans *et al.*, 1990); gelatin, for trout myosatellite cells (Powell *et al.*, 1989). Hong *et al.* (1996) and Goswami *et al.* (2012a) developed a feeder cell-free culture condition under which mid-blastula embryos (MBE) cells were grown on gelatin-coated surface. Primary cultures from the corpuscles of Stannius (Gellersen *et al.*, 1988; Wagner *et al.*, 1989) and the testis of rainbow trout (Loir, 1988) have been prepared respectively on a commercial extracellular matrix, Biomatrix, and on

fibronectin. The synthetic polypeptide, poly-D-lysine, has been coated onto Petri dishes for primary cultures of rainbow trout oligodendrocytes (Jeserich and Rauen, 1990) and astrocytes (Tocher and Wilson, 1990). For primary cultures of rainbow trout hepatocytes, a trout skin extract was utilised to obtain their firm attachment (Blair *et al*., 1990) and trout serum promoted their attachment and spreading onto type I collagen (Kocal *et al*., 1988). Matrigel was found to be detrimental to the development of *Salmo salar* fibroblastic cells and epithelial cells. Type I collagen improved the growth of *S. salar* epithelial cells but showed no pronounced effects on the fibroblast line (Butler and Nowak, 2004). Fin and heart of *Epinephelus fuscoguttatus* (Forsskål) cell line growth medium were supplemented with carboxy-methyl-chitooligosaccharide (Wei *et al*., 2009; 2010). Carboxymethyl chitosan (a kind of chitosan derivative) and N-acetylglucosamine hydrochloride (an acetyl form of glucosamine hydrochloride) were both found to have a positive effect on cell attachment and growth of *Penaeus chinensis* (Fan and Wang, 2002).

2.6.2 Physiological salines

A balanced salt solution (BSS) is composed of inorganic salts and may include sodium bicarbonate and, in some cases, glucose. Physiological salt solutions are essential in cell and tissue culture as economical solutions to keep the cells or tissues in live condition for a short period of time. They maintain the required pH, osmotic pressure and provide essential ions. They are used for washing, handling, manipulating and short-term holding of living cells. In addition, they are the inorganic foundation on which media are formulated.

Presently, wide arrays of physiological saline or balanced salt solutions are available. In fish culture, the available salt solutions *viz.*, Earle's balanced

salt solution (BSS), Hanks BSS and phosphate buffered saline (PBS) have been widely used (Wolf and Quimby, 1969). BSS forms the basis of many complete media and provides the necessary inorganic salts (Freshney, 2010). PBS is a common choice in primary isolations (Meguro *et al.*, 1991; Lai *et al.*, 2001; Chang *et al.*, 2001). PBS is also used regularly in the subculture of established fish cell lines such as SISK (Sahul Hameed *et al.*, 2006) to rinse cultures before passage.

Glucose, $NaHCO_3$, NaCl, calcium, magnesium or phosphate ions may sometimes be added to BSS, depending on the requirement. Salines may also be buffered with tris (hydroxymethyl) -aminomethane – hydrochloride (TRIS), N – 2- hydroxyethylpiperazine – N – 2' – ethanesulfonic acid (HEPES), N Tris (hydroxymethyl) methyl – 2 – amino ethanesulfonic acid (TES) or N, N – bis (2- hydroxyethyl) – 2 – aminoethane sulfonic acid (BES) (Eagle, 1971).

Hank's BSS is one of the widely used saline, which contains a small amount of sodium bicarbonate plus a phosphate buffer system, and exposure to air does not induce high pH values as quickly as it does in Earle's BSS. Dulbecco's phosphate buffered saline (DPBS) is similar to Hank's BSS but bicarbonate is omitted in the former and the levels of Na_2HPO_4 and KH_2PO_4 are increased to provide more buffering capacity. Saline solutions are usually prepared at ten times concentration in distilled water and stored in refrigerated temperature. When required, the stock solution is diluted with distilled water and autoclaved. At this stage bicarbonate and glucose can be added.

The concentration of saline used should be isosmotic with body fluid of animal from which the tissue is excised. Some papers have reported that, for cell cultures from marine organisms, additional NaCl is added. Clem *et al.* (1961) used both unmodified Hanks' and preparations which were

supplemented with 0.07 M additional NaCl. They reported significantly better results with the latter and established a permanent marine teleost cell line. Fernendez *et al.* (1993b) presented information on NaCl concentrations for freshwater fish showing that although high NaCl concentrations were used for establishment these levels were not required in successive passages. Fernendez *et al.* (1993a) also demonstrated optimum growth at lower NaCl concentrations for marine fish. Most cell lines do not rely on NaCl to be added to the media. Mammalian-type physiological salines can be adjusted to near-isotonic marine teleost osmolarity by adding 0.07M NaCl (conveniently with 20.6 ml of a 3.4 M (20% w/v) stock solution per litre (Wolf and Ahne, 1982). Salines used for elasmobranchs are supplemented with NaCl and urea (0.2 to 0.33M) (Wolf and Quimby, 1969).

2.6.3 Culture media and additives

The media developed for mammalian cell cultures has been found to be appropriate for the culture of fish cells as well. The complete medium for fish cell growth, as with mammalian cells, has two parts: a basal medium and a supplement. A basal medium is an aqueous solution of nutrients and buffering agents that contains a hexose, bulk ions, trace elements, amino acids and vitamins (Ham, 1984). Basal media that have been developed for mammalian cells have been successfully used for piscine cells (Wolf and Ahne, 1982; Nicholson, 1989). These include Medium 199, Eagle's MEM, RPMI-1640, L-15, F12 and others. The requirements for growth are satisfied by the basal medium and the appropriate hormones and growth factors, which are added as either defined or undefined supplements (Bols and Lee, 1991).

Earlier, Eagle's MEM had been the preferred choice of most fish tissue culturists (Fryer *et al*., 1965). Eagle's MEM supplemented with FBS comes

close to being an all-purpose culture medium for the cells of mammals, birds, reptiles, amphibians and of course fish (Wolf and Quimby, 1969). MEM was found to be best suited for fish cell lines when they compared the growth responses of 28 fish cell lines in different media at various temperatures and sodium chloride concentrations (Fernandez *et al.*, 1993a). Lakra *et al.* (2010c) concluded that an amino acid-rich nutrient medium such as L-15 that does not require CO_2 buffering (Leibovitz, 1963) has been successfully used with fish cell lines and due to this advantage, more than 80% of the cell lines established after 1994 used Leibovitz's L-15 media. However, some primary cell lines have had specific culture medium designed to optimise growth during development of the primary culture (Wang *et al*., 1995).

Serum contains growth factors, which promote cell proliferation, adhesion factors and antitrypsin activity, which promote cell attachment. Serum is also a source of minerals, lipids, and hormones, many of which may be bound to protein. The sera used most in tissue culture are fetal bovine serum (FBS) and calf (CS) serum (Freshney, 2010). Bradford *et al.* (1994) described that primary cell cultures need higher serum concentrations and seeding densities than cells in later passages. When used with synthetic media, the usual level of serum is 10-15%. Some fish cells will grow with as little as 2% serum (Jensen *et al*., 2012). Some have found it necessary to use as much as 20 or even 30% serum levels (Bryson *et al*., 2006; Lakra *et al.*, 2006). Fish serum was used (>1%) in combination with FBS in developing fish cell lines (Chen *et al*., 2004; Lakra *et al*., 2006a). Five per cent human serum was included along with FBS to develop the CyN cell line from *Cynoscion nebulosus* (Middlebrooks *et al.*, 1979). The widely used cell line RTG-2 was successfully adapted to a serum-free media (Kohlpoth and Rusche, 1997). Homologous fish serum or serum from species belonging to the same

evolutionary branch has desirable effects on attachment, differentiation and growth promotion than heterologous sera (Hashimoto *et al.*, 1997). There are several reports showing fish serum to be excellent (Babini *et al.*, 1961; Kunst, 1961; Tomasec *et al.*, 1964; Stephenson and Potter, 1967). Chou *et al.* (1989) used 10% DMSO and autologous serum for freezing melanised goldfish erythrophoroma cell line. The paper also highlighted the importance and desirability of using autologous serum for the cryopreservation of sensitive cell lines and further suggested that it may be able to prevent cells from undergoing spontaneous transformation in storage.

When comparing the efficiency of FCS over newborn calf serum (NCS), Blaxhall (1983 a, b) found the latter to have an inhibitory effect on mitosis in both trout and carp lymphocyte culture. The primary cultures require FCS for growth, but can be later adapted to NCS. However, a change in serum can lead to a reduction in growth (Wolf and Ahne, 1982). This could be due to cells having adapted themselves to a particular serum because of which the receptors for other sera would have been reduced or eliminated (Hashimoto *et al.*, 1997). A variety of sera have been used to promote the growth of cells such as homologous fish serum and calf serum (Hartely *et al.*, 1983), heterologous fish, human (Sigel and Beasley, 1973) chicken (Stephenson and Potter, 1967); horse, porcine (Ganassin and Bols, 1992), rabbit, sheep (Wolf and Quimby, 1969) and agamma globulin sera (Lannan *et al.*, 1984) with varying efficacies.

Fernandez *et al.* (1993b) reported use of increased NaCl concentrations for cell lines derived from marine fish. A few marine fish cell lines require NaCl (Middlebrooks *et al.*, 1979; Sobhana *et al*., 2008; 2009; Hasoon *et al*., 2011), though most of the published works on marine cell lines state a lack for such a need. Clem *et al*. (1961) were the first to establish monolayer cell

cultures from marine teleosts and obtained best results in commercial medium modified with 0.07 M NaCl. The JSKG cell line established from gonads of Japanese striped knife jaw, *Oplegnathus fasciatus* and PAS cell line from skin of purplish amberjack, *Seriola dumerili* were initiated at a higher NaCl concentration of 0.206 M, but gradually adapted to a low NaCl concentration of 0.116 M after several subcultures (Fernandez *et al*., 1993 b). However, Chang *et al.* (2001) successfully established SF cell line from Asian sea bass, *L. calcarifer* without using increased NaCl concentrations in the cell culture medium. Sahul Hameed *et al.* (2006) also concluded that for the establishment of SISK cell line from sea bass kidney, additional NaCl was not needed. However, using media with low concentration of NaCl was superior since high levels of salinity caused slow growth and absence of NaCl caused a mild cytoplasmic vacuolation and nuclear granulation.Various growth factors such as mammalian epidermal growth factor (mEGF) and basic fibroblast growth factor (bFGF) simultaneously or individually (Watanabe *et al*., 1987; Iida *et al*., 1998; Fan *et al*., 2003, 2010; Chen *et al*., 2004; Yu *et al*., 2005; Ye *et al*., 2006; Wei *et al*., 2009; Yan *et al*., 2011) had been used to stimulate growth of fish cell lines. bFGF and EGF have important regulatory abilities in cell proliferation, migration and differentiation. They probably activate tyrosine kinase by binding to tyrosine kinase receptor and speed cell proliferation *via* ras, MAPK and/or protein kinase C pathway (Boonstra *et al.*, 1995; Hrzenjak and Shain, 1995). Basic FGF is a potent mitogen for embryonic stem cells derived from *Oryzias latipes* (Hong *et al.*, 1996) and sea perch (Chen *et al*., 2003), lymphoid cells from *Penaeus monodon* (Hsu *et al*., 1995) and embryonic cells from *Paralichthys olivaceus* (Chen *et al*., 2004).

Antibiotics and antimycotics serve to avert initial contamination risks in primary culture and are usually discontinued after the establishment of the

cell line. Continuous use of these chemicals has been known to encourage the development of antibiotic-resistant organisms and hide cryptic contaminants (Freshney, 2010). A number of antibiotics used frequently in fish tissue culture include streptomycin SO_4 (100 µg mL^{-1}), penicillin-G (100 U mL^{-1}), gentamicin (50 µg mL^{-1}), kanamycin (100 µg mL^{-1}) and fungizones include amphotericin B (2.5 µg mL^{-1}) and nystatin (50 µg mL^{-1}).

Glutamine provides energy and carbon to cultured cells (Butler and Christie, 1994). Although glutamate can be metabolised in cells, glutamine with a half-life of 3-5 days is supplemented. The toxic effect of utilised glutamine is that it produces ammonia (Hassell *et al*., 1991). An alternative product that has been supplied by Invitrogen includes an alanyl-glutamine dipeptide which is acted on by dipeptidase. It is supplied under the name glutamax and is known to be stable. L- glutamine has been supplemented in fish cell lines along with DMEM, MEM, RPMI 1640 (Rio *et al*., 1973; Faisal *et al*., 1995; Béjar *et al*., 1997; Chang *et al*., 2001; Williams *et al*., 2003; Jensen *et al.*, 2012).

Phenol red is usually added as a pH indicator of the medium and accounts for the colour of culture media. This indicator is useful because it is particularly sensitive to slight pH changes around the growth optimum for cells. At lower pH, the phenol red becomes orange (pH 7.0) or yellow (pH 6.5). An overnight change in colour of the culture from red to yellow usually indicates bacterial contamination.

Embryonic stem (ES)-like cells derived from blastomeres of the early blastulae stage of Leopard danio, *Brachydanio frankei* were cultured *in vitro* in a medium containing Leibovitz's-15 supplemented with 10% fetal bovine serum, leopard danio embryo extract, sodium bicarbonate, sodium

selenite, basic fibroblast growth factor, epidermal growth factor, leukaemia inhibitory factor, 2-mercaptoethanol, sodium pyruvate, sodium selenite, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) and non-essential amino acids (Routray *et al.*, 2010). Similarly, Chen *et al.* (2003) used DMEM medium supplemented with HEPES, fetal bovine serum, sea bream serum, sea bream embryo extract, selenium, basic fibroblast growth factor and human recombinant leukemia inhibitory factor, 2-mercaptoethanol, sodium pyruvate and non-essential amino acids and cultured at 24˚C without CO_2.

2.6.4 Preparation of fish for primary culture

The initial stage to consider in fish primary culture is the isolation of the appropriate tissue. One of the main risks faced when isolating tissue is contamination from microbes and/or fungi. Internal organs are considered sterile with the exception of the digestive tract therefore as long as organs are removed aseptically the contamination risk is low. It is prudent however; to disinfect the area of incision or even the entire fish before dissection (Wolf and Quimby, 1969). There are numerous examples of disinfection procedures. 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, 1969). The fish is either immersed or bathed in such solutions for several minutes. To further reduce the risk of contamination from faecal matter, food can be withheld for several days prior to dissection (Sobhana *et al.*, 2008, 2009). Gill tissues can also be especially difficult to decontaminate. Butler and Nowak (2004) detail a method that prevented contamination of their primary gill cultures. Treating the fish in a similar manner as for internal organs appears to be the standard method for decontamination. Methods vary from complete immersion for a few minutes to immersion for hours. Generally there

is a greater use of antibiotic solutions to decontaminate tissue opposed to strong disinfectants as these can damage cells (Wolf and Ahne, 1982; Sobhana *et al.*, 2008, 2009).

2.6.5 Choice of tissues for primary culture

Tissues and cells are mostly chosen on the basis of the goal of study. Teleost fish cell lines have been developed from a broad range of tissues *viz.*, ovary, fin, swim bladder, heart, spleen, liver, eye muscle, vertebrae, and brain and skin (reviewed by Bols and Lee, 1991; Lakra *et al*., 2010c) and most fish cell lines originated from normal tissues. Embryos or fins are most frequently listed as the source of the tissues used in the primary culture. After ovary, the second most common tissue used for cultivation is fin, due to its high regenerative ability (Fryer and Lannan., 1994). Wolf (1988), summarising many years of experience with piscine cells, reported that immature gonads and ovaries were the most easily and consistently cultivable of all fish tissues, and gonadal cell lines have been described from many species. The first piscine cell line, RTG-2, was developed from pooled normal gonads of fingerling yearling rainbow trout (Wolf and Quimby, 1962). Cell lines have also been developed from ovary (Kumar *et al.*, 2001), skin and fin (Lakra and Bhonde, 1996), vertebrae (Pombinho *et al.*, 2004), pituitary gland pars intermedia (Chen *et al.*, 2010), muscle (Lai *et al*., 2008), liver (Williams *et al*., 2003), bulbus arteriosus of heart (Vo *et al*., 2014), peripheral blood leukocytes (Dewitte-Orr *et al*., 2006), scales (Akimoto *et al.*, 2000) and intestine (Kawano *et al.*, 2011). However, only one cell line XM (Barnes *et al*., 2006) was initiated from skin and fin tissue of fish melanoma; and in some cases, these cells remained tumorogenic *in vivo* following repeated *in vitro* passage (Lakra *et al*., 2010c).

2.6.6 Techniques for isolation of cells

When cells are isolated from donor tissue, they may be maintained in a number of different ways. A simple small fragment of tissue that adheres to the growth surface, either spontaneously or aided by mechanical means, a plasma clot, or an extracellular matrix constituent, such as collagen, will usually give rise to an outgrowth of cells. This type of culture is known as a primary explant, and the cells migrating out are known as the outgrowth. Cells in the outgrowth are selected, in the first instance, by their ability to migrate from the explant and subsequently, if subcultured, by their ability to proliferate. When a tissue sample is disaggregated, either mechanically or enzymatically, the suspension of cells and small aggregates that is generated will contain a proportion of cells capable of attachment to a solid substrate, forming a monolayer. Those cells within the monolayer that are capable of proliferation will then be selected at the first subculture and, as with the outgrowth from a primary explant, may give rise to a cell line. Tissue disaggregation is capable of generating larger cultures more rapidly than explant culture, but explant culture may still be preferable where only small fragments of tissue are available or the fragility of the cells precludes survival after disaggregation (Freshney, 2010).

2.6.6.1 Explantation

The simplest procedure is the explant method which involves finely chopping the tissue into fragments no larger than 1-2mm^3. These fragments are left to adhere to a tissue culture vessel with the culture vessel positioned so that excess fluid drains away. After the fragments have adhered, further media can be gently added to cover them completely (Wolf and Ahne, 1982). Cells outgrow from the explant eventually forming a culture. Freshney (2010)

suggests enhancing adherence of the explants by using serum or other attachment factors. Several cell lines have been established using this method (Béjar *et al*., 1997; Sahul Hameed *et al.*, 2006; Sobhana *et al*., 2009). The first documented success of developing primary cell cultures from fishes of Indian waters describes how primary cell cultures could be obtained from the kidney of the stinging catfish, *Heteropneustes fossilis* (Singh *et al*., 1995). Primary cultures from a variety of tissues such as heart tissue of Indian major carp (Rao *et al*., 1997), caudal fin of *Tor putitora* (Prasanna *et al*., 2000), caudal fin of rohu (Lakra and Bhonde, 1996) and ovary of *Clarias gariepinus* (Kumar *et al.*, 2001) have also been reported.

2.6.6.2 Enzymatic dispersion

In this method, tissues are minced and suspended in a digestion mixture, usually trypsin, but other proteolytic enzymes may be used (*e.g.* collagenase or pronase) (Wolf and Ahne, 1982). Cells are harvested by neutralising the enzyme with media containing serum, then pelleting the cells by centrifugation and re-suspending in fresh media. There is a great deal of variation to this basic method. Wolf and Quimby (1969) detail numerous variations of temperature, duration of digestion and digestive mixtures. Digestions can be short, extended (*i.e.* overnight) and range from temperatures of 4˚C to 20˚C. A higher temperature results in a faster digestion of the tissue and *vice versa* a colder temperature will take longer to disassociate cells from the tissue. Lai *et al.* (2001, 2003) used a trypsin solution for digestion at 4°C for one hour to establish cultures. Faisal *et al.* (1995) used a similar trypsin solution for thirty minutes at room temperature. Trypsin (0.25%) with EDTA is a common choice as an enzymatic dissociation solution (Faisal *et al*., 1995; Chi *et al*., 1999; Chang *et al*., 2001; Lai *et al*., 2001, 2003). There is also a great deal of variation in the harvesting method. There may be one harvest or

numerous harvests throughout the digestive period resulting in several pellets of cells which are then pooled. Chi *et al.* (1999) repeatedly harvested the cells at thirty minute intervals and pooled all harvests together at the end. Chang *et al.* (2001) also had more than one harvest with trypsinisation continuing for any remaining undigested tissue. Butler and Nowak (2004) established a method to isolate gill tissues in which two different enzyme solutions are used alternatively at thirty minute intervals. Cells are harvested from the supernatant after centrifuging rather than the pellet being re-suspended. Pombinho *et al.* (2004) initiated a bone derived cell line using 0.125% collagenase in HBSS at 37°C and harvested cells at 30 and 90 min intervals. Collagenase as the digestion component has also been used by Dannevig *et al.* (1995) and Bols *et al*. (1994) at a concentration of 50 mg mL^{-1} and 1mg mL^{-1} respectively. Viability assessment is fairly standard procedure; Freshney (2010) detailed a basic procedure using trypan blue and a haemocytometer.

2.6.6.3 Mechanical dispersion

Another common method of cell isolation is mechanical dispersion. By forcing the tissue through a sieve or syringe, individual or small clumps of cells are released from the bulk of the tissue. However this procedure causes a great deal of mechanical damage and produces lower yields than any other method (Freshney, 2010). Furthermore it is only recommended for softer tissues *e.g.* brain, spleen. Cell lines that have been established this way include leukocytes isolated from kidney using a sieve (Wang *et al*., 1995); brain tissue was mechanically dispersed by pipetting up and down using sterile glass pipettes to develop cerebellar-tegmental cultures (Servili *et al.*, 2009).

2.6.7 Subculture

The first subculture represents an important transition for a culture. The need of subculture implies that the primary culture has increased to occupy all of the available substrate. Primary cultures have variable growth fractions. The composition of cells in culture tends to change by adapting to the environment and especially, if the cells are not regularly passaged. Serial subculture of cell lines enriches a fraction of any cell population based on growth rate and viability (Freshney, 2010; Coriell, 1984). Cells in culture may be maintained by passaging there by preventing redifferentiation which is essential to determine, for each cell type, source, and application, a suitable number of passages during subculture. It has been found that the change in media significantly alter the morphology of the culture as reported by Pombinho *et al*. (2004) where the vertebra cell line phenotype changed to a homogenous polygonal morphology from a spindle-like phenotype when the cells were transferred from L-15 to DMEM. For all fish species cultured to date, cells are anchorage dependent, attaching and growing as a monolayer on the flask surface (Babich and Borenfreund, 1991). Monolayer cell cultures may be dispersed either mechanically or chemically for subculturing. Fish cells differ in the tenacity of their attachment to each other and to the substrate. In part, this reflects differences between cell lines; but media, culture conditions, and even culture age affect the ease or difficulty with which cells may be dispersed. Old cultures have been found to be more cohesive than young cultures, and cells grown in medium with 10-15% serum are more cohesive than cells grown in 5% serum (Wolf and Quimby, 1969). For cultures that are slow to reach confluency, 1 mM ATP was added to the medium since purine nucleotides stimulate the proliferation of the cell line (Ganassin *et al.*, 1994; Bryson *et al.*, 2006).

2.6.7.1 Enzymatic dispersion

At present, the use of a chelating agent with a tryptic enzyme is preferred to other enzymes. The concentration of trypsin used depends on the sensitivity of the tissues to the enzyme. A diluted solution of trypsin in either BSS or PBS can be used along with ethylenediaminetetraacetate (EDTA) or versene. When confluent monolayers are formed in primary culture, cells were carefully dislodged from the flask surface by treatment with trypsin. This usually takes 5-10 min and at that time a small amount of fresh medium is added and the cells dispersed by pipetting. The enzymic action is inhibited by serum, and the cells can be diluted as necessary. Middlebrooks (1979) used a trypsin-versene dispersant to subculture the cell line CyN developed from the muscle tissue of *Cynoscion nebulosus*. Heart tissue of the greasy grouper, was subcultured using 0.25 % trypsin (0.25 % trypsin and 0.2 % EDTA in PBS), a recombinant trypsin, trypLE (Invitrogen) was used to dissociate cells from brain tissues of *Dicentrarchus labrax* and cell lines LCE and LCF developed from the fry and fingerlings of *Lates calcarifer* was subcultured using TPVG solution (0.1% Trypsin, 0.2% EDTA and 0.2% glucose in PBS) (Sobhana *et al*., 2008; Servili *et al*., 2009; Lakra *et al.,* 2006 b).

2.6.7.2 Mechanical dispersion

There is only occasional justification for using mechanical means to disperse cell sheets. The objections to this method are that even under the best conditions many cells are destroyed and the dispersion itself is usually incomplete, the sheet being broken into fragments rather than individual cells. This of course compounds the problems of accuracy in enumeration. With human embryonal stems cells, enzymatic dispersal can

lead to the loss of stemness. Subculture must be done mechanically by subdividing a colony of cells and subculturing the pieces (Freshney, 2010). As reviewed Wolf and Quimby (1969), the cell sheet is scraped from the surface-with a rubber policeman or glass culture scraper-into a sufficiently small volume of fresh medium to permit vigorous pipetting for additional dispersion. Estimates of cell loss have been as high as 50% (Cerini, 1964; Malsberger and Cerini, 1965).

2.6.8. Maintenance of cells

Many different kinds of tissues and cells from freshwater and marine teleosts can be cultured on a routine basis. The maintenance of *in vitro* culture of fish cells closely follows that used with homoiotherm material; the major differences being, first, in temperature requirements and tolerances, and, second, in osmolarity of salines and media. For the most part, mammalian-type solutions are entirely satisfactory for many freshwater teleosts but for marine fishes, best results are obtained after the osmolarity is increased.

2.6.8.1 Temperature

Fish cells can tolerate an even wider temperature range, which has been defined as the endurance zone. The temperature chosen for a cell line depends on the purpose of the cultures, the species, and the availability of incubators. To maximise cell production, an optimal growth temperature should be selected. For cold-water fish, like salmon, this is in the 20–23 ˚C range; and for warm-water fish, like zebrafish, this is 26–30 ˚C. If the purpose is to just maintain the cultures for later use, lower temperatures allow the cells to slowly proliferate, reducing culture maintenance. Nearly all fish cells can be maintained at room temperature, but the variability of the ambient temperature

in laboratories makes this undesirable, and a cooled incubator is preferable. The incubator type depends on the basal media. With L-15 or CO_2-independent media, a regular incubator can be used. However, if the temperatures are high and the incubator is not humidified, medium can rapidly evaporate from Petridish cultures, increasing the osmolarity and slowing cell growth. Therefore, unvented flasks with the caps tightly closed are recommended. When buffering of the basal media is done only with sodium bicarbonate, a CO_2 incubator must be used (Bols *et al*., 2011). Cultured fish cells will tolerate considerable drops in temperature, can survive several days at 4 ˚C, and can be frozen and cooled to −196 ˚C (Freshney, 2010).

The principal advantage of vertebrate cell culture and notably fish cell culture is their innate ability to metabolise through a wide range of temperatures. In the absence of specific data, ecological niche typically occupied by fish can be used as a guideline for safe incubation temperature of cell and tissues. The adaptation of cells in cell culture can be aided by slight increases of incubation temperature at each subculturing or after several subcultures (Wolf and Ahne, 1982).

Fish cell culture is simpler as room temperature is adequate for incubation however; the number of times cultures are handled can be greatly reduced by incubation at lower temperatures (4- 15 ˚C). Optimal growth temperature of cells depends upon the fish species and its natural environment (Gabridge, 1985). Based on physiological requirements and environmental preferences, fishes are classified into cold water or warm water species and the temperature factor applies to the *in vitro* culture conditions. Cells from warm water fishes generally do well at 25 to 30˚C but may grow well at 15˚C through 35˚C and higher (Wolf, 1973). Two carp cell lines have been reported to grow optimally at 37˚C (Ku and Chen, 1992). For cold water fishes like

salmon and trout, the optimum temperature ranges from 15 to 20 ˚C. Generally, the *in vitro* growth temperatures are a few degrees above the preferred *in vivo* environmental temperature (Lakra and Bhonde, 1996). The range of temperature at which the fish cells grow, called the 'proliferation zone' (Bols *et al.*, 1992), is wider than that at which fish grows and this innate ability has allowed fish cell cultures to be used for a variety of allied studies (Wolf and Ahne, 1982).

2.6.8.2 pH

The pH of medium necessary for good growth of fish cells does not appear to be particularly critical, and most cells seem to fare well in the range of 7.2-7.8. Primary cultures and low densities of cells will usually do better at 7.3-7.4 than at pH 7.8. Routine passage of some cell lines can be made at pH 7.8 or even 8.0, but the lag phase may be extended. At the other end, old cultures can have a pH as low as 6.8 apparently without undue damage to the cells. Such cultures can be dispersed in fresh medium and growth will usually resume (Wolf and Quimby, 1969).

Basal media maintain their buffering capacity under normal atmospheric conditions (~0.03% CO_2) using salines or organic buffers such as HEPES. For instance, MEM is made up in Hank's salts, which maintains the pH under atmospheric conditions in CO_2 incubators. Leibovitz's L-15 maintains physiological pH through a combination of salts, high basic amino acid concentrations, and galactose in place of glucose. CO_2-independent medium (Gibco or Invitrogen) contains sodium bicarbonate. Most fish cell lines are grown in L-15, but a few fish cell lines maintained in CO_2-independent medium grew well. These basal media are used without a CO_2 incubator (Bols *et al.*, 2011).

Serum also modifies physicochemical properties such as viscosity and osmolality, protects labile essential nutrients (Ham, 1981), detoxifies by binding toxic metals and pyrogens, and acts as a pH buffer (Barnes and Sato, 1980).

2.6.8.3 Osmolality

Osmolalities between 260 mOsm kg^{-1} and 320 mOsm kg^{-1} are quite acceptable for most cells. Bryson *et al.* (2006) demonstrated that HEW cell line (developed from the embryos of Haddock) survived well within 250 – 400 mOsm kg^{-1}. Spot liver cell line formed confluent monolayers at a wide range of osmolalities from 295-335 mOsm kg^{-1} (Faisal *et al*., 1995). A slightly hypotonic medium may be better for Petri dish or open-plate culture to compensate for evaporation during incubation. Changes in osmolality are generally achieved by altering the sodium chloride concentration, for example, to compensate for different bicarbonate concentrations or to allow for the addition of HEPES. The addition of HEPES and drugs dissolved in strong acids and bases and their subsequent neutralisation can all markedly affect osmolality (Freshney, 2010).

2.7 Cryopreservation

Freezing and long-term storage of cultured cells at ultra-low temperature has been practiced successfully for mammalian cells, which works equally well with fish cells. Cell lines in continuous culture are prone to variation due to selection in early-passage culture, senescence in finite cell lines, and genetic and phenotypic instability in continuous cell lines. Even the best-run laboratory is prone to equipment failure and contamination (Freshney, 2010). Cross-contamination and misidentification continues to occur with an

alarming frequency (Capes-Davis *et al.*, 2010). In addition, it ensures that the lines are safely maintained and consistently distributed (Freshney, 2010).

Optimal freezing of cells for maximum viable recovery on thawing depends on minimising intracellular ice crystal formation and reducing cryogenic damage from foci of high-concentration solutes formed when intracellular water freezes (Freshney, 2010). Cryoprotectants, such as dimethyl sulphoxide (DMSO), reduce the amount of ice present during freezing and reduce solute concentration, thus reducing ionic stress. However, these compounds can themselves cause osmotic injury since they are hypertonic and can cause damage during their addition or removal. In general, cells are suspended in medium having 10% or more serum and 5-10% of either glycerol or DMSO as a protective additive. Cell lines from goldfish muscle and swim bladder tissue were cryopreserved in 10% DMSO and 10% FBS in L-15, (Rougée *et al.*, 2007). Chou *et al.* (1989) used 10% DMSO and autologous serum for freezing melanised goldfish erythrophoroma cell line. Chou *et al.* (1989) also highlighted the importance and desirability of using autologous serum for the cryopreservation of sensitive cell lines and further suggested that it may be able to prevent cells from undergoing spontaneous transformation in storage. Other cryoprotectants, include trehalose which enhances recovery and function of embryonic stem cells following cryogenic storage (Eroglu *et al.*, 2000; Dash *et al.*, 2008). The increase in serum concentration in freezing medium to 40, 50, or even 100% has also been carried out for increased survival rates. The fibroblastic-like cell line from caudal fin (RTF) of the red-line torpedo, *Puntius denisonii* (Day) was cryopreserved in 50% FBS and 10% DMSO in L-15 medium (Swaminathan *et al.*, 2012b). Most cultured cells survive best if they are cooled at -1˚C min^{-1} (Leibo and Mazur, 1971; Harris and Griffiths, 1977). This is probably a

compromise between fast freezing minimising ice crystal growth and slow cooling encouraging the extracellular migration of water.

When required, cells are thawed and reseeded at a relatively high concentration to optimise recovery. The ampoules are thawed as rapidly as possible to minimise intracellular ice crystal growth during the warming process (Freshney, 2010).

Several cell banks exist for the secure storage and distribution of validated cell lines. Initial seed stock should always be from a reputable cell bank such as, American Type Culture Collection (ATCC), where the necessary characterisation and quality control are routinely carried out (Freshney, 2010).

2.8 Authentication

The validity of outcomes proposed by the scientific community lies in the integrity of the cell lines used for testing. Hence, initiatives have called for standardised cell culture quality, including confirmation of cell line identity, through systematic processing and comprehensive testing to maintain high standards for cell line identity and reliability. The sequence of the mitochondrial CO1 gene can be used as a molecular barcode to differentiate animal species (Herbert *et al.,* 2003). Barcoding has also been employed to validate the identity of animal cell lines (Lorenz *et al*., 2005; Cooper *et al*., 2007) and is a recommended characterisation step for materials in biodiversity repositories (Hanner and Gregory, 2007). DNA barcoding through the Barcode of Life Database (BOLD), with universal primers for mitochondrial CO1 gene, used for fish species identification as reported by Ivanova *et al*. (2007), has also been applied for confirmation of the species of origin for several fish cell lines *viz*., brain (CB) and fin (CF) cell lines from *Rachycentron canadum*

(Cheng *et al*., 2010); fingerlings cell line (SBT-E1) from *Thunnus maccoyii* (Bain *et al.,* 2013); caudal fin (PDF) and heart (PDH) cell lines from *Puntius denisonii* (Lakra *et al.*, 2011); caudal fin (PSCF) cell line from *Puntius sophore* (Lakra and Goswami, 2011); fin (RTF) cell line from *Puntius denisonii* (Swaminathan *et al.*, 2012b) and intestine (RTgutGC) cell line from *Oncorhynchus mykiss* (Kawano *et al.*, 2011). Epithelioma papulosum cyprini (EPC) cell line, which was thought to be derived from the common carp (*Cyprinus carpio*) was proven to be originally derived from fathead minnow (*Pimephales promelas*) by DNA barcoding (Bols *et al.*, 2011).

2.9 Tissue markers

Intermediate filament proteins are the most widely used lineage or tissue markers. Both Diago *et al.* (1995) and Dannevig *et al*. (1997) investigated the antigenic profiles of salmonid cell lines using monoclonal antibodies (MAbs) directed against the corresponding antigens of mammalian origin. The rainbow trout pronephric stroma cell line (TPS) did not stain for keratins, collagen types I and III, vimentin or S-100 protein, whilst positive controls of normal rainbow trout tissue did cross-react with the mammalian antibodies (Diago *et al*., 1995). In the Atlantic salmon head kidney cell line (SHK-1), however, cells stained positively for cytokeratin and vimentin. These antigens are usually confined to epithelial and mesenchymal tissues and were used by the authors to establish the origin of the cell line (Dannevig *et al*., 1997). Butler and Nowak (2004) developed one epithelial cell line (RGE-2) which stained positively for cytokeratins and one fibroblast cell line (RGF) that stained strongly for fibronectin and collagen type I. The epithelial cell line RGE-2 also stained for cytokeratin K19 which is not common in epithelia *in vivo*, but is typically found in glandular-type epithelia and most carcinomas and is indicative of cells undergoing hyperproliferation (Lu *et al*., 2000). Cell

lines from the larvae of Atlantic sturgeon, *Acipenser oxyrinchus oxyrinchus* stained positive for the ectodermic glia fibrilliary acid protein (GFAP), vigilin (mRNA transport protein), and pan cytokeratin (Grunow *et al.*, 2011). Parameswaran *et al.* (2007) developed the Sahul Indian Milk Fish Heart (SIMH) fibroblastic cells which were strongly positive to fibronectin and desmin and the Sahul Indian Grouper Eye (SIGE) epithelial cells which were positive for pancytokeratin and Ki67, a proliferation marker.

Five single-cell clone lines (mRTP1B, mRTP1E, mRTP1F, mRTP1K, and mRTP2A) were developed from adult rainbow trout, *Oncorhynchus mykiss* pituitary glands. Immunocytochemical analysis with monospecific antisera of Gh and Prl (prolactin) showed that cells of all the five single-cell clones produced both Gh and Prl simultaneously emphasising its ability to produce pituitary-specific proteins (Chen *et al.*, 2010).

2.10 Chromosome content

Karyotyping is performed routinely to determine if the line has maintained a stable genotype so as to distinguish between normal and transformed cells. Cytogenetic analysis is also used to determine the karyotype of the cell substrate as a means of identifying the species, gender and ploidy.

The methods employed in initiating cell cultures, including digestion and centrifugation procedures, are well reviewed by Wolf and Quimby (1969). Techniques for harvesting cells and preparing chromosomes are essentially the same as those from tissues of live specimen, and are outlined in Denton (1973). Although chromosome studies using cell culture offer several advantages such as possibility in getting large numbers of metaphases; better chromosome morphology as compared to direct tissue preparation; several slides can be prepared from small individuals and the fact that specimens maintained in

laboratory aquaria for long periods may still be used (Amemiya *et al.*, 1984). Roberts (1964, 1966, 1967) effectively used cell culture as a tool for determining the chromosome numbers of fishes. Long- term cell cultures frequently become chromosomally heteroploid and are not recommended for general karyotyping (Regan *et al.*, 1968; Chen and Ebeling, 1975).

Wolf and Quimby (1969) stated that, 'among cell lines from all animals, attainment of the potential for indefinite subculturing is usually accompanied by alteration to a heteroploid chromosome constitution. In general, the adaptation of cell lines to *in vitro* conditions is often associated with dynamic chromosomal changes affecting chromosome number and/or morphology, giving rise in some cases to marker chromosomes which are line-specific (Ghosh and Chaudhuri, 1984). Béjar *et al.* (1997) karyotyped the continuous cell line SAF-1 developed from fin tissues of gilt-head seabream (*Sparus aurata* L.). The diploid number ranged from 33 to 70 with a modal peak at 48 chromosomes at passage 50. The chromosome number distribution at passage 70, displayed a 2n value ranging between 31 and 88 with the modal value at 48 chromosomes. Both distributions were asymmetrical with most of the 2n values appearing clustered below the modal value. *In vitro* conditions favour aneuploidy which is mainly due to chromosome missegregation during cell division by chromosome lagging and/or non-disjunction (Hsu, 1973). Seabass kidney cells (SISK) from kidney of seabass, *L. calcarifer*, at passage 37 showed a diploid number ranging from 30 to 56 with a modal peak at 48 chromosomes. The chromosome number distribution at passage 61, displayed a '2n' value ranging from 27 to 52 with the modal value at 46 chromosomes (Sahul Hameed *et al.*, 2006). Differences in the modal chromosome count imply the loss of partial or entire chromosomes.

The major and minor rRNA regions are known for high instability (Miller, 1983) due to their DNA arrangement in the form of tandem repeats. This instability can result in rearrangements revealed as chromosome polymorphisms in the rRNA regions, which have been described in fish cell lines (Sánchez *et al*., 1993). Béjar *et al.* (2005) ascertained the ribosomal RNA regions by fluorescence *in situ* hybridisation technique (FISH) in gilthead seabream (*Sparus aurata*) cell line. In the SAF-1 cells the major rRNA genes (labelled by nick translation with biotin-16-dUTP) appeared as one signal on the telomeres of the short arms of the largest biarmed chromosomes. Hybridisation with the 5S rDNA probe (probes were obtained by PCR from genomic DNA of *Diplodus puntazzo*) produced a single signal in an interstitial position of a medium-sized acrocentric pair. These indicated that the rRNA regions in SAF-1 have the standard species-specific pattern, suggesting genetic stability for these regions.

2.11 Transfection of fish cell lines

Fish cells stably expressing exogenous genes have potential applications in the production of fish recombinant proteins, gene-function studies, gene trapping, and the production of transgenic fish. Transfection of cells with plasmids encoding a gene of interest coupled to a reporter gene, *e.g.* green fluorescent protein (GFP) has become a pivotal technique for the study of gene expression, protein trafficking and localisation (Tsien, 1998). DNA transposons, Sleeping Beauty (SB) and Frog Prince (FP) transposons, derived, respectively, from fish and frog genomes were assessed for their transposases activity in fish cell lines from genetically distant species CHSE-214 from chinook salmon embryos (*Oncorhynchus tshawytscha)* (Fryer *et al*., 1965), RTG-2 from rainbow trout (*Oncorhynchus mykiss*) gonads (Wolf and Quimby, 1962), EPC from a skin tumor of carp (*Cyprinus carpio*) (Fijan *et al*., 1983),

and SAF-1 from fin fibroblasts of the gilthead seabream (*Sparus aurata*) (Béjar *et al.,* 1997). Their transpositional ability was evaluated by the plasmid-based excision assay, the colony formation assay, and the footprint patterns. The results revealed that while both transposases are active in all cell lines, the transposition rates and the precision of the transposition were overall higher with FP than SB (Gallardo-Gálvez *et al*., 2011). Production of transgenic fish that express fluorescent marker genes under the control of PGC-specific promoters has been reported, and may provide a valuable complement to the ES cell-based work (Krovel and Olsen, 2002).

2.12 Contamination

A cell culture contaminant can be defined as some element in the culture system that is undesirable because of its possible adverse effects on either the system or its use. Contamination truly endangers the use of cell cultures as reliable reagents and tools. Cell cultures are biological entities that respond to their environment and consequently, to environmental contamination. The potential effects on the contaminated cell culture system may increase exponentially over time. The potential risks to the validity of experimental results and conclusion increase likewise with the duration of any unrecognised contamination.

2.12.1 Sources of contamination

Contamination may enter the cell culture system as a physical, chemical or biological component (Coriell, 1984; McGarrity *et al.,* 1985; Ryan, 1994).

2.12.1.1 Physical contamination

Physical components of cell culture systems known to have an impact are temperature, radiation, irradiation and vibration (Adams, 1980; Harakas *et al.*, 1984; Ryan, 1994). Exposure of media, media components and cell cultures to radiation, irradiation (*i.e.*, fluorescent and ultraviolet light), temperature extremes can elicit a number of metabolic responses in cell cultures, such as cell cycle synchrony, reduced cell growth and cell death. Other sources include plasticisers in plastic tubing and storage bottles and impurities in gases used in CO_2 incubators, supplies such as pipettes and culture vessels, equipment such as tissue culture hoods and incubators.

2.12.1.2 Chemical contamination

Chemical contamination is best described as the presence of any non-living substance that results in undesirable effects on the culture system. Hormones and other growth factors found in serum can cause changes that, while not necessarily harmful to cultures, may be unwanted by researchers using the system. Even essential nutrients become toxic at high concentrations.

Potential chemical contaminants include: 1. Metal ions, endotoxins and other impurities in media, sera and water. Highly purified water can leach potentially toxic metal ions from glassware or metal pipes. 2. Deposits on glasswares, pipettes and instruments left by disinfectants or detergents, antiscaling compounds in autoclave water, residues from aluminium foil or paper. 3. Free radicals generated in media by the photoactivation of tryptophan, riboflavin or HEPES exposed to fluorescent light. These media components can be photoactivated producing hydrogen peroxide and free radicals that are toxic to cells, and 4. Residues from germicides or pesticides used to disinfect incubators, equipment and labs (Ryan, 1994).

2.12.1.3 Biological contamination

Biological contamination represents the greatest threat to the cell culture system because living organisms metabolise and replicate. Replication increases the titre of the contaminant. Consequently, the risk of spreading the infection *via* cross-contamination to other cell cultures increases. Metabolism increases the array of potential effects on the cell culture system by reducing essential nutrients and by introducing exotic byproducts such as unique enzymes, antigens and toxins. Contamination may enter the system in the form of other cultured cells, bacteria, molds, yeasts, viruses, protozoa, insects, mycoplasma and other cell lines (Atkin, 1983; McGarrity *et al*., 1985; Ryan, 1994).

2.12.2 Detection of contamination

When most bacterial contamination occurs, it usually occurs within a few days and is typically obvious to the naked eye. Distinct changes to the medium such as turbidity, presence of particles visible in suspension, and a rapid decline in pH (yellow colour, indicating acidity) are all indicators of bacterial contamination. When antibiotics are routinely used in culture, resistant organisms may develop into slow growing, low level infections that are very difficult to detect by direct visual observation. Similar detection problems can occur with fastidious bacteria species that grow very slowly.

Fungal contaminants may or may not cause a change in the pH of the medium and can be distinguished from bacteria by checking for the presence of filamentous structures in the suspension. Yeast cells are larger than bacteria, but may not appreciably change the pH of the medium, and will appear as separate round or ovoid particles. Microbacterial media which can be used to test for bacterial and fungal contamination include blood agar, thioglycollate broth, tryptic soy broth, BHI broth, Sabouraud broth, YM broth, and nutrient

broth with 2% yeast extract. However, some microbial contaminations are not apparent.

Mycoplasmas are common and widespread contaminants of cell cultures. An infection of cell cultures may persist for an extended time without apparent cell damage and can affect virtually every parameter within a cell culture system (McGarrity *et al*., 1985) such as changes in cell metabolism, alterations in cell karyotype and retardation of cell growth (Boyle *et al*., 1981; McGarrity *et al*., 1984; Sasaki *et al*.,1984). Cell lines are screened for mycoplasma contamination by direct (agarose and broth culture) and indirect methods fluorescent staining, PCR, ELISA assay, immunostaining, autoradiography (Freshney, 2010; Lincoln and Gabridge, 1998) and DNA probes (Yogev and Razin, 1996). Most cell culture laboratories have incorporated PCR- based mycoplasma testing (Uphoff and Drexler, 2002, 2004).

Fluorescent staining of DNA by Hoechst 33258 (Chen, 1977) is one of the easiest and most reliable methods and reveals mycoplasmal infections as a fine particulate or filamentous staining over the cytoplasm with a 50x or 100x objective.

Presently there are no reliable methods for eliminating viruses from a culture and disposal or tolerance is the only option. Detection techniques include: 1. *In vitro* test for adventitious virus. This test involves the inoculation of different cell lines – a human, a primate and a bovine (if bovine material was used for the production, otherwise a cell line from the species of origin of the cell substrate), and the production cell line (co-cultivation test). 2. *In vivo* tests where animals are used to identify the presence of virus. The animals are treated using different inoculation routes (the health of these animals should be monitored and any abnormality should be investigated to

establish the cause of the illness). 3. Electron microscopic examination to detect adventitious viruses in the case of rather high virus loads). 4. Specific virus tests: *In vivo* inoculation tests (mouse antibody (MAP), rat antibody (RAP), hamster antibody (HAP) - examination of serum antibody levels against specific viruses or enzyme activity after a specified period), different specific PCRs. 5. Retrovirus (eventually after induction of endogeneous retroviruses: RTase (detect the presence of all viruses which contain reverse transcriptase enzyme) (Berthold *et al.*, 1996; Merten, 2002).

2.12.3 Cross contamination and overpassaging

During the development of tissue culture, a number of cell strains have evolved with very short doubling times and high plating efficiencies. Although these properties make such cell lines valuable experimental material, they also make them potentially hazardous for cross-infecting other cell lines. With the advent of improved karyotyping methods, such as Giemsa banding which fine-tuned the identification of chromosomes by allowing distinction of various regions, later isoenzyme profiling (O'Brien *et al.*, 1980), molecular studies based on DNA polymorphisms which enables distinct DNA fingerprinting patterns to be generated for individual cell lines (Stacey *et al.*, 1992), further confirmed the contamination of many cell lines. A few fish cell lines have also been reported that have been cross contaminated:

1. **OLGA-PH-J/92 [OL-J/92] (ATCC® CRL-2576™) -** This cell line was originally deposited as a crayfish cerebral ganglion cell line. However, cytochrome c oxidase subunit I (COI) testing at ATCC cannot confirm the crayfish origin. Based on this analysis, the distribution of ATCC® CRL-2576™ has been discontinued.

2. **EPC (ATCC® CRL-2872™)** - Cytochrome c oxidase subunit I (COI) testing at ATCC revealed that EPC, originally deposited as a Carp cell line *(Cyprinus carpio),* was in fact a Fathead Minnow cell line *(Pimephales promelas).* Since the time of deposit, isoenzymology testing has correctly and consistently identified EPC (ATCC® CRL-2872™) as a fish cell line. However, isoenzymology does not allow for speciation between genus, and information regarding the species of fish was previously only provided by the depositor. These observations were confirmed *via* COI testing of the original stock available to ATCC.

Scientists working on cell lines derived themselves or received from a colleague, should routinely carry out basic authentication tests such as spectral karyotyping, STR profiling, isoenzyme analysis, and contamination tests (Riley *et al.*, 1991; Markovic and Markovic, 1998; Drexler *et al.*, 1999; Vierck and Dodson, 2000; Rush *et al.*, 2002; Wenzel and Daniel, 2005). Researchers could also acquire cell cultures from reputable sources, such as nationally and internationally recognised cell banks where tested, identity-verified, contamination-free lines are distributed (Markovic and Markovic, 1998, Hartung *et al.*, 2002; Buehring *et al.*, 2004).

The basis for any research, development, or production program involving cell cultures is the selection of an identity-verified and low-passage cell line. The use of similar and identified passage numbers throughout a project ensures reproducible results and comparisons between laboratories. Divergent effects accumulate by long-term culturing on cell line morphology, development, and gene expression. As a result of selective pressures and genetic drift, cell lines, when kept in culture too long, exhibit reduced or altered key functions and often no longer represent reliable models of their original source material. The faster growing cells in culture eventually overrun

slower proliferators in the population. In addition, cell lines maintained in culture over a long period of time may experience mutations that alter the original functional characteristics of the cell lines identified at earlier passage levels (Hughes *et al.*, 2007). Besides the impact on cell line characterisation, the use of over-passaged cell lines poses an increased risk of microbial, viral or cellular cross-contamination.

Mitalipova *et al.* (2005) showed techniques used for cell passaging can also have an effect on the genetic stability of stem cells. They demonstrated that bulk passage methods after as few as 23 passages compared to manual passage methods, leads to aneuploidy detectable by karyotyping. Bulk passage methods include use of either enzymatic disaggregation (collagenase/trypsin) or nonenzyme-based methods using cell association buffer. Maitra *et al.* (2005) reported that following long-term culturing, a number of late-passage hES cell lines from different sources had at least one genetic abnormality commonly observed in human cancer cells, including DNA copy number and promoter methylation. To confirm the use of authenticated cell lines, full cell line documentation, including the source and passage numbers used during experiments, should be submitted for scientific publications. Cell lines are critical components of experiments and should be considered as standard research reagents and given the same care and quality control measures that surround the use of kits, enzymes, and other laboratory products commercially obtained.

2.13 Continuous cell lines

Research has led to the establishment of cell lines from a wide range of fish species and by 1980. Sixty one cell lines originating from 36 fish species belonging to 17 families were reported in the course of the last four decades

(Fent, 2001). Currently, 283 cell lines have been established from finfish around the world (Lakra *et al.*, 2010c).

Primary culture is the first in the series of selective processes that may or may not give rise to an established cell line (Freshney, 2010). Only those cells which survive isolation and adhere will form the basis of a culture. Cells which then proliferate are selected over those cells which survive but do not grow. Once confluence is reached and the culture is passaged the same selection process is repeated. Unfortunately this will not change the fact that most cell lines are still finite. Normal cells can only divide a limited number of times (Freshney, 2010). This is known as the Hayflick limit. Finite cell lines only have 20-80 population doubles before cell death occurs. The protective ends of the chromosomes, the telomeres, gradually shorten with each cell cycle and when a critical telomere length is reached, the cell is unable to divide. The cell enters an irreversible state of quiescence (Barker *et al.*, 2000). This is thought to involve the tumour suppressor gene p^{53} which arrests cell cycle progression. The gene p^{53} arrests the cell cycle in order to allow the cell to survey its DNA for damage; if DNA is undamaged the normal cycle resumes (Luft *et al*., 1998). This progression of events whereby cells move from actively dividing to a non-dividing state is known as senescence. A cell line to become established or continuous, it must undergo transformation or immortalisation.

2.14 Immortalisation of cells

Transformation usually refers to the genetic alteration of a cell resulting from the introduction, uptake and expression of foreign DNA. The use of the term transformation within cell culture has slightly different meaning to this. Generally it refers to an alteration in growth characteristics (*e.g*. loss of

contact inhibition, low serum requirement, continuous growth) which may or may not correlate with immortalisation (Freshney, 2010). Normal cells are converted into cells that will divide without limit hence bypassing senescence and cell death.

Fish cell lines though developed from normal tissues are eventually found to show characteristics of transformed cells leading to immortalisation (Fryer and Lannan, 1994; Butler and Nowak, 2004). Continuous cell lines appear to arise spontaneously more readily from fish than from higher vertebrates (Nicholson, 1989). However transformation can also be induced through chemical mutagens, infection with a transforming virus or through transfection (Takarada *et al*., 1989; Guo *et al*., 2003; Butler and Nowak, 2004; Freshney, 2010). Media, growth factors and other supplements will simply optimise the conditions for growth and provide more opportunities to develop a continuous cell line, but do not ensure development of an immortalised cell line.

A marker of totipotency is telomerase activity. High telomerase activity has been associated with undifferentiated cells (Mantell and Greider, 1994) and the ability of a cell culture to proliferate indefinitely (Holt *et al*., 1996). In fish cell lines for which telomerase activity has been analysed so far (Barker *et al*., 2000; Béjar *et al*., 2002; Ossum *et al*., 2004; Béjar *et al*., 2005), telomerase activity is a common feature suggesting that cells of piscine origin support the telomerase-related immortalisation model. Cell lines, whether finite or continuous, have advantages over primary cell cultures. The cell lines become fairly homogeneous during early passaging and can be cryopreserved indefinitely (Wolf, 1979). This makes them a much more reproducible and convenient source of cells.

2.15 Application of fish cell lines

Piscine cell cultures are increasingly being applied to studies of fish, which, as the largest and most diverse group of vertebrates, are important model systems in embryology, neurobiology, endocrinology and environmental biology (Powers, 1989). Cell lines from teleosts would be invaluable in advancing basic knowledge in comparative endocrinology, physiology, and immunology and in providing practical information that could be used to enhance the health, growth, and reproduction of fish in aquaculture (Bols, 1991). Bols and Lee (1991) extensively reviewed literature on the *in vitro* use of piscine cells as primary cultures as well as cell lines.

2.15.1. Virology and vaccine development

DeWitte-Orr (2006) described the utility of cell lines into five major areas for the understanding of viruses. Firstly, cell lines are needed to produce sufficient virus to characterise the biophysical and biochemical properties of a virus. Examples of such characterisation would include describing viral proteins and genome organisation (Winton *et al.*, 1987). Secondly, cell cultures are needed to study the single-cell reproductive cycle of virus. This would include understanding the molecular events involved in the entry, replication, assembly and release of viruses (Duncan, 1996; Rivas *et al.*, 1998). Thirdly, cell lines can contribute to preventing and controlling viral diseases by being a source of viruses for vaccines and an experimental system for the development of antiviral drugs (Corbeil *et al.*, 2000). Fourthly, in some cases, cell lines can advance an understanding of viral pathogenesis. For example, CHSE-214 was used as a tool for studying the characteristics of IHNV persistent infections found both in the cell lines and in whole fish (Engelking and Leong, 1981). Finally, cell lines are powerful diagnostic tools,

for discovering new viruses and identifying viral pathogens from environmental samples (Amend, 1975).

The Asian region contributes to over 80% of the total fish production and has also established over 60% of the currently established fish cell lines (Lakra *et al.*, 2010c). With the main intention being to evaluate the susceptibility of the cell lines to various virus affecting the species along with studies on virology and vaccine development at the cellular level. A few cell lines developed include *Epinephelus* sp. (Chi *et al*., 1999; Qin *et al*., 2006; Zhou *et al*., 2007; Wen *et al*., 2008; Ku *et al*., 2009; Wei *et al*., 2010; Huang *et al*., 2009), *L. calcarifer* (Chang *et al*., 2001; Lakra *et al*., 2006b; Sahul Hameed, 2006; Lai *et al*., 2008; Hasoon *et al.*, 2011; Lei *et al.*, 2012), *Pagrus major* (Imajoh *et al*., 2007; Ku *et al.*, 2010) and *Paralichthys olivaceus* (Tong *et al.*, 1997; Kang *et al*., 2003)

Susceptibility of cell lines to viral infection is the basis for isolating and characterising fish viruses. The cell line from the snout of red spotted grouper, *Epinephelus akaara*, was susceptible to turbot *Scophthalmus maximus rhabdovirus* (SMRV) and also frog *Rana grylio* virus 9807 (RGV_{9807}). The anti-RGV_{9807} and anti-SMRV serum produced signals in the cytoplasm of virus-infected GSC cells (Zhou *et al.*, 2007). It is characteristic of ranavirus and rhabdoviruses that their replications occur in an area of the cytoplasm that acts as a virus "factory" (Ni *et al*., 1996; Huang *et al*., 2006). *Siniperca chuatsi*, mandarin fish fry (MFF-1) cell line was used for the study of Infectious spleen and kidney necrosis virus (ISKNV). Immunofluorescence assay results showed that most of the rounding cells were stained by the recombinant MBP-ISKNV-VP23 antibody specific for ISKNV-VP23 (Dong *et al*., 2008).

2.15.2. Immunology

Cellular and molecular aspects of fish immunology have been investigated using both primary cell cultures and immortalised cell lines (Barnes *et al*., 2006). Three major B cell lineages have been described in teleost, which are those expressing either IgT or IgD, and the most common lineage which coexpresses IgD and IgM. The evolution of B cells from fishes and mammals have been revised recently (Sunyer *et al*., 2012) and also the effects of aquatic toxicants in the fish's innate immune system have been reviewed by Bols *et al.* (2001). A benefit of cell lines is cell type specificity, which allows researchers the ability to study the responses of one cell type in a controlled environment. RTS11, a rainbow trout macrophage-like cell line, has proven to be a valuable tool for studying immune cell– specific responses *in vitro*. RTS11 was initiated from a long-term haemaopoietic cell culture of a rainbow trout spleen (Ganassin and Bols, 1998). RTS11 cells secrete lysozyme and are able to phagocytose (Ganassin and Bols, 1998) and express major histocompatibility complex (MHC) class II β at the transcript level (Brubacher *et al*., 2000). RTS11 is also able to respond to lipopolysaccharide by expressing interleukin (IL)-1 β, tumour necrosis factor (TNF)-α and cyclooxygenase (cox)-2 (Brubacher *et al*., 2000). The role of two retinoid-related orphan receptor (ROR)-g homologues (ROR-gammaa1 and -gammaa2) genes expressed in rainbow trout skin was studied *in vitro*. Studies using trout cell lines demonstrated that ROR-g is induced significantly by LPS and down regulated by the presence of Poly I:C and recombinant interferon (IFN)-g. *In vivo* studies demonstrated that its expression was significantly higher in vaccinated versus unvaccinated fishes following bacterial (*Yersinia ruckeri*) challenge, but it was down regulated after a viral (VHSV) infection. This data suggest a potential role of trout ROR-g, a putative

TH17 transcription factor, in protection against extracellular bacteria (Monte *et al.*, 2012).

2.15.3 Biomedical research

Hightower and Renfro (1988) reviewed fish cell lines with a perspective on biomedical research which included epithelial ion transport, endocrinological studies, cellular stress (heat shock) response, thermo-tolerance, cancer biology, and environmental toxicology. Rakers *et al*. (2011) demonstrated that it was possible to integrate freshly harvested rainbow trout (*Salmo gairdneri*) scales into fish skin cell cultures, and antibody staining indicated that both cell types proliferated and started to build connections with the other cell types. This may be the first step to generate an "artificial skin" with two different cell types, and, in the future, similar studies could lead to the development of a three-dimensional test system (Rakers *et al.*, 2011). Melanoma cell cultures have been studied to unravel the processes leading to proliferation and differentiation of melanoma cells (Wakamatsu *et al*., 1984) and carcinogenesis (Fryer *et al*., 1981; Poulet *et al*., 1993). One of the rarest applications of fish cell culture is the use of Brokmann bodies (islets) from the teleost fish for transportation in to diabetic animals to normalise blood glucose level (Morsiani, 1995).

2.15.4 Transgene expression

Fish cell lines have been used to express exogenous DNA for a variety of objectives. Many have focused in identifying promoter or enhancer elements that function in fish cell culture, testing their relative strength for possible use in transgenic fish production (Friedenreich and Schartl, 1990). Other objectives include the selection of pluripotent cells, investigation of stress protection mechanisms and functional characterisation of recombinant fish proteins (Hong *et*

al., 2011; Rau *et al.*, 2004; Santos *et al.*, 2008). Promoters obtained from fishes that have been used to achieve transient, stable or inducible expression in fish cell cultures include those derived from the metallothionein, beta actin and alpha-globin genes (Olsson *et al*., 1990; Fu *et al*., 1991; Moav *et* al., 1992). The luciferase reporter gene under the control of dioxin- responsive enhancers was introduced into RTH-149 cells to generate the recombinant cell line, designated remodulated lightning trout or RTL 2.0 (Richter *et al*., 1997). A gene transfer approach using rainbow trout gonad (RTG-2) cells also has been developed to create an *in vitro* assay system for the detection of estrogenic compounds in environmental samples (Ackerman *et al*., 2002; Rutishauser *et al*., 2004). Similarly, the zebrafish embryo cell line, ZEM2s (zebrafish embryo 2-serum adapted), was transfected with plasmids containing a reporter gene under the control of either aromatic hydrocarbon, heavy metal, or electrophile response elements (Carvan *et al.*, 2000), so that expression from these transgenes indicated induction upon exposure to compounds belonging to each class of inducer. In another application of this technology, plasmid-based metallothionein expression was investigated in transfected CHSE-214 cells (Kling and Olsson, 2000), identifying this system in protecting cells against oxidative stress.

2.15.5 Biotechnology

Fish cell cultures have been used as biotechnological tools in aquaculture (Bols, 1991). Viral diseases affecting fish have brought into focus the importance of biotechnological products like vaccines (Noga and Hartmann, 1982; Yang *et al.*, 1992) and interferons both *in vivo* and *in vitro* (Rio *et al.*, 1973). Cloning has also been attempted to address questions concerning the evolution of B cell function (Miller *et al.*, 1994). Embryo cell cultures were used to drive stem cells for fish transgenesis (Collodi *et al*.,

1992) and also pluripotent embryonic stem cells for genome manipulation with many applications in marine biotechnology (Sun *et al*., 1995).

Caipang *et al*. (2005) studied the antiviral mechanism of JF IRF-1 using transfection experiments in the hirame natural embryo (HINAE) cell line (Kasai *et al.*, 2001) and the epithelial papilloma of carp (EPC) cell line (Fijan *et al*., 1983).

Epithelial integrity has been studied using apolipoprotein in rainbow trout gill cell cultures as a study in functional proteomics (Smith *et al.*, 2005). Culture conditions affect induction of vitellogenin synthesis by estradiol-17β in primary cultures of tilapia hepatocytes (Kim and Takemura, 2003). The incorporation and metabolism of (n-3) and (n-6) polyunsaturated fatty acids (PUFA) supplemented to growing cultures were studied in rainbow trout (RTG-2) and turbot (TF) cell lines (Tocher *et al*., 1989). Wang *et al.* (2003) studied the regulation of follistatin expression by gonadotropin in a primary culture of zebrafish ovarian follicle cells using semi-quantitative RT-PCR.

2.15.6 Toxicology

There are two types of cellular models, *i.e*. undifferentiated and differentiated cells, used in acute toxicity testing (Ekwall, 1983). Of these two types, the simple systems measuring basal cytotoxicity are probably the more useful in the sense that a central toxic effect is measured. Four useful parameters to evaluate common cellular responses include cytotoxicity, cell growth, genotoxicity and xenobiotic metabolism. Cell lines are more amenable to toxicogenomic technologies and up or down regulation of genes or proteins. The homogeneity of cell lines makes these responses to toxicants easier to detect and with less variability than whole organisms (Castaño *et al.,* 2003; Schirmer, 2006). Short-term primary cultures and cell lines from fish tissues have provided

successful cell culture systems for environmental toxicology (Babich and Borenfreund, 1991; Castaño and Tarazona, 1995; Castaño *et al*., 1996; Segner, 1998; Segner and Cravedi, 2001). RTgill-W1 cells have been used to evaluate the toxicity of industrial effluents, including petroleum refinery effluents, oil-sands process–affected waters and the toxicity of several compounds (Dayeh *et al*., 2005; Lee *et al*., 2008). Genotoxicity was assessed using comet assay, which detects DNA strand breaks. PLHC-1 hepatoma fish cell tested using both model chemicals (benzo[a]pyrene (B[a]P) and ethyl methanesulfonate (EMS) and extracts of sediment samples obtained with solvent dichloromethane/methanol. The highest level of DNA damage was determined after exposure to sediment extract obtained with the solvent mixture dichloromethane/methanol which extracts a wide range of contaminants (Srut *et al*., 2011). *In vitro* cytotoxicity of triton X-100 was assessed in a protozoan, two fish cell lines (RT gillW1 and RTL-W1) and mammalian cell lines using resazurin (commercially available as Alamar blue, AB), neutral red (NR; 3-amino-7-dimethylamino-2-methylphanzine hydrochloride) and propidium iodide which respectively monitored energy metabolism, lysosomal activity and membrane integrity. Protozoan, *Tetrahymena thermophile* was found to be most sensitive and may be used as an alternative to fish in toxicity testing (Dayeh *et al*., 2004).

Schirmer (2006) reviewed the so far established cell cultures and found that vertebrate cell lines can be compared well with fish lethality in their relative sensitivity towards the toxicity of chemicals. Given strong correlations of r-values for cell lines versus fish acute toxicity, cell cultures could provide excellent tools as cell-based biosensors (Brennan *et al.*, 2012; Kubisch *et al.*, 2012). Ecotoxicological effects following exposure to heavy metals *in vitro* have been reported by a number of researchers (Mazon *et al*., 2004; Shuilleabhain *et al*., 2004; Taju *et al*., 2013; Rakers *et al*., 2014; Nguyen *et al*., 2015).

2.16 Repository of fish cell lines

Approximately 10 times as many fish cell lines have been described in the literature as have been placed in repositories. Lists of these have been prepared at several points over the last 30 years (Wolf and Mann, 1980; Fryer and Lannan, 1994; Lakra *et al*., 2010c). While 29,000 species of bony fish in over 500 families have been classified, cell lines have been developed from only about 75 species in 35 families. In the past, methods of confirming that a cell line was indeed from the stated starting species were problematic, but now species confirmation can be done reliably by DNA barcoding. Over 10 different cell lines have been developed from each of the several species, including catfish (*Ictalurus punctatus*), rainbow trout (*Oncorhynchus mykiss*), and zebrafish (*Danio rerio*). The total number of fish cell lines in the literature is now reaching close to 200, but many of these have likely been lost. Yet others are still available from the community of scientists developing and using piscine cell lines (Bols *et al.*, 2011).

At the fall of 2002, the German Collection of Microorganisms and Cell Cultures had 4 cell lines representing 2 species and Istituto Zooprofilattico Sperimentale (IZSBS). Centro Substrati Cellulari, Italy, had 12 fish cell lines representing 8 species and Riken Cell Bank (RIKEN). The Institute of Physical and Chemical Research, Japan, had 12 fish cell lines representing 5 species. To date, American Type Culture Collection (ATCC) has listed 43 cell lines of aquatic animals, and only 17 fish cell lines are usable and available for dissemination to the researchers globally. The European Collection of Cell Cultures (ECACC) currently holds 21 fish cell lines. Altogether ~283 cell lines have been established from finfish around the world. If all the established cell lines would have been deposited in repositories, it would be beneficial to the international research community in order to use those cell lines as they are the

best alternative to the whole animal research (Bols *et al*., 2005; Lakra *et al*., 2010c).

More recently, researchers interested in preserving rare and endangered fish species or strains have identified the importance of banking fish cell cultures as a source of genetic material to compensate for the difficulty with cryopreserving fish sperm, oocytes and embryos (Rawson, 2012).

Currently, in India the Department of Biotechnology (DBT), New Delhi has funded through the counsel of the task force on Aquaculture and Marine Biotechnology and established a National Repository for fish cell lines (NRFC), based at the ICAR-National Bureau of Fish Genetic Resources, Lucknow. NRFC hosts about 50 cell lines which have been developed at various laboratories and will be available for academic and industrial work (Nagpure *et al.*, 2016).

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Chapter

3

Materials and Methods

Contents

3.1 Materials

3.1.1 Cell culture media, additives and supplements

Amphotericin B (Sigma Aldrich, USA)

DMEM (Delbecco's Minimum Essential Medium) (Invitrogen, USA)

EDTA (GIBCO, USA)

Fetal bovine serum (Thermo Scientific, USA)

Leibovitz's L-15 (Invitrogen, USA)

M199 (Invitrogen, USA)

Penicillin G (Sigma Aldrich, USA)

Streptomycin (Sigma Aldrich, USA)

Trypsin (GIBCO, USA)

3.1.2 Reagents and molecular biologicals

2- propanol (Sigma Aldrich, USA)

10x PCR buffer (Sigma Aldrich, USA)

100 bp DNA ladder (Bangalore Genei, India)

Acetic acid (SRL)

Agarose (Lonza, USA)

Bovine serum albumin (Sigma Aldrich, USA)

Colchicine (MERCK)

Dimethyl sulphoxide (Sigma Aldrich, USA)

DNA polymerase (Thermo Scientific, USA)

dNTPs (Sigma Aldrich, USA)

Ethidium bromide (Sigma Aldrich, USA)

Goat serum (PAN)

Magnesium chloride (Sigma Aldrich, USA)

Methanol (MERCK)

Paraformyldehyde (Sigma Aldrich, USA)

Phenol: Chloroform: Isoamyl alcohol ($CHCHI_3$/ IAA) 24:1 (Sigma Aldrich, USA)

Potassium chloride (SRL)

Prolong Gold antifade reagent (Invitrogen, USA)

Proteinase K (Sigma Aldrich, USA)

RNase (Sigma Aldrich, USA)

SDS (Sigma Aldrich, USA)

Sodium hypochlorite (SRL, India)

Tris-HCl (Sigma Aldrich, USA)

Tris-Acetate EDTA buffer (Sigma Aldrich, USA)

TritonX 100 (Sigma Aldrich, USA)

Trypan blue (SRL, India)

3.1.3 Plasticwares and disposables

1.5 mL centrifuge tubes (Genaxy, USA)

15 mL centrifuge tubes (Corning, USA)

50 mL centrifuge tubes (Corning, USA)

2 mL cryovials (Corning, USA)

Cryocanes (Nalgene, USA)

Cell culture dishes – 6 well, 12 well and 24 well (Corning, Eppendorf, Greiner, BD Falcon)

Cell culture flasks - 25cm^2 and 75cm^2 (Corning, Eppendorf, Greiner, BD Falcon)

Cryosleeves (Nalgene, USA)

Chamber Slides (Thermo Scientific, USA)

Glass slides (Labtek, India)

Membrane filters 0.45µm, 0.2µm and 0.1µm

Serological pipettes (Eppendorf/Corning)

3.1.4 Equipment

4˚C Refrigerator (LG, India)

-20˚C Freezer (Siemens, Germany)

Autoclave (KEMI, India)

CO_2 incubator (NuAire, USA)

Gel documentation system (Biorad, USA)

Hemacytometer, double chamber (Sigma, USA)

Hot air oven (KEMI, India)

Hot plate (Tarson, India)

Liquid nitrogen storage cans or dewars (Thermo Scientific, USA)

Microscope, inverted (Nikon, Japan)

Milli-Q Synthesis water system (Millipore, USA)

Mini gel electrophoresis system (SCIE-PLAS, UK)

Mr. Frosty cryogenic controlled rate freezing container (Lonza, USA)

Nanodrop spectrophotometer (Thermo Scientific, USA)

PCR thermocycler (Thermo Scientific, USA)

pH meter (Eutech, Singapore)

Research microscope (Leica, Germany)

Refrigerated centrifuge 5801R (Eppendorf, Germany)

Refrigerated incubator (Barnstead Labline, USA)

Shaker incubator (Barnstead Labline, USA)

Sterilisation filter units 500 mL and 250 mL (Nalgene, USA)

Ultra low freezer (Thermo Scientific, USA)

Vertical laminar flow hood (Labline, India)

Water bath (Barnstead Labline, USA)

3.1.5 Experimental fish

Normal and apparently healthy specimens of *P. caeruleus* (body weight: 3±0.25g; total length: 4.5 cm) were collected from the coastal waters of Mandapam in Tamil Nadu along the southeast coast of India and transported live under oxygen packing to the wet laboratory of the ICAR-Central Marine Fisheries Research Institute, Kochi, Kerala.

3.2 Methods

3.2.1 Initiation of primary cultures

3.2.1.1 Acclimatisation of fishes

The fishes were acclimatised for a period of one week under captive conditions, (temperature 26˚C - 28˚C and salinity 30-32‰) in circular fibre glass tanks having *in situ* biological filtration system on a diet of marine, pelleted dry feed (Varna). About 10% of the rearing water along with faecal matter and excess feed was siphoned off daily and was replaced with fresh seawater. The fishes were subsequently transferred to rectangular perspex tanks holding 20 L of well aerated and dechlorinated seawater (30 ‰).

3.2.1.2 Preparation of fish and tissue collection

The fishes were starved for two days prior to primary culture to reduce the gut bacterial load and to avoid the possibility of gross contamination from faeces and unutilised feed. Fishes were then maintained overnight in sterile, aerated seawater containing 1000 IU mL^{-1} penicillin and 1000 µg mL^{-1}

streptomycin. Prior to sacrifice, the fishes were tranquilised by plunging in iced water for 1 - 2 min, then disinfected in sodium hypochlorite (500 ppm available chlorine) for 10 s, washed in sterile seawater and swabbed with 70% propanol. For sacrifice, an incision was made at the dorsal artery and the tissue samples from various organs such as fin, gill, caudal peduncle, heart, liver, spleen, kidney and brain were aseptically excised. Similar tissues were pooled and transferred to sterile petriplates containing phosphate buffered saline (PBS, pH 7.2) having 500 IU mL^{-1} penicillin, 500 µg mL^{-1} streptomycin and 1.25 µg mL^{-1} amphotericin B. Subsequently, the tissues were washed thrice in the same medium prior to primary culture. The tissues collected were minced into small fragments using a sterile surgical scalpel and again washed in serum- free medium containing 500 IU mL^{-1} penicillin, 500 µg mL^{-1} streptomycin and 1.25 µg mL^{-1} amphotericin B. Primary culture was done using explantation and trypsinisation techniques (Table 2.1).

Table 3.1. Techniques employed to develop primary cell culture systems from the caerulean damsel, *P. caeruleus*

Tissues taken	Techniques employed to initiate primary cultures	Number of attempts
Fin	Explantation and trypsinisation	3
Gill	Explantation and trypsinisation	3
Caudal peduncle	Explantation and trypsinisation	1st and 2nd attempts - explantation; 3rd attempt trypsinisation
Liver	Explantation and trypsinisation	1st attempt - explantation; 2nd and 3rd attempts : trypsinisation
Heart	Explantation	3
Brain	Explantation	3
Kidney	Explantation	3
Spleen	Explantation	3

3.2.1.3 Tissue culture media

Three different media *viz*., Leibovitz's L-15, Dulbecco's modified Eagle's medium (DMEM) and Medium 199 (M199) was used in the study. When DMEM and M199 were used, the cultures were incubated in a CO_2 incubator (NuAire, USA) with 5% CO_2 supply. The medium was reconstituted in Milli Q synthesis grade water (pH 7.2± 0.2), filter sterilised (0.2µm), dispensed into sterile screw cap bottles and stored at 4ºC until use. For preparation of the growth medium, sterile FBS was used (20% v/v). Antibiotics such as penicillin and streptomycin (100 IU mL^{-1} and 100 µg mL^{-1} respectively) and the fungizone, amphotericin B (0.125 µg mL^{-1}) were supplemented.

3.2.1.3.1 Additives/Supplements

3.2.1.3.1.1 Fetal bovine serum (FBS)

Serum is the major supplement to the growth media. For preparation of the growth medium, sterile, mycoplasma screened FBS was used at a concentration of 20% (v/v) after inactivation at 56˚C for 30 min in a water bath.

3.2.1.3.1.2 Fish muscle extract (FME)

Homologous fish muscle extract was prepared as described by Kumar *et al*. (1998). Twenty grams of muscle tissue was extracted from *P. caeruleus*, then homogenised in 200mL of PBS (pH 7.2) and centrifuged at 4629g for 5 min. The supernatant was inactivated at 56˚C for 30 min and centrifuged again at 4629g for 10 min to remove the coagulated proteins. The supernatant was then sterilised by passing through a membrane filter (0.2µm) and stored at 4-20˚C till use. FME was added at the rate of 20% to the growth medium.

3.2.1.3.1.3 Fish serum

Both homologous and heterologous fish sera were prepared from the fishes *P. caeruleus* and *Epinephelus malabaricus* respectively. Blood was allowed to clot by leaving undisturbed at room temperature for 15-30 min and stored at 4˚C in glass test tubes. The clot was removed by centrifuging at 4629g for 10 min at 4˚C. Fish serum was inactivated at 56˚C for 30 min and sterilised by membrane filtration (0.2µm) and stored at -20˚C. Heat inactivated sterilised fish serum was incorporated at a rate of 1% in the growth medium.

3.2.1.3.1.4 Conditioned media

Culture media from several flasks containing good growth of cell monolayer was pooled and later filter sterilised by membrane filtration (0.2 µm). Sterile conditioned medium was incorporated at the rate of 50% of the growth medium.

3.2.1.3.1.5 Antibiotics and fungizone

Antibiotics such as penicillin (100 IU mL^{-1}) and streptomycin (100 µg mL^{-1}) and fungizone, 0.25 µg mL^{-1} amphotericin B were used in the preparation of the complete growth medium.

3.2.1.4 Explantation

The tissue samples were minced into $1mm^3$ pieces in Leibovitz' L-15 with 500 IU mL^{-1} penicillin, 500 µg mL^{-1} streptomycin and 2.5 µg mL^{-1} amphotericin B. Approximately 25 tissue fragments were explanted uniformly into serum coated 25 cm^2 tissue culture flasks (BD Falcon Primaria) following a 4-5 h incubation at room temperature for the tissues to attach. Similarly, the explants were also placed in flasks that were coated with gelatin and laminin respectively. Excess high antibiotic media was aspirated and 2 mL of fresh

growth medium containing 20% FBS, 200 IU mL^{-1} penicillin, 200 µg mL^{-1} streptomycin and 0.25 µg mL^{-1} amphotericin B was added to each flask and incubated at 28 ± 2°C in a refrigerated incubator (Barnstead Labline, USA).

3.2.1.5 Trypsinisation

The tissue samples were minced into 1-2 mm^3 pieces in Leibovitz' L-15 with 500 IU mL^{-1} penicillin, 500 µg mL^{-1} streptomycin and 2.5 µg mL^{-1} amphotericin B using sterile scalpel/scissors and transferred to sterile glass beakers containing 'trypsin digestion solution' (0.25% trypsin - 0.02% EDTA) in D-PBS without Ca^{2+} and Mg^{2+}. The softer tissues (*i.e.*, spleen, heart, kidney, liver and brain) were gently agitated for 10 min whereas fin, caudal peduncle and gill tissues were agitated for 20 min with a sterile magnetic stirrer bar at 28 ± 2°C. After settling of the larger undigested tissue pieces, the supernatant containing the dissociated cells was transferred into an equal volume of growth medium (L-15) containing 20% FBS, 100 IU mL^{-1} penicillin, 100 µg mL^{-1} streptomycin and 0.25 µg mL^{-1} amphotericin B and mixed well to inhibit trypsin activity. The resultant cells were centrifuged at 200 g for 10 min (Eppendorf, 5810R) and the pellet re-suspended in fresh complete medium (pH 7.2±0.2). Prior to seeding, the concentrations of viable cells were determined by using trypan blue exclusion method using a haemocytometer (Phillips, 1973). The cells were seeded at a concentration of 1 x 10^6 cells mL^{-1} into 25 cm^2 tissue culture flasks (Falcon Primaria).

3.2.1.6 Microscopic examination

The cells in primary culture were monitored on attachment, growth, proliferation as well as signs of deterioration such as contamination by fungus or bacteria using an inverted microscope (Nikon TS 100). Morphology of the cells was observed and recorded using a dedicated digital camera attached to

the inverted microscope. All cultures were examined during the initial 24-72 h post- seeding.

3.2.2 Development of cell lines

3.2.2.1 Subculture

At 2 days post-seeding flasks were gently washed twice with complete growth medium to remove non-adherent cells. Media were subsequently changed every 3-5 days and replaced by complete growth medium supplemented with 50% v/v conditioned medium from the primary cultures passed through a 0.2 µ filter. This step was followed until the cells were passaged for the first time. Thereafter cells were allowed to proliferate until confluent monolayers were formed.

For subculturing, cell monolayers were rinsed with sterile D-PBS and then incubated with 1-2 mL of trypsin- EDTA (0.05% trypsin and 0.02% EDTA in D-PBS) until the cells got detached from the flask surface. To the detached cells, fresh medium was added to neutralise the action of trypsin before being distributed to new flasks. The flasks were then incubated at 28±2ºC and observed for growth, cell attachment and formation of confluent monolayers using an inverted microscope (Nikon TS 100).

When a new confluent monolayer was obtained, the content of one flask was transferred after trypsinisation to two new flasks (1:2). The spilt ratio increased to 1:3 and the serum was reduced to 10% FBS and gradually to 5% in the culture medium as the cultures were progressively passaged. Similarly, beyond 10 passages penicillin/ streptomycin as well as amphotericin B were omitted. The cultures were monitored daily and subcultured on reaching confluence.

3.2.2.2 Cryopreservation

The cells in logarithmic phase were harvested and counted using a hemocytometer. The viability before freezing was checked employing trypan blue dye exclusion technique. The harvested cells were resuspended in cold freezing medium, Leibovitz's L-15 medium containing 40% FBS and 10% dimethyl sulphoxide (DMSO; Sigma, USA) to a final concentration of 3 x 10^5 viable cells mL^{-1}. The cell suspension was dispensed into sterile plastic cryovials labelled with cell line code, passage number and date. First, the sealed cryovials were kept at 4ºC for 30 min to allow DMSO to equilibrate and then transferred to a Mr. Frosty freezing container (Nalgene, USA), and refrigerated at -80ºC overnight (a process in which the temperature decreased at a rate of -1ºC min^{-1}). Finally, the cryovials were transferred to liquid nitrogen containers (-196ºC; Thermo Fischer Scientific, USA) for long term storage.

3.2.2.2.1 Revival of cryopreserved cells

For recovering cells, the cryovials were taken out from liquid nitrogen, rapidly thawed in a water bath at 42ºC, the freezing medium was removed by centrifugation and the pellet resuspended in 2.5mL of Leibovitz's L-15 medium supplemented with 20% FBS. The suspension was pipetted gently to disperse the cells prior to seeding into 25 cm^2 culture flasks, and incubated at 28 ± 2ºC.

3.2.2.3 Determination of optimal growth conditions

3.2.2.3.1 Comparison of media and supplements

Attachment and growth response were investigated using three kinds of culture media. Disassociated cells were harvested by centrifugation at 279g for 7min and were seeded into three sets of three flasks each. Each of the three flasks was incubated with a different culture media (DMEM, L-15 and M199)

supplemented with FBS (20%), penicillin (100 IU mL^{-1}) and streptomycin (100 µg mL^{-1}) and the fungizone amphotericin B (0.25 µg mL^{-1}). Attempts to improve cell growth with the use of homologous serum (1%), fish muscle extract (FME) (20%) and heterologous serum (1%) (FS) were undertaken along with the media comparison experiments. The resulting cultures were examined and the optimal media was used as the basic culture media for all remaining tests.

3.2.2.3.2 Determination of optimal serum concentration(s)

Cells at a concentration of 3 x 10^4 were seeded in 25cm^2 cell culture flasks having L-15 containing 10% FBS, 100 IU mL^{-1} penicillin, 100 µg mL^{-1} streptomycin and 0.25 µg mL^{-1} amphotericin B and incubated at 28±2°C overnight. The next day, medium was removed, the cells were washed once with PBS and fresh culture medium containing 2, 5, 10 and 20% FBS was added, with each FBS concentration in triplicate flasks. The flasks were incubated at 28±2°C and observed for 7 days. At 24 h intervals, the relative number of viable cells in triplicate flasks in each set was estimated microscopically using a haemocytometer.

3.2.2.3.3 Determination of optimum temperature(s)

To assess the growth response at different temperatures, cells at a concentration of 3 x 10^4 were seeded in 25cm^2 cell culture flasks having L-15 containing 10% FBS, 100 IU mL^{-1} penicillin, 100 µg mL^{-1} streptomycin and 0.25 µg mL^{-1} amphotericin B and incubated at 28±2°C overnight. The next day, cells were incubated (refrigerated incubator, Barnstead Labline, USA) at 16˚C, 20˚C, 24˚C, 28˚C and 32˚C in 25 cm^2 cell culture flasks at an initial concentration of 3x10^4 cells mL^{-1} in L-15 media containing 10% FBS. Every 24^{th} h post-subculture, cell monolayers in duplicate flasks at each temperature

were harvested using trypsin-EDTA and cell density was estimated using a haemocytometer.

3.2.2.4 Quantitation

3.2.2.4.1 Growth curve

The three cell lines derived from the fin (PC1F1Ex), liver (PC1L1Tr) and caudal peduncle (PC1CpTr) cells at the same passage 60 were used to plot the growth curve. A confluent culture was harvested and transferred to fresh culture medium to make a final concentration of approximately 1 x 10^5 cells mL^{-1} and seeded into T-25 cm^2 culture flasks (Corning, USA). At each 24h interval, cells from three flasks were harvested using trypsin-EDTA and counted using a hemocytometer. The growth curves were plotted and the population doubling times of the 3 cell lines were calculated during the exponential growth phase of the cells. All experiments were repeated at least twice using the same density of cells.

3.2.2.4.2 Plating efficiency

Plating efficiency experiments were performed according to the methods described by Ham and Puck (1962), cells were trypsinised, counted and diluted so as to plate 2000, 1000, 500, 200, 100 and 50 cells per well in 6 cm dishes (Corning, USA), in 1mL L-15 medium containing 10% FBS. Triplicates were used for each cell density. The medium was replaced thrice a week. At 14 days, the cultures were rinsed, fixed with anhydrous methanol and stained with Giemsa stain, and the colonies were counted by field of view with a grid superimposed on the culture plate. Triplicate experiments were performed independently.

Plating efficiency was calculated using the formula:

$$Plating\ efficiency = \frac{No.of\ colonies}{No.of\ cells\ seeded}\ x100$$

3.2.2.4.3 Seeding efficiency

To optimise the seeding density, PC1F1Ex, PC1L1Tr and PC1CpTr cells were seeded onto T-25 cell culture dishes at defined densities of 2 mL of various densities of cells per flask (1 x 10^4, 3 x 10^4, 1 x 10^5, and 1 x 10^6). Cells were enumerated using a hemocytometer, 6 to 8 h after seeding.

$$Seeding\ efficiency = \frac{No.of\ cells\ attached}{No.of\ cell\ seeded}\ x\ 100$$

3.2.3 Characterisation of the cell lines

3.2.3.1 Staining with giemsa to visualise cell morphology

Monolayers with 90% confluence were rinsed with PBS, fixed in methanol (Merck) for 10 min, thereafter stained with undiluted Giemsa (Merck) for 2 min (Freshney, 2010). The flasks were then rinsed in distilled water and examined under an inverted microscope (Nikon TS100) and photomicrographs were taken using a dedicated digital camera attached to the microscope.

3.2.3.2 Chromosome analysis to determine transformation

PC1F1Ex at passage 70, PC1CpTr at passage 65 and PC1L1Tr at passage 73 were used for the study. The cell lines were grown in 2% L-15 and were in the log phase of growth. Higher passage cultures were taken to determine transformation by determining deviation from the original chromosome number of the species. Cultures of the caerulean damsel, in the logarithmic phase of growth, were incubated for 2 h with colchicine (GIBCO, USA) at a final concentration of 1 μg mL^{-1}. Following this, the cells were

detached from the culture flasks and pelleted by centrifugation at 279g for 7 min (Eppendorf-5810R, Gemany) before being gently resuspended in a hypotonic solution of 0.075 M potassium chloride. The cell suspension was incubated for 30 min at 28˚C and then fresh fixative solution (0.5mL of Carnoy's fixative; 3:1 methanol to acetic acid) was added. The cell suspension was then pelleted by centrifugation at 279g for 7 min. The cell pellet obtained was then resuspended in fresh fixative and the cells were centrifuged again. This process was repeated three times. Finally, aliquots of the cell pellet were dropped onto chilled microscope slides and stained with 5% Giemsa stain. Metaphase spreads were visualised using a compound microscope (Leica LB2, Germany). Chromosome counts were performed for 50 metaphase plates.

3.2.3.3 Analysis of cell type (epithelial/fibroblast) markers using immunofluorescence staining

The cells were grown in 4 well chamber slides (NUNC, USA) to exponential growth phase, fixed with 7.4% p-formaldehyde for 20 min, permeabilised in 0.1% Triton X-100, blocked with 5% goat serum and incubated for 1h at RT with primary antibodies. The following primary antibodies were applied: Mouse monoclonal antibodies directed against fibroblast markers *viz.*, fibroblast surface protein (Sigma Aldrich, USA), monoclonal anti-vimentin antibody (Sigma Aldrich, USA) and the epithelioid cell markers like cytokeratin (Sigma Aldrich, USA). The respective primary antibodies were diluted in a ratio of 1:75 in 0.1% bovine serum albumin (BSA) in PBS. Subsequently, samples were incubated with the secondary antibodies, goat antimouse IgG FITC (Sigma Aldrich, USA), at a dilution of 1:50 for 1h at RT. Nuclei were stained with 4', 6'- diamidino-2-phenylindole (DAPI) (Sigma Aldrich, USA) for 15 min before mounting with Prolong Gold antifade reagent (Invitrogen, USA). Negative controls were incubated with

blocking buffer instead of primary antibody. The slides were examined using a fluorescence microscope (Leica, Germany).

3.2.3.4 Authentication of the cell lines by sequence analysis of mitochondrial CO1 gene

Authentication of the cell lines to confirm the species of origin was done by amplification and sequencing of the mitochondrial cytochrome c oxidase subunit I (COI) gene as described by Cooper *et al.* (2007) with minor modifications using a PCR primer cocktail developed for fishes as described by Ivanova *et al.* (2007). The universal primer pair described by Ward *et al.* (2005) were used (Table 3.2). Cells at passage 70 were collected by enzymatic dispersion as described for the propagation of cells. Template DNA for PCR analysis was extracted from the cell lines and from the donor fish muscle tissue samples by salting out and phenol chloroform method respectively (Sambrook and Russell, 2006).

Briefly, the samples were homogenised separately in NTE buffer (0.2 M NaCl, 0.02 M Tris–HCl, 0.02 M EDTA, pH 7.4) and centrifuged at 3000g at 4°C, after which the supernatant fluids were placed in fresh centrifuge tubes together with an appropriate amount of digestion buffer (100 mM NaCl, 10 mM Tris–HCl, pH 8.0, 50 mM EDTA, pH 8.0, 0.5% sodium dodecyl sulfate, 0.1 mg mL^{-1} and 0.4 mg mL^{-1} proteinase K). After incubation at 65°C for 2 h, the digests were deproteinised by successive phenol/chloroform/isoamyl alcohol extraction.

Each PCR reaction was carried out with a total reaction mixture of 25µL containing both forward and reverse primers (10 µm, 0.5 µL each), $MgCl_2$ (25mM, 1.5 µL), dNTPs (2 mm, 2.0 µL), PCR buffer (10X, 2.5 µL), Taq DNA polymerase (1U, 0.5 µL), template DNA (0.3–0.4 µg) and nucleic

acid-free water. PCR cycling profile included an initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 45s, annealing temperature of 50°C (for CO1) for 30s, extension at 72°C for 45s and a final extension at 72°C for 5 min. The PCR products were electrophoresed in 1.5% agarose gels (Lonza, USA) containing ethidium bromide (Sigma, USA) and visualised in a gel documentation system (Biorad, USA). The amplified products were cleaned up using PCR product purification kit (Qiagen, USA) and were sequenced with the same set of primers in Applied Biosystems AB 3730 XL capillary sequencer following manufacturer's instructions at the sequencing facility. The raw DNA sequences were edited using the software BioEdit sequence alignment editor version 7.0.5.2 (Abbott Molecular, Carlsbad, CA) (Hall, 1999). Sequence analysis for species identification used both the Barcode of Life Data (BOLD: Ratnasingham and Hebert, 2007) system (http://www.barcodinglife.org) as well as the NCBI BLAST search engine (http:// www.ncbi.nlm.nih.gov/BLAST).

Table 3.2: CO1 gene primers used in barcoding to confirm the species of origin

Gene	Primer Sequence		Reference
COI	F1	5'TCAACCAACCACAAAGACATTGGCAC 3'	Ward *et al.* (2005)
	R1	5'TAGACTTCTGGGTGGCCAAAGAATCA 3'	

The partial sequence (640bp) of the CO1 gene of the cell lines (PC1CpTr, PC1F1Ex and PC1L1Tr) and caerulean damsel muscle tissue were deposited in the International Sequence Database (INSD) of the National Centre for Biotechnology Information (NCBI).

3.2.3.5 Detection of inherent contaminants

The cell lines developed from the caerulean damsel (PC1CpTr, PC1F1Ex and PC1L1Tr) were checked for presence of inherent viruses or mycoplasmas by Transmission electron microscopy and the cells were also screened for mycoplasmas using the MycoFluor™ Mycoplasma Detection Kit (Sigma, USA)

3.2.3.5.1 Transmission electron microscopy

Confluent flasks were trypsinised and the harvested cells were given a wash with 0.1 M phosphate buffer (PB). The pellet was agitated, fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M PB for 10 h at 4˚C. The suspension was mixed occasionally to avoid clumping of cells. After incubation, the fixative was removed and the cells were washed thrice with PB. Cells were post-fixed in 1% OsO_4 for 1h at 4˚C, dehydrated in an ascending grade of acetone, infiltrated and embedded in araldite CY 212 (TAAB, UK). Thick sections (1 µm) were cut with an ultramicrotome (Leica Ultracut – UCT), mounted on to glass slides, stained with aqueous toluidine blue and were observed under a light microscope for gross observation of the area and quality of the tissue fixation. For electron microscope examination, thin sections (70-80 nm) were cut and mounted onto 300 mesh-copper grids. Sections were stained with alcoholic uranyl acetate and alkaline lead citrate, washed gently with distilled water and observed under a Morgagni™ 268D transmission electron microscope (Fei Company, The Netherlands) at an operating voltage 80 kV. Images were digitally acquired using a CCD camera (Megaview III, Fei Company) attached to the microscope.

3.2.3.5.2 Screening for mycoplasma

The status of mycoplasma contamination of cultured cells and routinely used media was determined by MycoFluor™ Mycoplasma Detection Kit (M-7006, Sigma, USA), according to the manufacturer's standard protocol. Briefly, cells were seeded into 4 well chamber slides to form monolayers with 90% confluence. The cells were then rinsed with PBS, fixed in methanol:acetic acid, stained with mycofluor reagent and incubated for 10 min at RT. The chamber wells were detached after removal of the mycofluor reagent and coverslip with 10µL of Prolong Gold antifade mounting reagent was added and sealed in place with the sealant. The slides were examined under ultraviolet fluorescence (Leica LB2) at 100X magnification. The microscopic appearance of the cell lines were compared with that of the MORFS (Microscopic Optical Replicas for Fluorescence assays) and controls, examining for extra-nuclear fluorescence. To prepare positive control slides with mycoplasma MORFS, live cells are fixed in methanol:acetic acid and 5 µL of MORFS stock suspension is added to the chamber slide and sealed as mentioned above. To prepare control slides, 5 µL of MORFS stock suspension is added to a coverslip and mounted on to a clean slide and sealed as mentioned.

3.2.4. Evaluation of the established cell lines for gene expression studies and cytotoxicity assays

3.2.4.1 Gene expression studies using GFP reporter gene

The caerulean damsel cell lines were tested for transfection efficiency using pcDNA3-EGFP plasmid (Addgene) which contains a cytomegalovirus (CMV) promoter, a SV40 polyadenylation signal and a gene for the resistance to neomycin. The cell lines PC1F1Ex, PC1CpTr and PC1L1Tr were seeded at a density of 0.5-2 x 10^5 cells in 500 µL of growth medium in 4 well chamber

slides (NUNC, USA). Monolayers with 90% confluence were transfected using 2 different transfection reagents: JetPRIME (Polyplus transfection, France) and Lipofectamine 3000 (Thermo Scientific, USA), according to the respective manufacturer's protocols: Briefly, for transfection with Jet PRIME reagent, 1.5 µg of DNA was diluted into 50 µL of JetPRIME buffer and 3 µL of JetPRIME transfection reagent were added into the diluted DNA mixture. Then, the two solutions were mixed together and incubated at room temperature for 10 min.

In the case of transfection with Lipofectamine 3000, 1.5 µg of DNA and 3µL of P3000 reagent were diluted in 50µL of Opti-MEM. Also, 0.75µL of Lipofectamine 3000 reagent was diluted in 25µL of Opti-MEM. Then, the two solutions were mixed together and incubated at room temperature for 15 min.

Subsequently, each transfecting solution was directly added to respective cell cultures grown to 80% confluence. Medium was completely replaced 24h post-seeding. The cells were incubated for 72 h, and then mounted in Prolong Gold antifade reagent. Finally, green (EGFP) fluorescent signals were visualised and images were captured using a Leica LB2 microscope and analysed using imaging software.

3.2.4.2 Cytotoxicity assays to evaluate susceptibility to bacterial extracellular products (ECPs)

3.2.4.2.1 Bacterial growth conditions

A fish pathogenic strain of the bacterium *Vibrio alginolyticus* (Lab Strain V4) was used for the study. Bacteria were cultured in nutrient agar (NA, Hi Media, India) slants containing 3% NaCl at 28˚C for 18 h. Single colonies from agar slants were transferred to 5 mL tryptic soy broth (TSB, Hi Media, India) and grown at 28˚C for 18 h.

3.2.4.2.2 Extraction of ECPs from *Vibrio alginolyticus* (Lab strain V4)

The ECP from *V. alginolyticus* (V4) were obtained by a cellophane overlay method as described by Liu (1957). In brief, this growth technique allows the bacteria to draw nutrients freely from below the cellophane sheet, and at the same time prevent mixing of ECP proteins released by the bacteria with the proteins present in the underlying culture medium. Tryptic soy agar (TSA) plates overlaid with sterile cellophane sheet were inoculated with bacterial suspension from the 18 h TSB culture using sterile cotton swab. The plates were incubated at 28˚C for 48 h. Bacteria grown on the cellophane sheet overlay were washed into 2 mL of PBS in to a sterile centrifuge tube and centrifuged for 30 min at 13000 *g*. Following centrifugation, the supernatant containing ECP was filter sterilised firstly using a 0.4 µm membrane filter followed by 0.2 µm filter and stored at -80˚C until required. The protein concentration of bacterial ECP was determined using a Nanodrop spectrophotometer (Thermo Scientific, USA).

3.2.4.2.3 Cell culture

All the three cell lines developed from *P. caeruleus* were tested for susceptibility to bacterial ECP from *V.* alginolyticus. Cells in the logarithmic growth phase having 80-90% confluency were used for cytotoxicity assay. Cells were exposed to six different concentrations of bacterial ECP (1µg ml^{-1}, 3 µg ml^{-1}, 5 µg ml^{-1}, 7 µg ml^{-1}, 9 µg ml^{-1}, 11 µg ml^{-1}). Six replicate wells were used for each control and test concentration per microplate. Exposure to bacterial ECP was conducted for 48h on each of the three cell lines. After this incubation period, morphological damage was evaluated in comparison to the controls. The test medium was removed; cell monolayers washed with phosphate buffered saline (PBS) and prepared for the cytotoxicity assays. Phase contrast micrographs were taken after 48h and the morphologies were compared to the control cells.

3.2.4.2.4 XTT assay to determine viability after exposure to bacterial ECPs

XTT (2, 3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]- 2H- tetrazolium hydroxide, sodium salt) (Sigma, USA) was prepared at 1mg mL^{-1} in prewarmed (37˚C) DPBS. PMS (Sigma) was prepared at 5mM (1.53 mg mL^{-1}) in PBS. The 5mM PMS solution was stable at 4˚C for at least 3 months. Fresh XTT and PMS were mixed together at the appropriate concentrations. For a 0.025mM PMS-XTT solution, 25µL of the stock 5mM PMS were added per 5mL of XTT (1mg mL^{-1}). Fifty microliter of this mixture (final concentration, 50µg of XTT and 0.38µg of PMS per well) were added to each well on day 2 after cell inoculation. After 6h incubation at 28˚C, the plates were mixed on a mechanical plate shaker and absorbance at 450nm was measured with a spectrophotometer (Cary 50, Varian).

The spectrophotometric readings from cell viability assays were recorded as absorbance units (OD). The background absorbance of each dye (no-cell controls) was subtracted from the ODs from wells with cells. Cell survival was expressed as "% to the control", where the control was set to 100%. Means from independent experiments were calculated and plotted using GraphPad Prism version 7.0 (GraphPad Software, San Diego, California, USA). For calculation of the concentration that led to 50% reduction in cell viability (EC_{50}), the non-linear regression sigmoidal dose-response curve fitting module using the Hill slope equation of GraphPad Prism was applied and concentration-response curves were drawn. The bottom and top values of the concentration-response curves were fixed to 0 and 100% respectively. Concentration-response curves were based on XTT assay and six different concentrations. EC_{50} values are expressed as mean of three independent replicates.

3.2.5 Statistical analyses

The data from quantitation studies were statistically evaluated by one-way analysis of variance (ANOVA) using SPSS/17.0 software. Comparison of means was carried out at 5% level of significance ($p<0.05$).

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Chapter

4

Results

Contents

4.1 Preparation of tissues to initiate primary cultures

The donor fish was acclimatised for a week, to reduce stress which is known to cause the release of organic materials (secretions and excretions, such as mucus and excrements). Although fed to satiation daily, two-three days before dissection the fish were starved to reduce the gut bacterial load and the possibility of gross contamination from faeces and unutilised feed. The fish were further treated with sterile seawater containing antibiotics overnight and dipped in sodium hypochlorite (500 ppm available chlorine) to decontaminate the external surface prior to dissection for the removal of tissues. Each tissue was washed three times with media containing high concentrations of antibiotics and antimycotics devoid of serum, in order to avoid contamination.

4.2 Development of primary cultures

Three attempts were made in total to initiate cell culture systems from the tissues of the caerulean damsel. Attempts were made to initiate primary cultures from fin, gill, caudal peduncle, heart, liver, spleen, kidney and brain tissues.

4.2.1 Development of primary cultures by explant method

The different tissue explants exhibited variation in the degree of attachment to the culture flasks on incubation. Gill, fin, caudal peduncle, heart, kidney, liver and brain tissue explants showed good attachment and emergence of cells. However, only cells from gill, fin, heart and brain tissues formed confluent monolayers. Table 4.1. shows details about the primary cultures developed by explantation method.

Table 4.1. The response of various tissues of *P. caeruleus* towards development of cell culture systems by explantation method

Tissue	Cell attachment time (hours post-seeding, hps)	Time of confluency (days post-seeding, dps)	Nature of culture	Passage no. (p)/ Generational doublings (gd)	Morphology of cells
Fin	<24	20 - 25	Continuing	7 (p)	Fibroblastic
Gill	48	20-25	Finite	28 (gd)	Mixed (Epithelial and fibroblastic)
Heart	48	15-20	Finite	24 (gd)	Fibroblastic
Brain	24-48	-	Finite	12 (gd)	Fibroblastic
Kidney	24-48	-		Cells failed to form a monolayer	-
Spleen	24-48	-		Cells failed to form a monolayer	-

4.2.1.1 Fin

Attachment of the fin tissues, to the culture flasks after incubation was good. Emergence of cells from the explants was observed within 24h. Migration and multiplication of cells into colonies was gradually noticed. Different types of cells emerged from the spreading tissue outgrowths, such as fibroblast-like, epithelioid cells and a few polygonal cells. By 20^{th} -25^{th} day of culture, the continuously increasing patches of adherent cells joined to form a monolayer (Fig. 4.1 *a-h*).

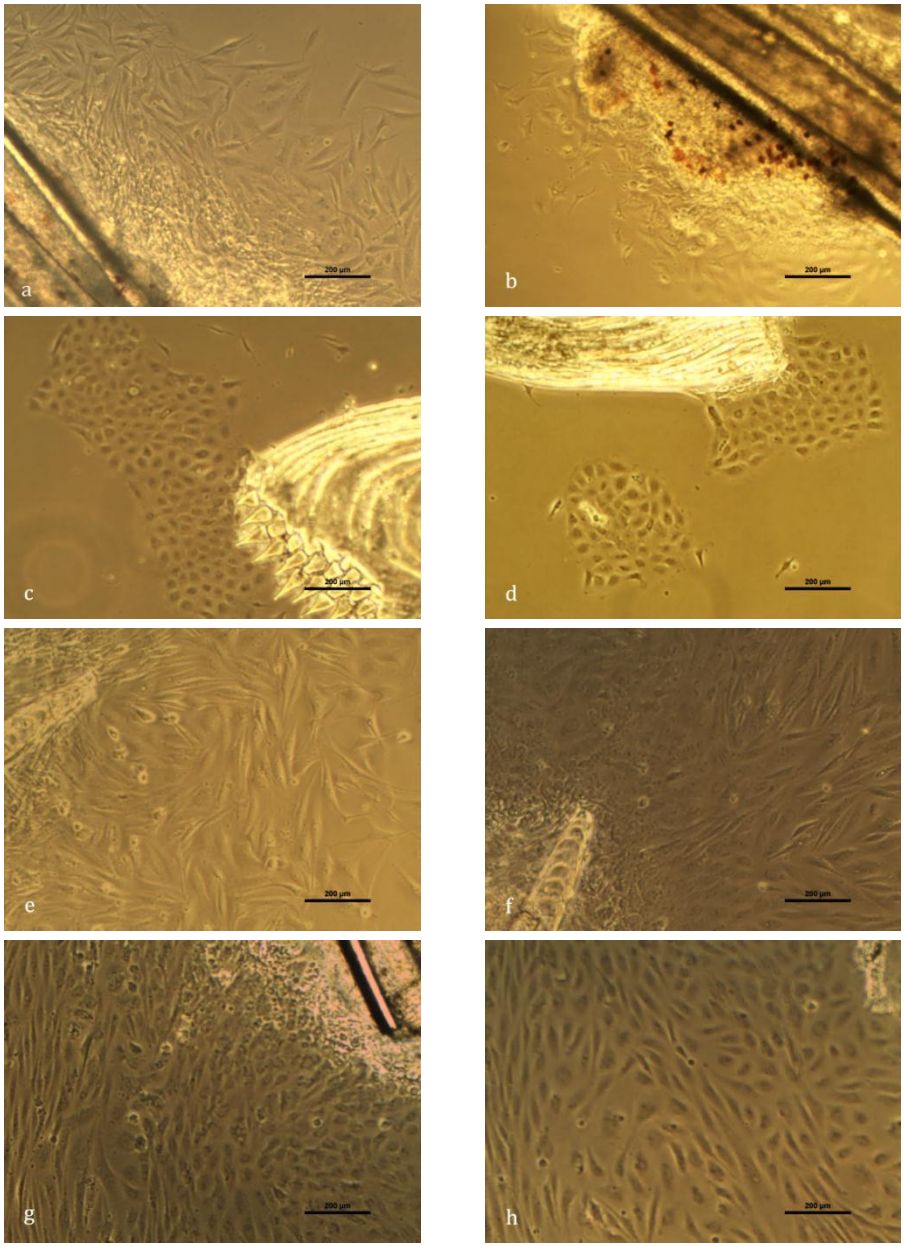

Fig. 4.1 Representative images showing emergence of cells from fin tissue explants of the caerulean damsel, *Pomacentrus caeruleus.*

Attachment of fin explants to the flask surface with cells emerging within 24h (*a-e*). The cells from fin explant showing epithelioid, fibroblast like and polygonal shaped morphology. Patches of adherent fin cells can be seen joining to form a confluent monolayer (*f-g*). Cells from fin explants predominated by fibroblast-like cells as the culture progressed (*h*).

4.2.1.2 Gill

Post-incubation, explants of gill tissues got readily attached to the culture flasks. Different types of cells including erythrocytes were found emerging from the explants within 48 h of incubation. Erythrocytes were seen lasting only a few days around the gill explants. Cells were also observed to spread and attach forming patches. These cells formed a confluent monolayer of epithelioid-like and fibroblast-like cells (Fig. 4.1 *i-l*).

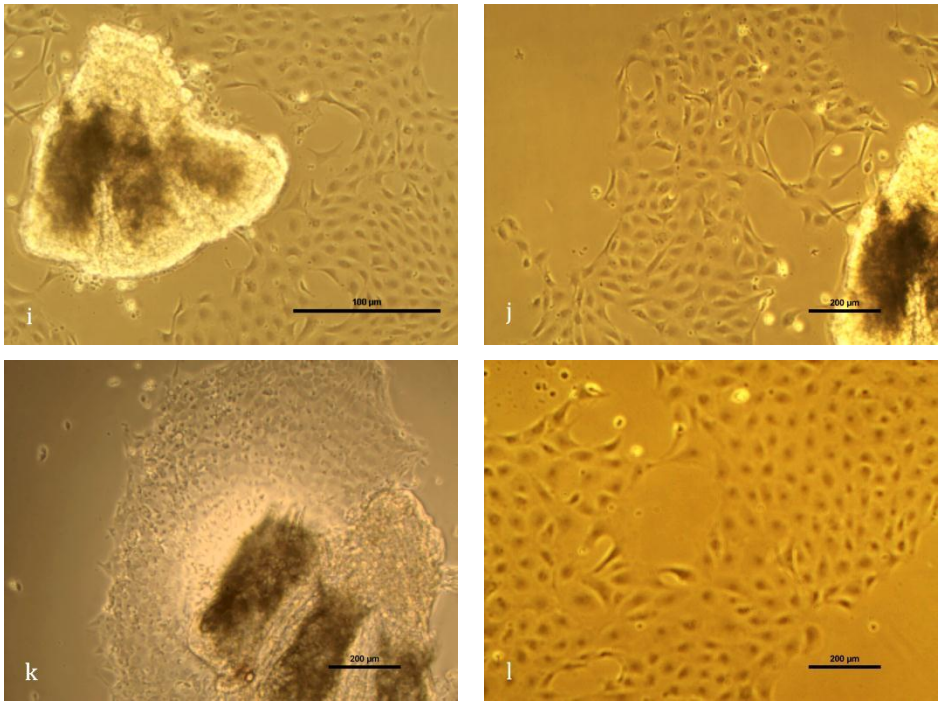

Fig. 4.1 Attachment and spreading of cells from the gill explant of *P. caeruleus* on day 2 post-explantation. Explants become firmly attached (*i-k*), Multiple patches of gill cells forming a confluent monolayer (*l*).

4.2.1.3 Caudal peduncle

Cells were observed to spread and attach to the culture flasks from the second day. The cells formed heterogeneous clusters of both epithelial-like and fibroblast-like cells. Initially outgrowth was observed from the explants

(Fig. 4.1*m-n*), followed by rapid proliferation of cells with the cultures reaching close to confluency. Repeated attempts resulted in bacterial contamination as evidenced by a change in media colour to yellow with drop in pH, the cultures had to be discarded.

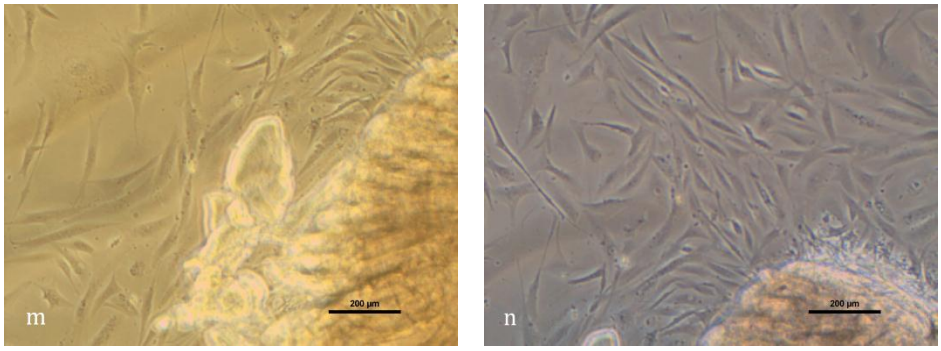

Fig. 4.1 Migration with attachment of cells from the caudal peduncle explants to the culture flask from the second day onwards (*m-n*)

4.2.1.4 Heart

Attachment of heart tissue explants to the culture flask was good. New cells around the explants were observed 2 days post-explantation. Cells were found to spread and attach to the culture flask from the third day, gradually the cells migrated and multiplied to form a monolayer (Fig. 4.1 *o* - *p*).

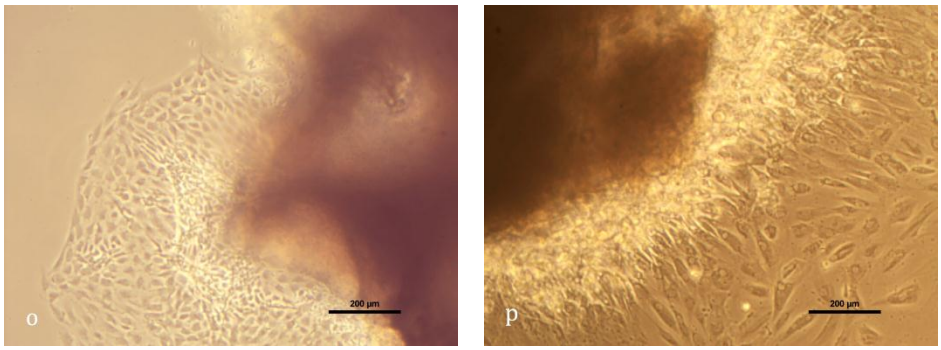

Fig. 4.1 Heart cells were found restricted to the vicinity of the explant initially but gradually migrating and proliferating to form a monolayer (*o-p*)

4.2.1.5 Liver

Post-explantation, liver tissue explants attached well to the culture flask. Emergence of cells was observed from the peripheral region of the explants by the third day (Fig. 4.1*q*- *r*). Initially the cells migrated, attached, but grew very slowly after which they gradually stopped growing and cell death ensued within a month of initiation of the culture.

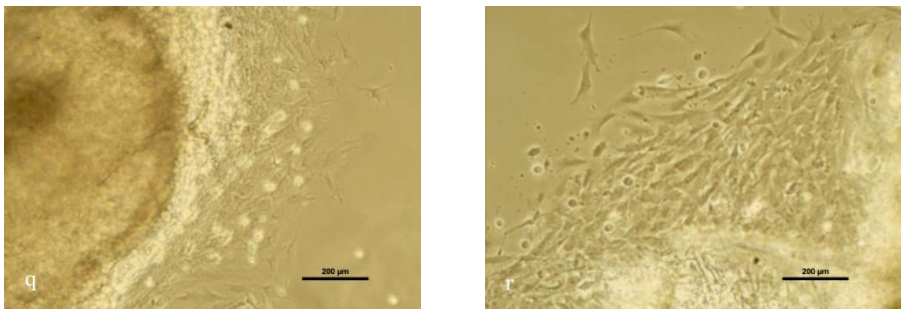

Fig. 4.1 Emergence of liver cells observed from the peripheral region of the explant within 72h (*q*-*r*)

4.2.1.6 Brain

Freshly explanted tissues showed attachment to the flask but further development could not be noted (Fig. 4.1 *s*).

4.2.1.7 Kidney

Attachment of few kidney tissues to the culture flask after incubation was observed but emergence of cells was restricted to the vicinity of the explant as seen in Fig. 4.1 (*t*- *u*). Erythrocytes were found to last only a few days as in the case of gill explants. But, rounding of cells occurred and no further progress was observed.

4.2.1.8 Spleen

Spleen tissues were weakly adherent to the flasks, with the emergence of numerous erythrocytes and few cells, but further multiplication could not be achieved (Fig. 4.1 *v*).

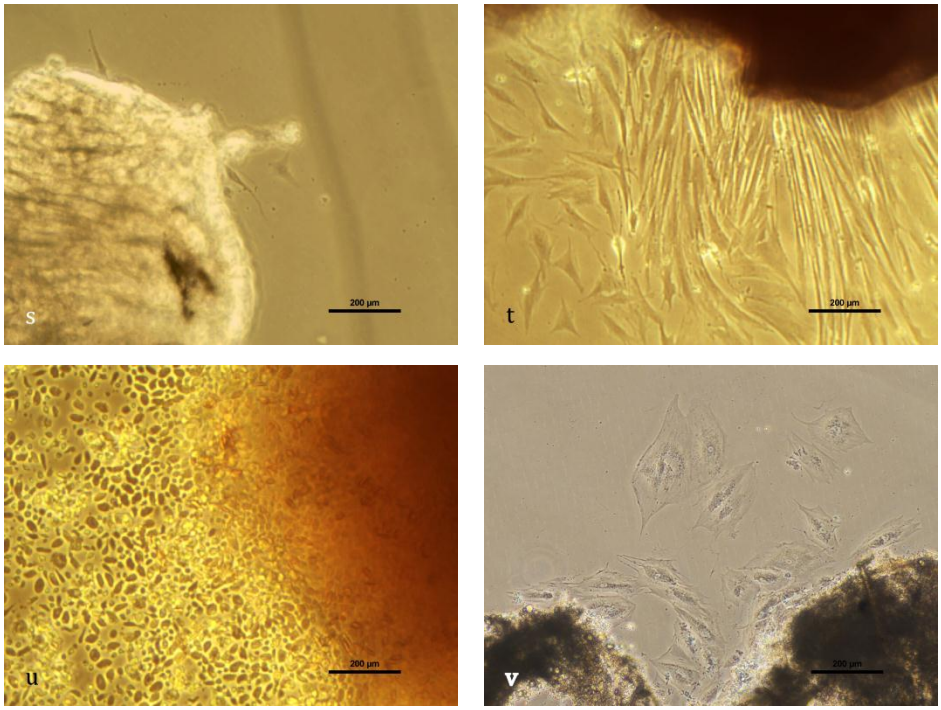

Fig. 4.1 Attachment of the brain explant to the flask surface (*s*), Emergence of cells around the vicinity of the kidney explant (*t*). Erythrocytes are seen emerging from the kidney explant (*u*), Emergence of cells from spleen explant (*v*)

4.2.2 Development of primary cultures by trypsinisation method

Dissociation of tissues using trypsin-EDTA (0.05% trypsin and 0.02% EDTA) yielded dispersed cells. The resuspended cells consisted of individual cells and cell clumps. Similar to the explant technique, the dispersed cells showed variation in their degree of attachment to the growth surface. Among the trypsinised tissues, cells from caudal peduncle and liver exhibited good attachment which grew to confluency. *In vitro* cell culture systems from the caudal peduncle and liver tissues of *P. caeruleus* were established by means of trypsinisation technique. Caudal peduncle tissues were found to attach as well as multiply better compared to liver cells. The dispersed cells were obtained from trypsinised tissues of the fin, gill, heart and brain. These cultures could not be

developed further due to their poor proliferative capacity. Table 2.3. includes details about the primary cultures developed by trypsinisation method.

Table 4.2. The response of various tissues of *P. caeruleus* towards development of cell culture systems by trypsinisation method

Tissue	Cell attachment time (hours post-seeding, hps)	Time of confluency (days post-seeding, dps)	Nature of culture	Passage no. (p)/ Generational doublings (gd)	Morphology of cells
Caudal peduncle	12	8	Continuing	10 (p)	Fibroblastic
Liver	48-72	40	Continuing	5 (p)	Fibroblastic

4.2.2.1 Liver

After a substantial period of growth lag, disaggregated liver cells started proliferation. Addition of FBS along with conditioned and fresh media promoted gradual progression in the growth and proliferation of cells. The development into monolayers took 40 days. Cells were heterogeneous in nature comprising predominantly of epithelial-like cells (Fig. 4.2*a-b*).

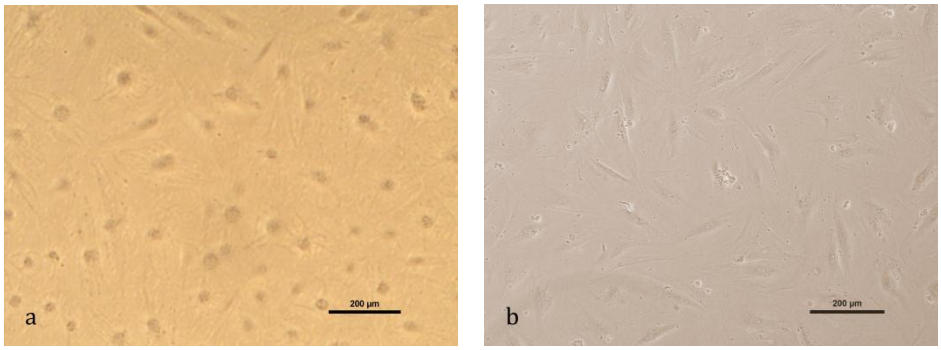

Fig. 4.2 Representative images showing multiplication of cells from trypsinised tissues of the caerulean damsel, *P. caeruleus.* Trypsinised liver cells were heterogeneous in nature, consisting of both epithelial-like and fibroblast-like cells and formed a monolayer (*a-b*)

4.2.2.2 Caudal peduncle

The dispersed cells easily attached within 12h after which emergence of cells could be perceived. Non-adherent cells round or oval in shape with irregular cell membrane were observed floating in the medium; which were discarded and fresh medium was added. The adherent cells maintained a constant or rising potential growth rate over time. By 6^{th} -8^{th} day of culture, the continuously increasing cells almost occupied the entire area of the culture flask. Confluent monolayers were observed in all the culture flasks by two weeks (Fig. 4.2 *c* - *d*).

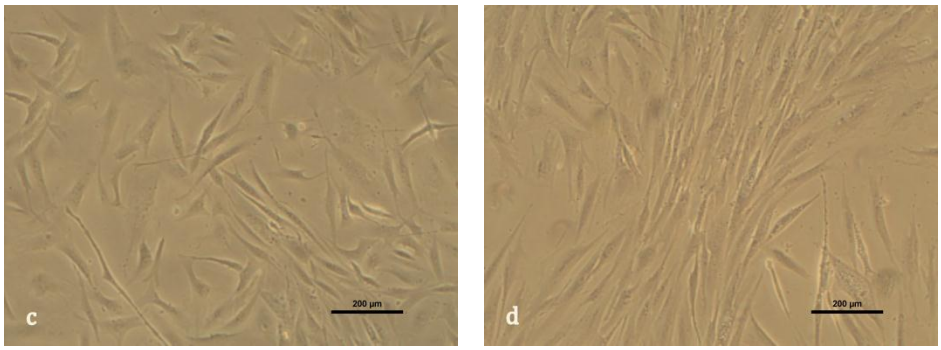

Fig. 4.2 Caudal peduncle cells proliferating to form a monolayer (*c*-*d*)

4.3 Subculture, passaging and derivation of cell lines

The primary cell cultures that proliferated to confluency were subcultured, split in to new flasks and passaged further for deriving *in vitro* transformed cell lines. During the initial passages, cells were developing in patches having heterogeneous morphology, comprising thin bipolar cells, fibroblast-like and epithelial-like cells. Gradually, the bipolar and the epithelial-like cells disappeared from fin, caudal peduncle and liver cultures, leaving a relatively uniform population of fibroblast-like cells. The cell shape varied depending on the cell density in the culture vessels. As the cell density increased, cells became more fibroblast-like. The approximate width of the cell body ranged from 4.0 to 8.0 µm depending on density of the cultures. The

cell cultures that were successfully subcultured, reattached and proliferated in two new flasks after passaging and were considered potential cultures to develop cell lines. Continuous use of penicillin, streptomycin or kanamycin showed that the cultures were more prone to contamination as compared to a periodic alternation of antibiotics. Similarly, use of fungizone was also studied. It was noted that the fungizone nystatin had a deleterious effect on primary cultures and also on slow growing cultures. The cells failed to multiply, with necrosis and rounding being observed. Hence, amphotericin B was preferred over nystatin as the fungizone. The cultures were also intermittently maintained in media devoid of antibiotics and fungizone to prevent the development of antibiotic resistant strains. However, antibiotics and fungizone were omitted from the cultures beyond the 10th passage.

4.3.1a Fin

After primary culture, surface-adherent fin cells emerged from the explant margin and occupied most of the surface of culture plates forming a complete monolayer of heterogeneous cells. They have been successfully subcultured, and have developed a fibroblast-like morphology. The culture from fin was designated PC1F1Ex.The cultures required 20% FBS till around 30th passage which gradually reduced to 10% till the 40th passage and subsequently to 5 and 2%. Fin cell lines is to date being passaged in L-15 medium supplemented 2% FBS (Fig. 4.3 *a-j*).

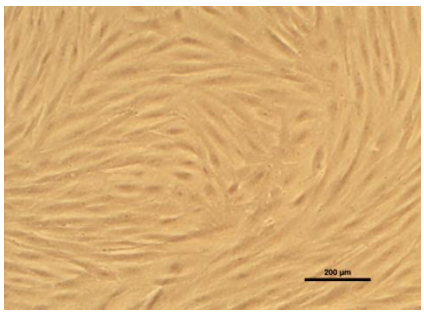

Fig. 4.3*a* PC1F1Ex 5P

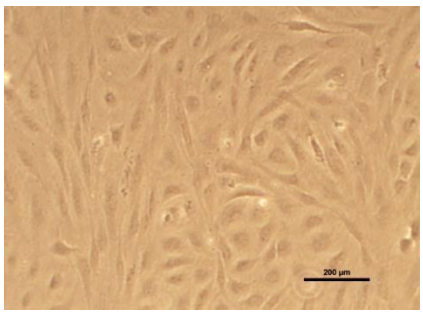

Fig. 4.3*b* PC1F1Ex 9P

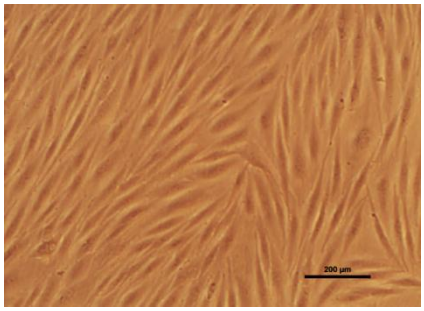

Fig. 4.3*c* PC1F1Ex 25P

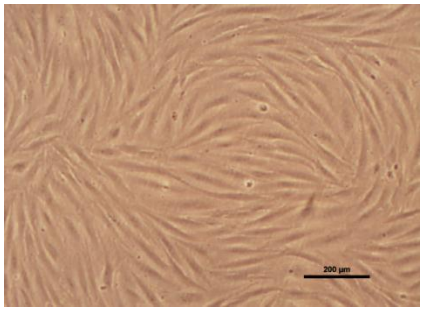

Fig. 4.3*d* PC1F1Ex 30P

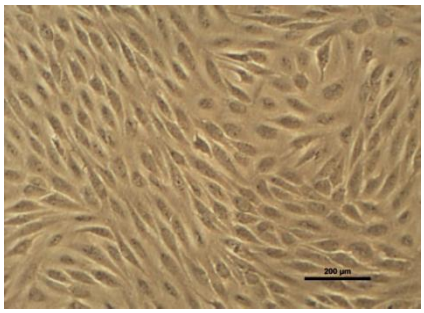

Fig. 4.3*e* PC1F1Ex 46P

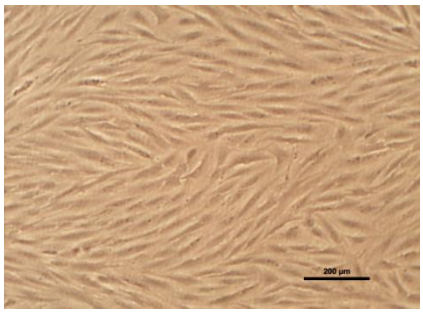

Fig. 4.3*f* PC1F1Ex 53P

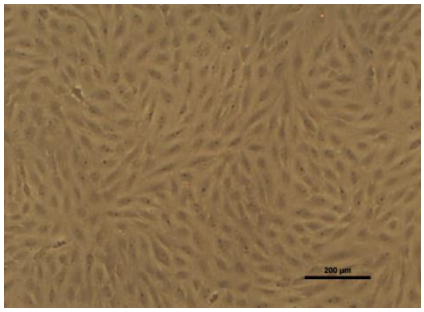

Fig. 4.3*g* PC1F1Ex 66P

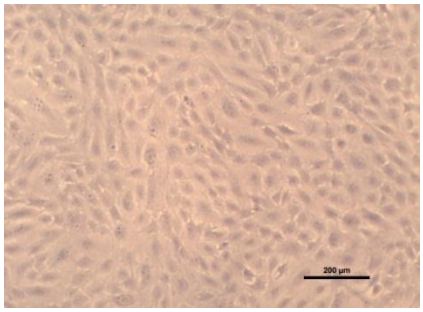

Fig. 4.3h PC1F1Ex 77P

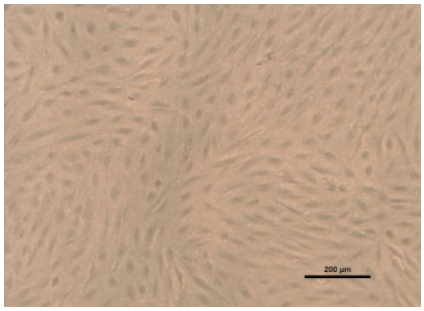

Fig. 4.3*i* PC1F1Ex 83P

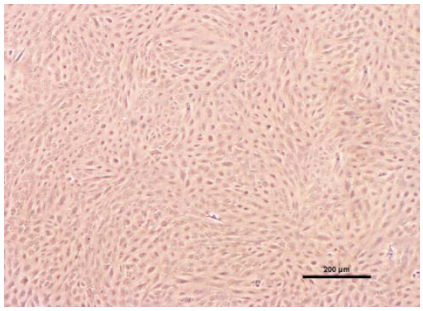

Fig. 4.3 *j* PC1F1Ex 120P

Fig. 4.3 Representative images showing fin cell lines, PC1F1Ex, at different passages. *Scale=200µm*

4.3.1b Gill

The *in vitro* cell culture developed from gill explants was designated PC1G2Ex. The gill cells were a heterogeneous mixture of cells in the initial passages. The cell monolayers on trypsinisation detached easily and were split in to new culture flasks which proliferated well comprising predominantly of epithelioid-like cells in subsequent subcultures. The cell monolayers that had developed persisted for approximately 28 generational doublings, but were further unsuccessful due to poor reattachment and cultures were lost (Fig. 4.4 *a*-*b*).

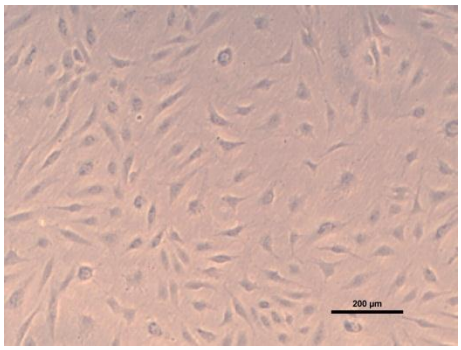

Fig. 4.4*a* PC1G2Ex 4P

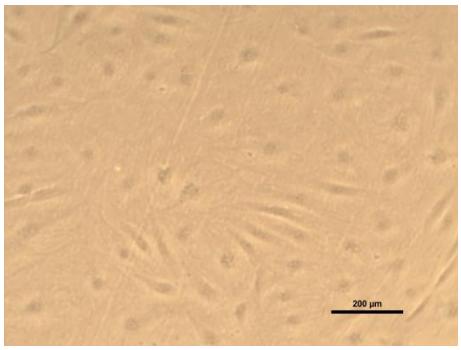

Fig. 4.4*b* PC1G2Ex 5P

Fig. 4.4 Photomicrographs of gill cells at early passages. *Scale=200µm*

4.3.1c Heart

There was success with heart explant attachment and initial growth. The cell monolayers that had developed persisted for approximately 24 generational doublings before senescence. Although cells did re-attach after the first passage, they failed to grow to confluency a second time and detached after 2-3 days.

4.3.1d Caudal peduncle

Cultures from caudal peduncle tissues of caerulean damsel were quite successful. There was variation in the morphology of cells in primary cultures. Most cultures were flat monolayers of epithelial-like cells. These cells progressed into a fibroblastic-like morphology gradually. The fibroblastic cultures reached confluency within 2 weeks and were passaged regularly. Cultures detached on trypsin treatment during the first passage and reattached with more than 50% of the cells attaching to the surface of the flasks. The cells adapted well *in vitro* and required low serum levels by the 10^{th} passage. The cell culture has been designated PC1CpTr and is being successfully subcultured till date (Fig. 4.5 *a-j*).

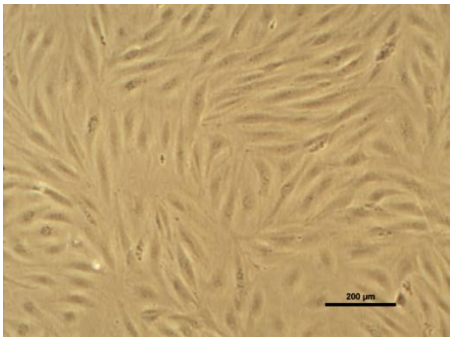

Fig. 4.5*a* PC1CpTr 13P

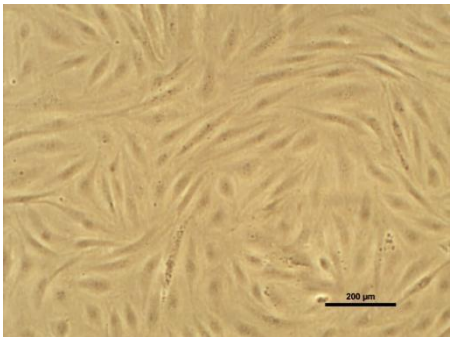

Fig. 4.5*b* PC1CpTr 19P

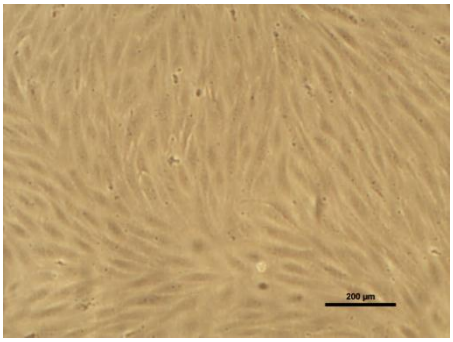

Fig. 4.5*c* PC1CpTr 21P

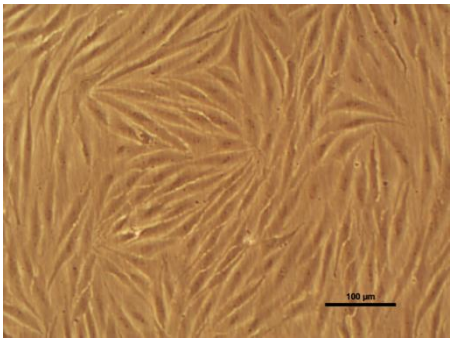

Fig. 4.5*d* PC1CpTr 30P

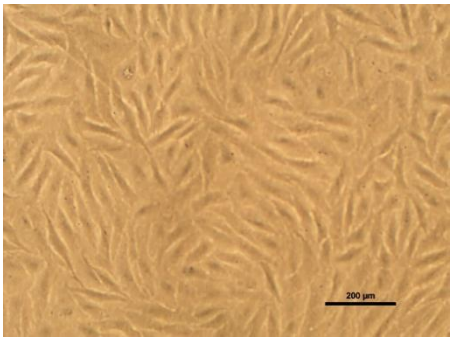

Fig. 4.5*e* PC1CpTr 49P

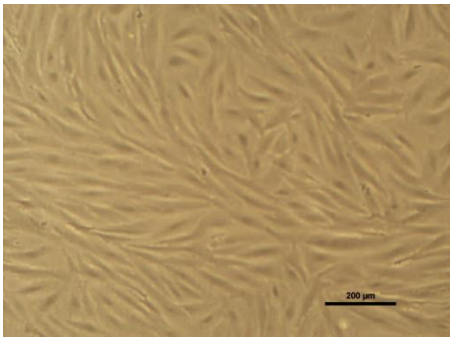

Fig. 4.5*f* PC1CpTr 54P

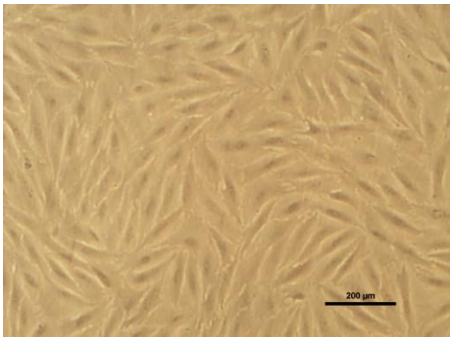

Fig. 4.5*g* PC1CpTr 63P

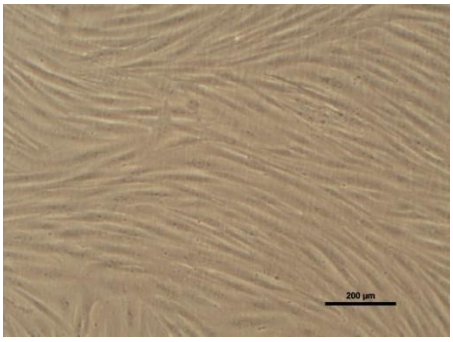

Fig. 4.5*h* PC1CpTr 75P

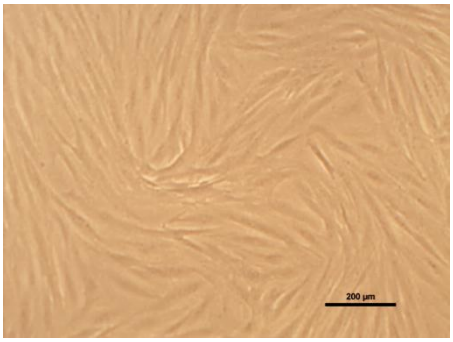

Fig. 4.5*i* PC1CpTr 83P

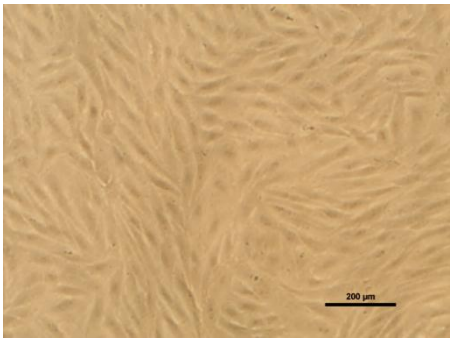

Fig. 4.5*j* PC1CpTr 92P

Fig. 4.5 Photomicrographs of caudal peduncle cell line PC1CpTr at different passages. *Scale=200µm*

4.3.1e Liver

Primary cultures from trypsinised liver tissues were very slow to develop and were maintained for long periods before the first subculture was carried out. Initially the cultures had high serum requirement (>20%), but

presently the cultures require only 2% serum to grow to confluency similar to other cultures developed from the caerulean damsel in this study. The liver cell culture has been designated PC1L1Tr and is being successfully passaged till date (Fig. 4.6 *a-j*).

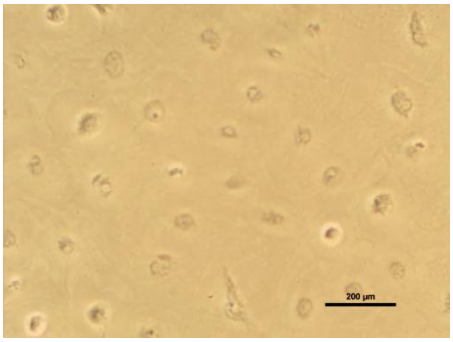

Fig. 4.6*a* PC1L1Tr 6P

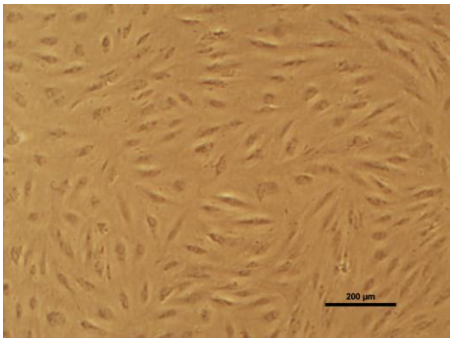

Fig. 4.6*b* PC1L1Tr 20P

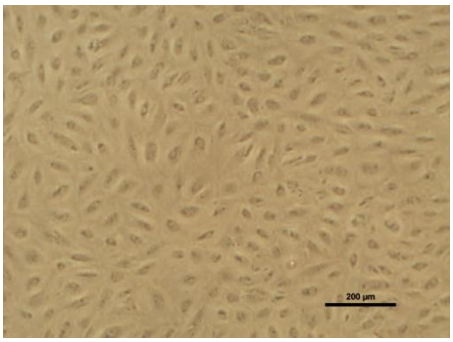

Fig. 4.6*c* PC1L1Tr 29P

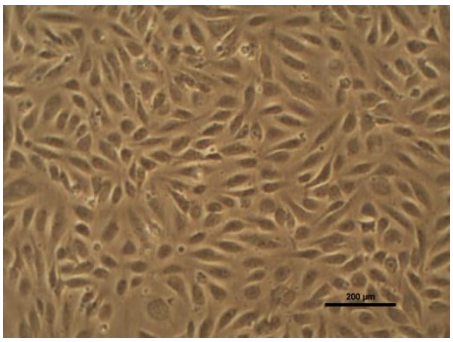

Fig. 4.6*d* PC1L1Tr 44P

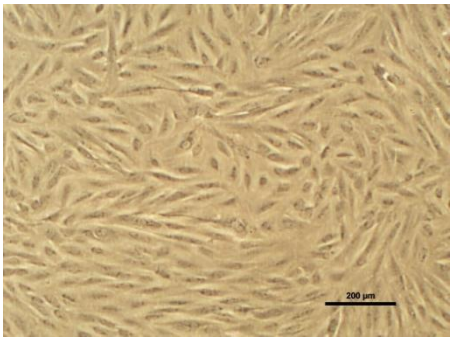

Fig. 4.6*e* PC1L1Tr 55P

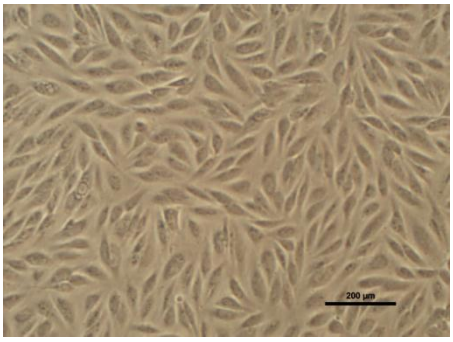

Fig. 4.6*f* PC1L1Tr 65P

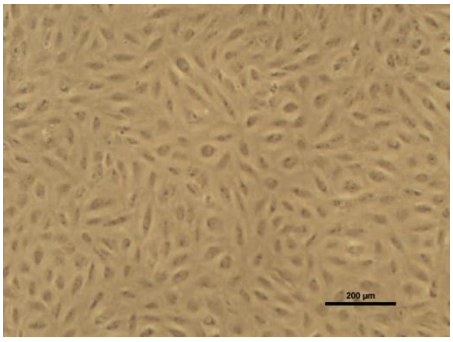

Fig. 4.6*g* PC1L1Tr 78P

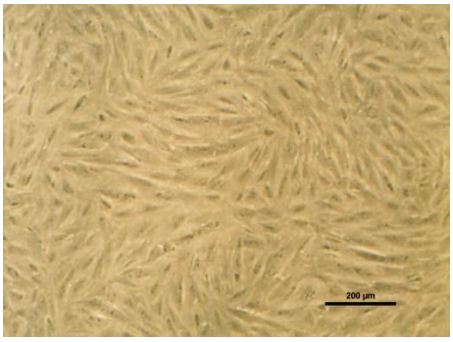

Fig. 4.6*h* PC1L1Tr 83P

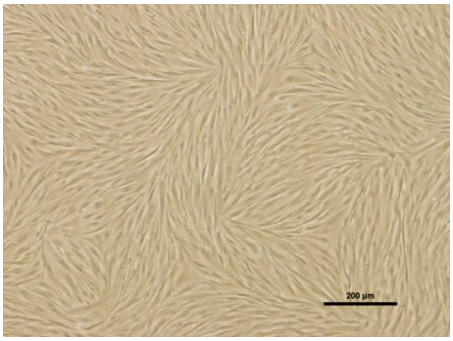

Fig. 4.6*h* PC1L1Tr 103P

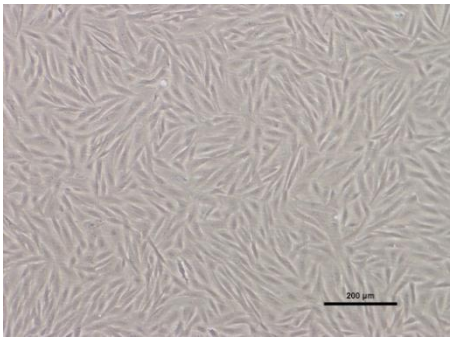

Fig. 4.6*j* PC1L1Tr 119P

Fig. 4.6 Photomicrographs of liver cell line PC1L1Tr at different passages. *Scale=200µ*

4.3.2 Cryopreservation and revival of cells

The cells were cryopreserved at every 20 passage levels using 10% DMSO as the cryoprotectant. The cells recovered after storage in liquid nitrogen showed 70-80% viability and grew to confluency within 3-5 days. Following storage, no obvious alteration in morphology or growth pattern was observed for the cells on revival.

4.3.3 Determination of optimal growth conditions

4.3.3a Effect of different media and additives

Best growth and survival were observed using Leibovitz's L-15 medium for fin, liver and caudal peduncle cultures. L-15 when supplemented with FBS supported a higher percentage of attachment of explants, cell radiation and

proliferation. The tissues in the present study seemed best suited for growth in L-15 supplemented with 20% FBS for primary cultures which gradually reduced to 10% and presently the cells are being maintained in 2-5% FBS. Although when DMEM and M199 with 5% CO_2 was used, the cultures did not yield good results as compared to L-15 medium.

Addition of homologous fish muscle extract did not contribute to any significant improvement in growth or formation of monolayers. Neither homologous nor heterologous fish serum (inactivated) helped in improving cell growth and monolayer formation.

Therefore, Leibovitz's L-15 supplemented with FBS was used as the medium of choice for the propagation of the cultures. All further experiments conducted were also carried out in the same medium.

4.3.3b Effect of serum (FBS) levels on cell proliferation

The cell cultures grew well at a serum concentration of 20% during the initial passages and no obvious cell proliferation could be observed when there was no FBS in the media. As the number of passages increased, the requirement for serum gradually decreased. Low serum levels resulted in vacuolation of cells and gradual peeling off of the monolayer. The growth rates of the cultures, once they had emerged, progressively increased and continued to do so in L-15 with 20% FBS. The caudal peduncle cell culture, PC1CpTr showed a drop in the requirement for serum; with only 5% serum requirement by the 20^{th} passage. PC1L1Tr, liver cultures required 10% serum by the 20^{th} passage, which further decreased to 2% by the 50^{th} passage. In comparison, fin culture, PC1F1Ex showed the highest requirement for serum. The cultures reached confluency in 7 d only when supplied with L-15 medium containing 20% serum for passages below 40. Although after the 50^{th} passage, the cells were capable of growing in

10% serum and could be alternated with 5% serum for growth every two weeks (Fig. 4.7 a-c). Beyond the 70th passages in PC1F1Ex, the cells were found to grow well in L-15 medium supplemented with 2% FBS with a split ratio of 1:3 and grew to confluency in 7 days post-subculture.

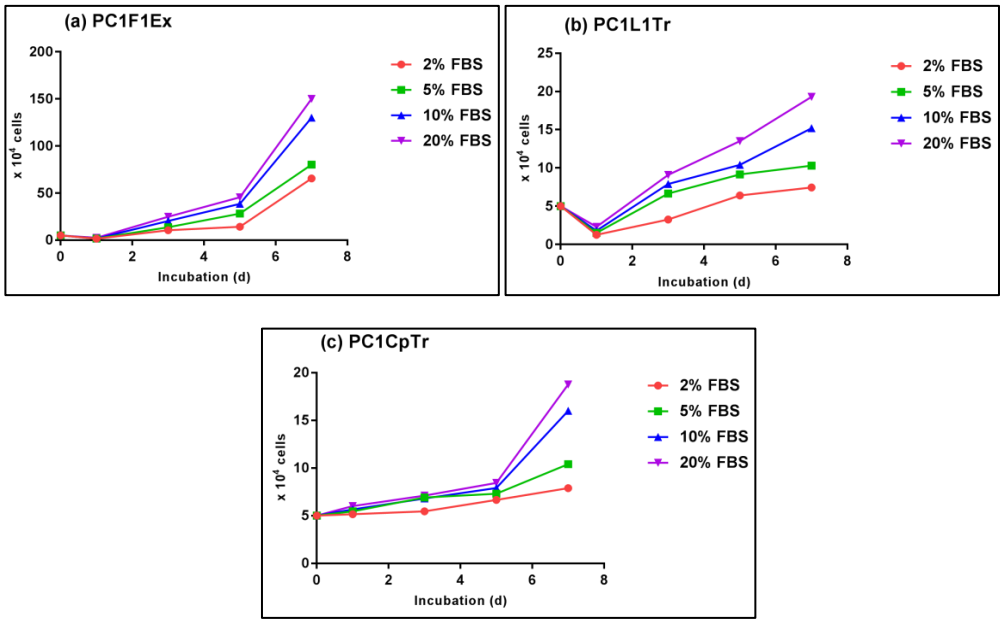

Fig. 4.7 Growth response graph of (a) fin, (b) liver and (c) caudal peduncle cell lines of *P.caeruleus* at varying serum levels and at a constant temperature of 28ºC. The cells were grown in 25cm^2 flasks and plated with L-15 containing 2% (●), 5% (■), 10% (▲) and 20% (▼) FBS over a one week period. The cells were harvested by trypsinisation and cell numbers determined using a hemocytometer.

4.3.3c Effect of temperature on cell proliferation

At 4th, 6th and 8th d after subculture, the cell lines at different temperatures were trypsinised after which cell numbers were counted. The results showed that the cell lines were able to grow between 22 ˚C and 30 ˚C. However, maximum growth was recorded at 28 ˚C. Though cells were found to grow at all temperatures evaluated but when temperature fell below 18 ˚C, data suggested that the multiplication period lengthened, confluency was not

achieved by 7 d. At 20 ˚C, confluency was reached by 7d. In contrast, between 24 ˚C and 32 ˚C confluent monolayers were achieved by 48-72 h (Fig. 4.8*a-c*). Temperature studies carried out using L-15 medium with 5-10% FBS showed that an ambient temperature of 28± 2°C is the most suitable temperature for optimum growth and proliferation of the cultures. Fin cells proliferated in FBS concentrations above 5%, however, 10% FBS was found to be optimal for growth of the cells. Liver and caudal peduncle cells adapted to media containing 5% FBS.

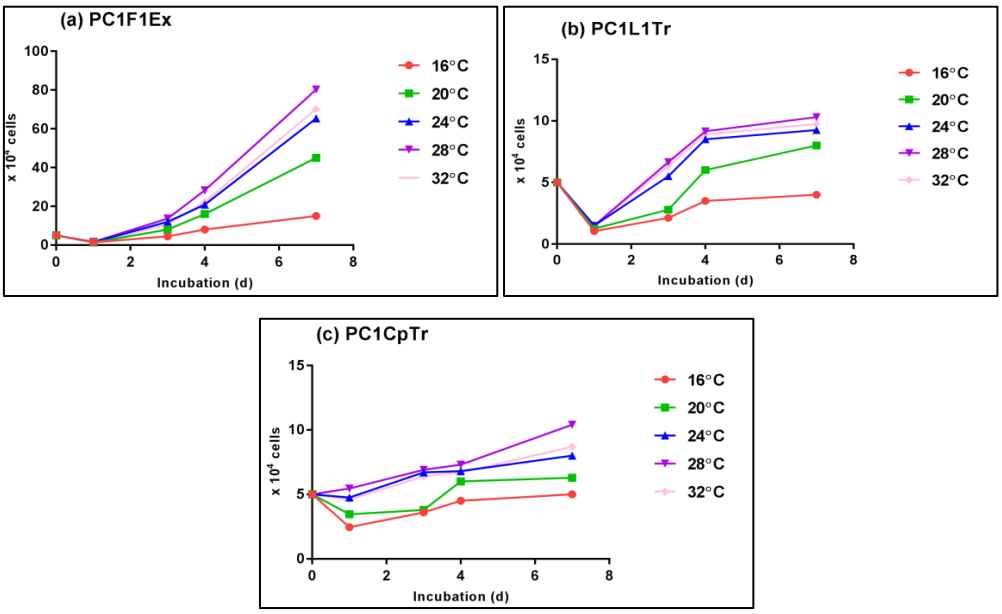

Fig. 4.8 Growth response graph showing effect of varying temperatures on proliferation of (a) fin, (b) liver and (c) caudal peduncle cell lines of *P. caeruleus* that were grown in L-15 medium containing 5% FBS. The cells were maintained at 16 ºC (●), 20 ºC (■), 24 ºC (▲), 28 ºC (▼) and 32 ºC (♦) over a 1 week period. The cells were harvested by trypsinisation and cell numbers determined using a hemocytometer.

4.3.4 Quantitation

4.3.4a Growth curve

Cultured cells exhibited peak growth from day 5 to day 11, thereafter a plateau phase could be observed by 12 through 21 day as from the day of subculture. The population-doubling time of PC1F1Ex, PC1CpTr cells at passage 73 was 48h and for PC1L1Tr was 36h. This growth rate continued to increase until it equalled or exceeded that of the original culture (Fig. 4.9).

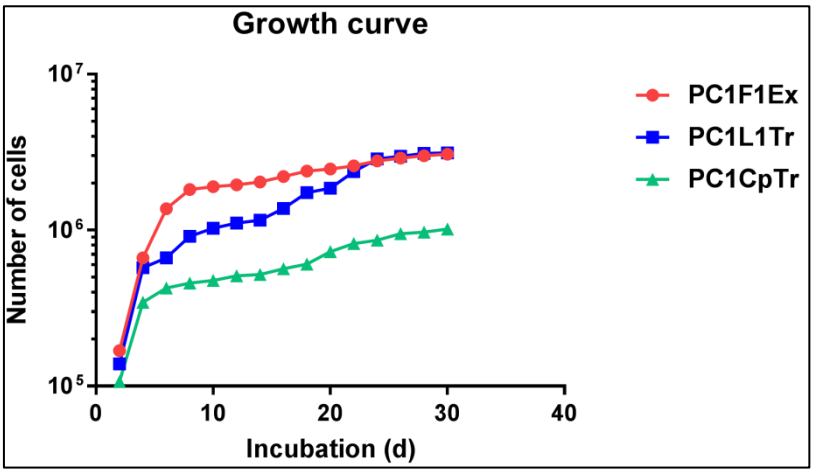

Fig. 4.9 Growth curve of the cell lines (a) PC1F1Ex (●), (b) PC1L1Tr (■) and (c) PC1CpTr (▲)

4.3.4b Plating efficiency

Maximum plating efficiency was observed when cell were seeded at a concentration of 1 x 10^3 cells in 6 well plates (Table 4.3). When higher number of cells was seeded, clumping of cells was observed whereas 1 x 10^3 cells resulted in single cells attaching and forming colonies. For low seeding, initial rounding was observed and the remaining cells dispersed to form colonies. There were no significant differences in replicates ($p>0.05$).

Table 4.3. Plating efficiency of PC1F1Ex, PC1CpTr and PC1L1Tr

Cell Line	Seeding density (No. of cells)					
	2 x10^3	**1 x 10^3**	**5 x 10^2**	**2 x 10^2**	**1 x10^2**	**5 x 10**
PC1F1Ex	17.7% ±1.5	21% ± 1.5	19.5% ±1.65	14.28% ±0.5	16.3% ±1.66	10% ± 0.7
PC1CpTr	16.1% ±0.5	23% ±3.5	22.63% ±2.76	15.8% ±1.35	15.1% ±3.1	7% ±0.5
PC1L1Tr	9.5% ±0.5	14.76% ±2.5	12.4% ±0.5	10.1%±1.3	11.33% ±0.3	8± 1.4

4.3.4c Seeding efficiency

The seeding efficiency in 25cm^2 tissue culture flask after 6h for an initial inoculum density of 2mL of 2.5 x 10^5 cells mL^{-1} was 75.6% for PC1F1Ex, 72.8% in PC1L1Tr and 50% for PC1CpTr . For 2mL of 1.25 x 10^5 cells mL^{-1} was 80% for PC1F1Ex, 70.4% in PC1L1Tr and 20% for PC1CpTr.

4.4 Characterisation of the cell lines

4.4.1 Determination of cell morphology by giemsa staining

Giemsa stained monolayers of the cell lines PC1F1Ex, PC1L1Tr and PC1CpTr were evaluated by light microscopy at high (x200) and low (x100) magnifications.

Cells in the intact giemsa stained monolayers of PC1F1Ex showed a bipolar elongated appearance with a narrow diameter, mostly densely packed with little intercellular space. At log phase, cells were observed to be randomly oriented in nature having an elongated morphology and growing attached to the substrate (Fig. 4.10*a- b*).

For PC1CpTr, the cells were found to be usually bipolar in nature (Fig. 4.10*b-c*) forming characteristic parallel arrays and whorls. Depending on the density of the cells, they tend to be tightly packed or in random orientation.

In case of PC1L1Tr, the cells were seen to be bipolar or multipolar, which tend to be tightly packed in parallel arrays. At confluence, the cells

were shown to have smaller nuclei and narrow diameter. The cell morphology was found to be characteristically spindle-like which grows attached to the surface in parallel arrays (Fig. 4.10*d-e*).

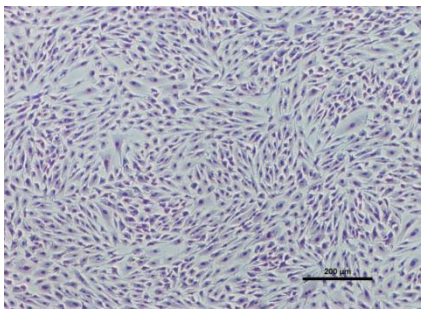

Fig. 4.10*a* PC1F1Ex 50P 10x *Scale=200µ*

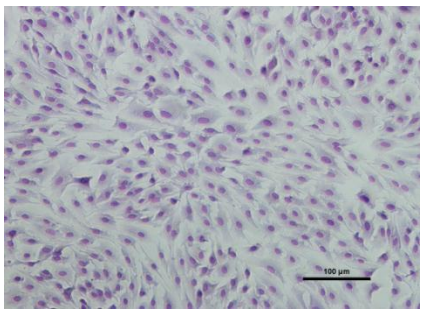

Fig. 4.10*b* PC1F1Ex 50P 20x *Scale=100µ*

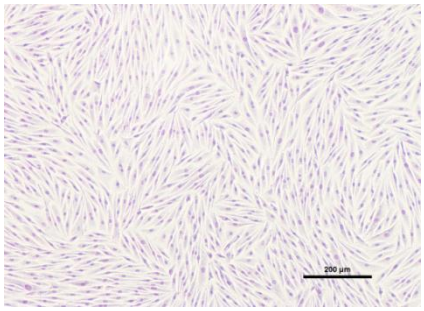

Fig. 4.10*c* PC1CpTr 60P 10x *Scale=200µ*

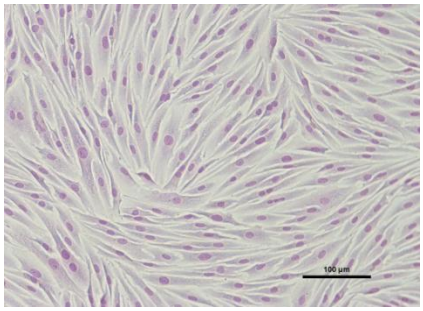

Fig. 4.10*d* PC1CpTr 60P 20x *Scale=100µ*

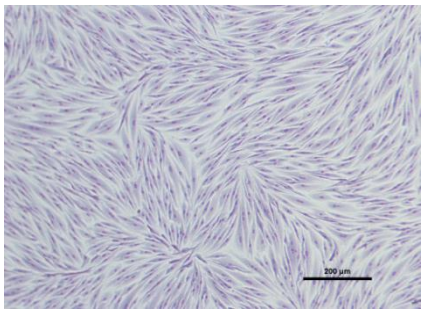

Fig. 4.10*e* PC1L1Tr 70P 10x *Scale=200µ*

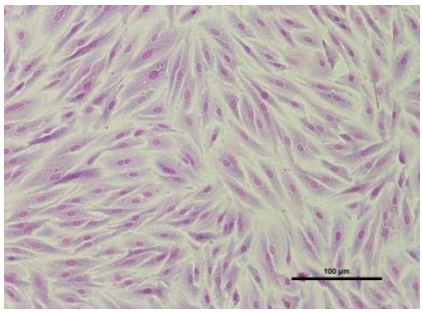

Fig. 4.10 *f* PC1L1Tr 70P 20x *Scale=100µ*

Fig. 4.10 Morphology of the cell lines visualised using Giemsa stain.

4.4.2 Determination of transformation by chromosome analysis

Fifty randomly selected metaphase spreads from each cell line of *P. caeruleus* was counted. All the three lines were found to be aneuploid. The

chromosome number of the fin cells counted from the PC1F1Ex cell line varied from 41 to 60 (Fig. 4.11 *a-b*).

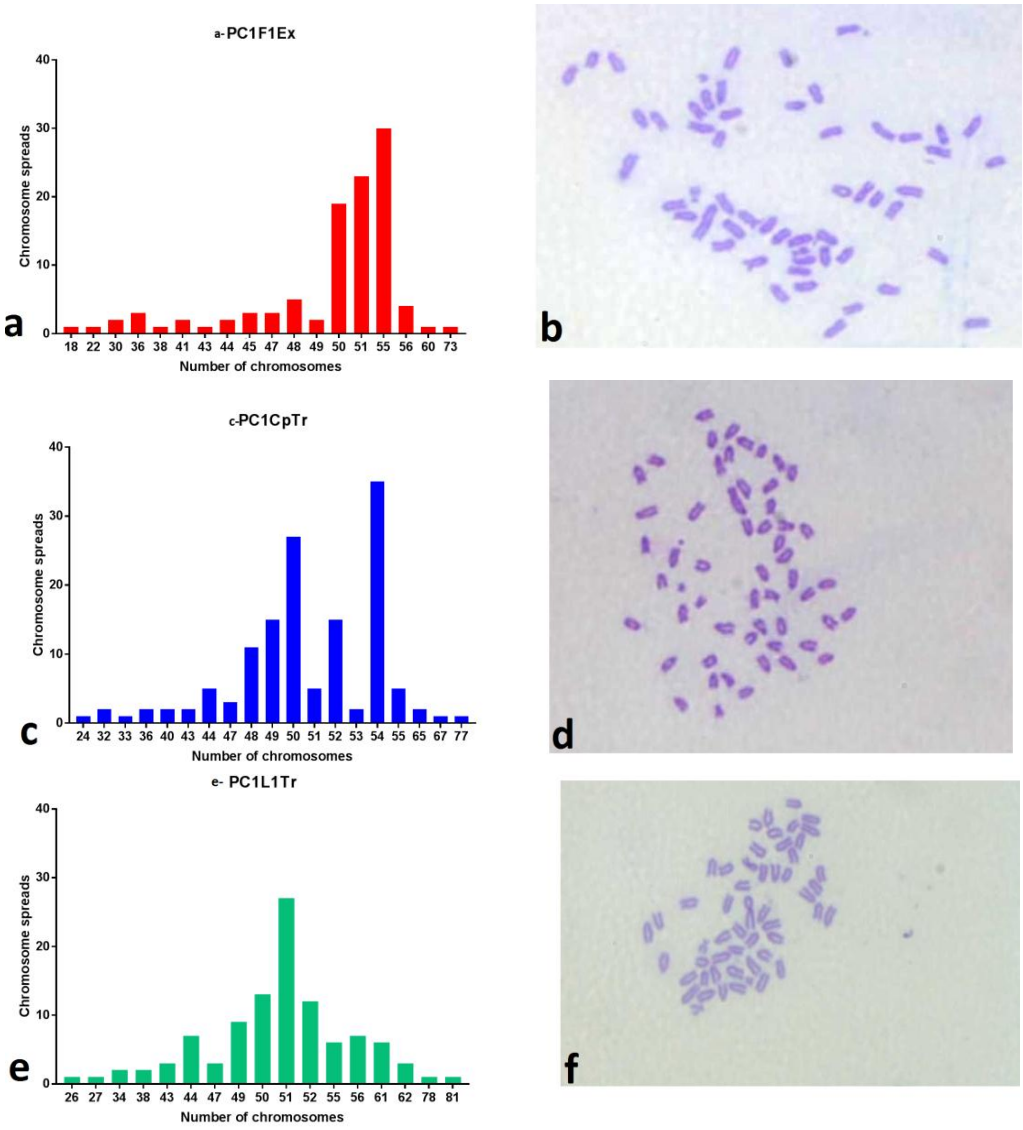

Fig. 4.11 Chromosome analysis of the cell lines. a) Frequency distribution of chromosomes in PC1F1Ex at passage 50; b) representative metaphase spread in PC1F1Ex (100x). c) Frequency distribution of chromosomes in PC1CpTr at passage 56; d) representative metaphase spread in PC1CpTr (100x). e) Frequency distribution of chromosomes in PC1L1Tr at passage 60; f) representative metaphase spread in PC1L1Tr (100x)

From the PC1CpTr caudal peduncle cell line, fifty randomly selected metaphase cells were counted, revealing a range from 36 to 65 chromosomes with majority of the counted cells falling between 50 and 54 chromosomes (Fig. 4.11 *c-d*). For PC1L1Tr liver cell line, chromosomal count ranged from 43 to 62 (Fig. 4.11 *e- f*).

4.4.3 Analysis of cell type by immunofluorescence staining

Positive controls confirmed the cross-reactivity of the primary antibodies to the mammalian cell type markers in the caerulean damsel cell lines. Cells in the monolayers of all the three cell lines developed from *P. caeruleus* showed strong immunoreactivity to the antibodies directed against the fibroblast marker, vimentin (Fig. 4.12 *a-c*) and no immunoreactivity to the epithelial marker, cytokeratin (Fig. 4.12 *d-f*). These results therefore lend strong support that the cell lines PC1F1Ex, PC1L1Tr and PC1CpTr are fibroblastic in nature.

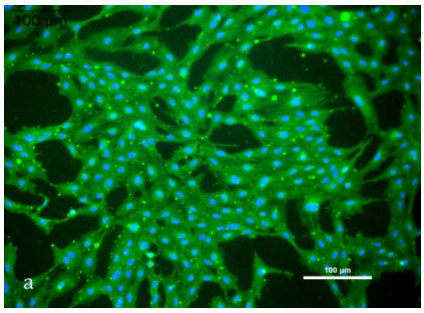

Fig. 4.12*a* PC1F1Ex 69P (20x)

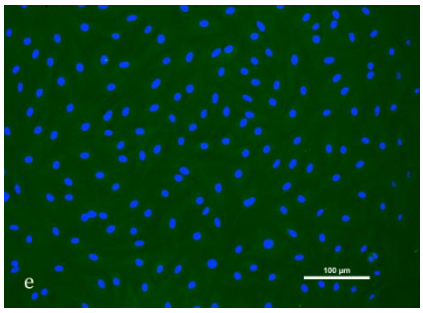

Fig. 4.12*d* PC1F1Ex 69P (20x)

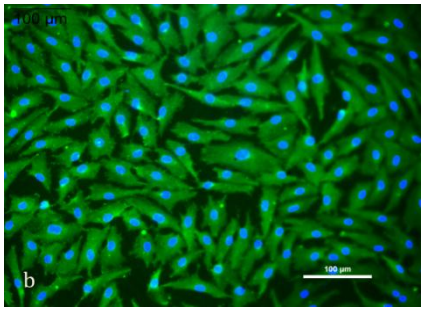

Fig. 4.12*b* PC1CpTr 53P (20x)

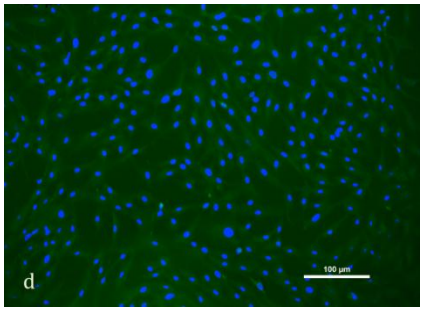

Fig. 4.12*e* PC1CpTr 53P (20x)

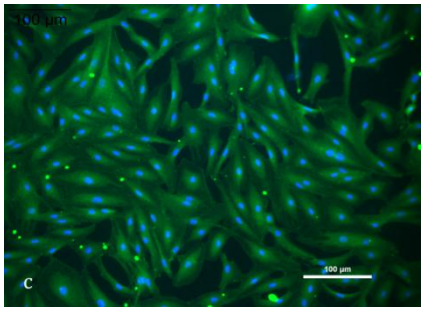

Fig. 4.12*c* PC1L1Tr 65P (20x)

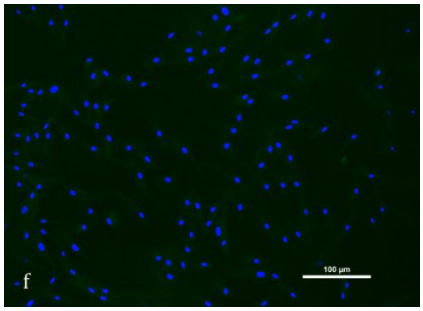

Fig. 4.12*f* PC1L1Tr 65P (20x)

Fig. 4.12 Assessment of selected protein markers in PC1F1Ex, PC1CpTr and PC1L1Tr cells. Cells exhibited positive expression for intracellular vimentin: a) PC1F1Ex b) PC1CpTr c) PC1L1Tr. No reactivity to cytokeratin: d) PC1F1Ex e) PC1CpTr and f) PC1L1Tr. Cell nuclei were counterstained with DAPI (blue). *Scale= 100µm*

4.4.4 Cell line authentication by PCR amplification and sequence analysis of CO1 gene

Amplification of the COI gene from the cell lines and caerulean damsel muscle tissue gave products of ~640bp. The analysis of COI gene sequences from the cell lines and caerulean damsel muscle tissue was done with an objective to authenticate the cell lines by confirming species of origin. PCR amplification of the partial CO1 sequence of the cell lines yielded 99% match when aligned with CO1 gene of *P. caeruleus* in the NCBI database (Accession No. KJ129032.1). The partial sequences of the CO1 gene of the three cell lines and the tissue have been registered in NCBI GenBank with accession numbers as shown in the table.

Table 4.2: Accession numbers of the caerulean damsel tissue isolate and cell lines

Cell line/Tissue isolate	GenBank Accession No.
Caerulean damsel muscle tissue	KY982625
PC1CpTr cell line	KY982626
PC1F1Ex cell line	KY982627
PC1L1Tr cell line	KY982628

4.4.5 Detection of inherent contaminants

4.4.5a Bright field (Phase contrast) microscopy

The cultures that showed deterioration in growth due to fungal or bacterial contamination were discarded; no attempts were made to disinfect the cultures. Vacuolation, granulation and/ or clumping of cells were also observed in cultures that were contaminated. A sharp drop in pH was clearly indicative for bacterial contaminants and mycelium with hyphae was observed in fungus contaminated cultures. However, whenever a bacterial, yeast or fungal contamination was observed in routinely maintained cultures, they were immediately discarded.

4.4.5b Transmission electron microscopy (TEM)

TEM results revealed that all the three cell lines were free of viruses, viral related particles or mycoplasmas. (Fig. 4.13).

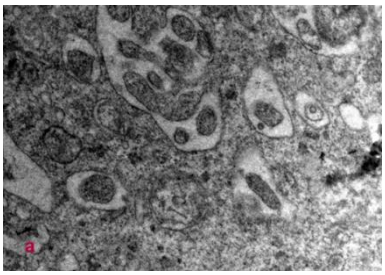

Fig. 4.13a PC1F1Ex

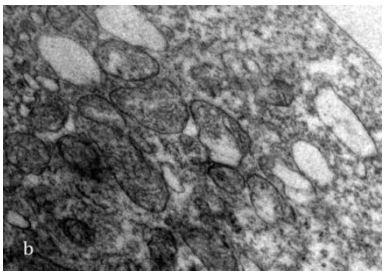

Fig. 4.13b PC1CpTr

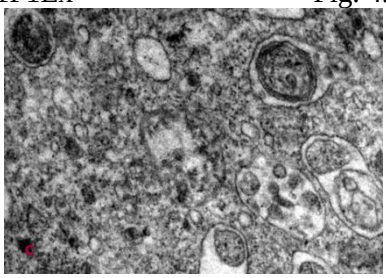

Fig. 4.13c PC1L1Tr

Fig. 4.13 Transmission electron micrographs demonstrating ultrastructure of cells from caerulean damsel cell lines a) PC1F1Ex at passage 57; b) PC1CpTr at passage 53 and c) PC1L1Tr at passage 60.

4.4.5c Screening for mycoplasmas

Screening using MycoFluor™ mycoplasma detection kit also ruled out mycoplasma contamination of the cell lines.

4.5 Applications: Gene expression studies and cytotoxicity assays using bacterial extracellular products

4.5.1 Gene expression studies using GFP reporter gene

The PC1F1Ex, PC1CpTr and PC1L1Tr cell lines were successfully transfected with pcDNA3-EGFP plasmid using JetPRIME as well as Lipofectamine 3000 transfection reagent. The expression of EGFP in the cell lines could be detected as early as 48h after transfection by the observation of a clear green fluorescent signal under a fluorescent microscope. The estimated transfection efficiency was 10% and 12.5% respectively for JetPRIME (Fig. 4.14a-c) and Lipofectamine 3000 (Fig. 4.14d-f) reagents, which indicates the ability of these cells to be transfected and cytomegalovirus (CMV) promoter can drive the expression of EGFP gene in *P. caeruleus* cell lines, PC1F1Ex, PC1CpTr and PC1L1Tr.

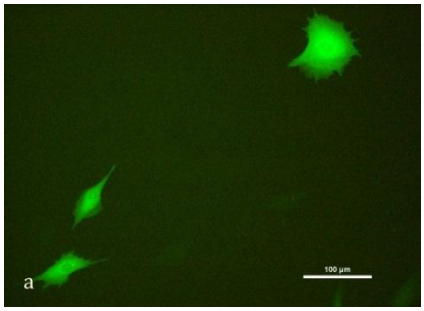

Fig. 4.14*a* PC1F1Ex

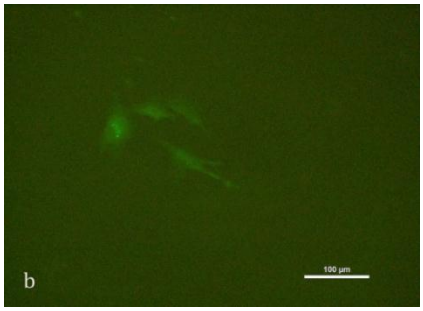

Fig. 4.14*b* PC1CpTr

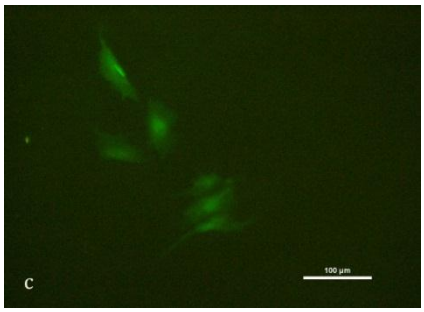

Fig. 4.14c PC1L1Tr

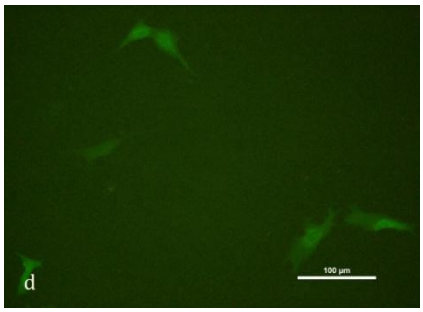

Fig. 4.14*d* PC1F1Ex

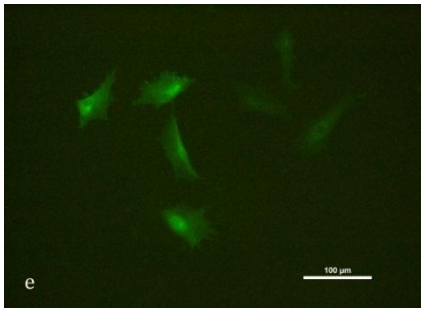

Fig. 4.14*e* PC1CpTr

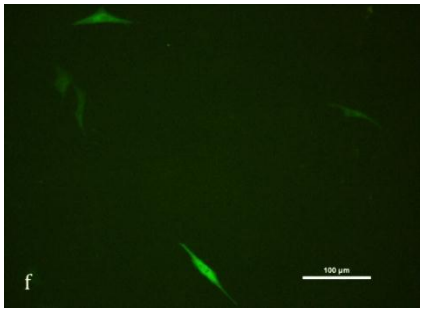

Fig. 4.14*f* PC1L1Tr

Fig. 4.14 Expression of GFP reporter gene after tranfection with lipofectame 3000 reagent and plasmid pcDNA3-EGFP: a) PC1F1Ex, b) PC1CpTr and c) PC1L1Tr. After transfection with jetPRIME reagent: d) PC1F1Ex, e) PC1CpTr and f) PC1L1Tr. Fluorescence was analysed using Leica LB2 microscope 48h after transfection. *Scale= 100µm*

4.5.2 Susceptibility to bacterial ECP from *V. alginolyticus*

All the three cell lines elicited a dose-dependent decline in cell viability after 48h of exposure. Whereas the control cells exposed to L-15 alone appeared to maintain their typical fibroblastic morphology. The initial observations were vacuolation in the cells exposed to ECP from the least (1µg mL^{-1}) to the highest concentration (11µg mL^{-1}). The cells were rounded, cytolysed or both with the consequences of monolayer disruption. The cells appeared to be shrunk or collapsed, observed as a star-like appearance. An increase of dying cells that had granulated and fragmented was observed and the integrity of the monolayers was clearly affected. The 'lowest observed effect concentration' for the cell lines was 1µg mL^{-1}. Figure 4.15 depicts the effect of *V. alginolyticus* on caerulean damsel cell lines at different concentrations.

Similar morphological changes were detected in the three cell lines. In PC1F1Ex, the ECP caused vacuolation, rounding, detachment and finally monolayer destruction at the highest concentration in less than 18h. Dose dependent response was observed, with the least effect observed at lower concentrations (Fig. 4.15a i-vii).

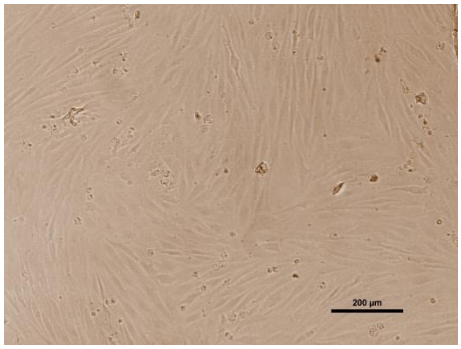

Fig. 4.15*a i* CONTROL

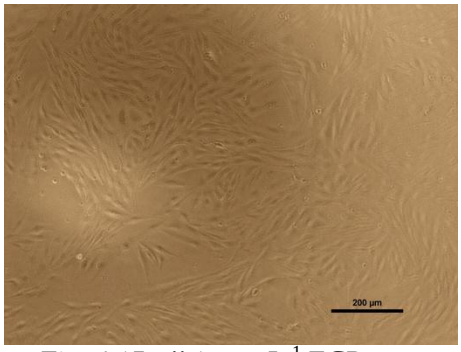

Fig. 4.15*a ii* 1µg mL^{-1} ECP

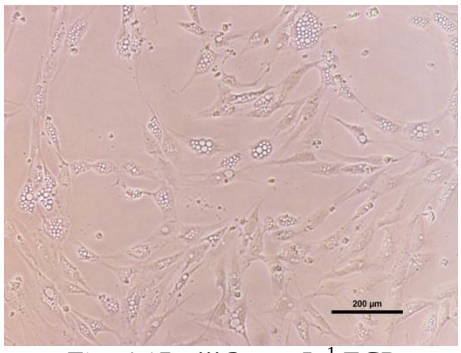

Fig. 4.15*a iii* 3µg mL^{-1} ECP

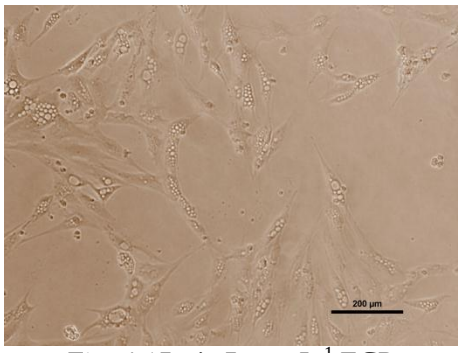

Fig. 4.15*a iv* 5µg mL^{-1} ECP

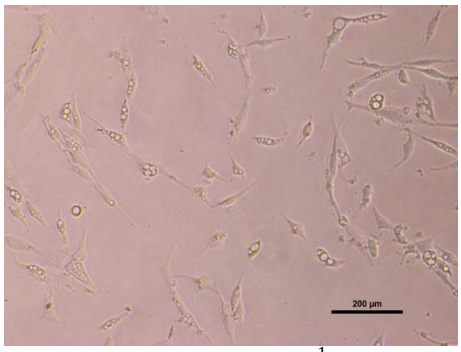

Fig. 4.15*a v* 7µg mL^{-1} ECP

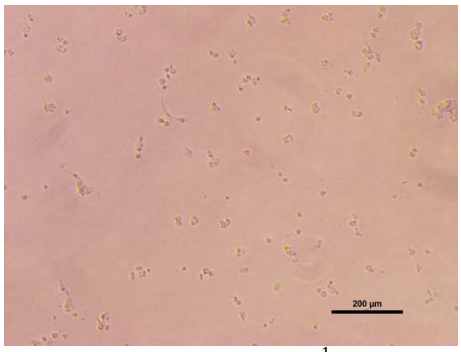

Fig. 4.15*a ii* 9µg mL^{-1} ECP

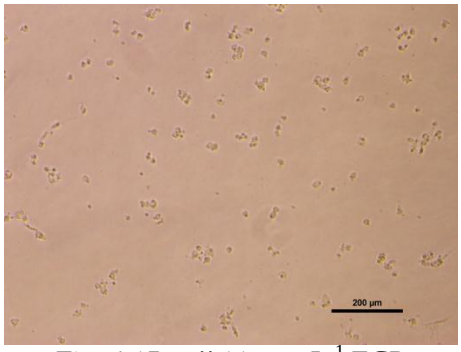

Fig. 4.15*a vii* 11µg mL^{-1} ECP

Fig. 4.15 Light microscopy appearance of caerulean damsel cell lines following 48h exposure to ECP of *V. alginolyticus* and concentration-response curves of ECP from *V. alginolyticus* using XTT assay.

Fig. 4.15a Dose dependent toxicity of ECP from *Vibrio alginolyticus* as observed by morphological changes in PC1F1Ex cells. PC1F1Ex cells at passage 45 were plated at 10,000 cells per well in 96 well TC plates using L-15 containing 5% FBS. PC1F1Ex cells were exposed to six different concentrations of ECP: 1µg ml^{-1}, 3 µg ml^{-1}, 5 µg ml^{-1}, 7 µg ml^{-1}, 9 µg ml^{-1}, 11 µg ml^{-1} and observed at time intervals. Phase contrast micrographs were taken after 48 h and the morphologies were compared to the control cells. A decrease in cell viability correlated to an increase in concentrations of ECP. (i) control cells maintained in L-15 containing 5% FBS displaying an intact fully confluent monolayer; (ii) cells exposed to 1µg ml^{-1} showed intact cells; (iii) cells exposed to 3 µg ml^{-1} displaying vacuolation and cells did not form a monolayer; (iv) cells exposed to 5 µg ml^{-1} showed vacuolation and cells did not form a monolayer; (v) cells exposed to 7 µg ml^{-1} displaying vacuolation, shrinking and clear loss of cell monolayer integrity; (vi) cells exposed to 9 µg ml^{-1} exhibited cell death; (vii) cells exposed to 11 µg ml^{-1} presented complete cell death. *Scale bar =200 µm.*

Similarly, in PC1CpTr, the cells showed shrinking and detachment from the second day onwards with gradual cell death observed at lower concentrations. The highest dose of 11µg mL $^{-1}$ resulted in complete cell death within 24h (Fig. 4.15 *b i-vii*).

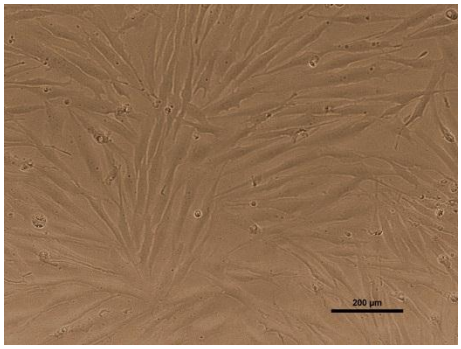

Fig. 4.15*b i* CONTROL

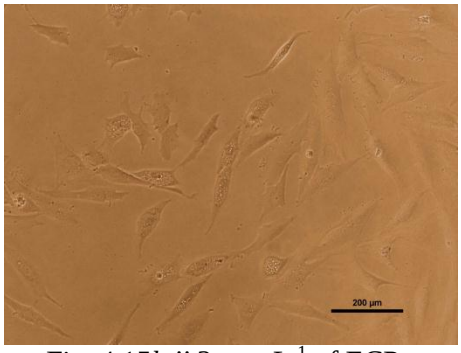

Fig. 4.15*b ii* 3µg mL^{-1} of ECP

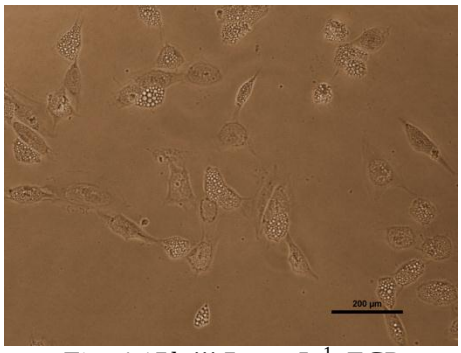

Fig. 4.15*b iii* 5µg mL^{-1} ECP

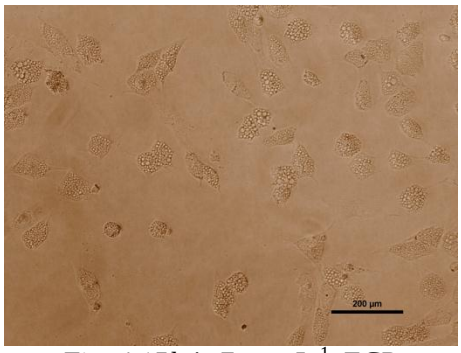

Fig. 4.15*b iv* 7µg mL^{-1} ECP

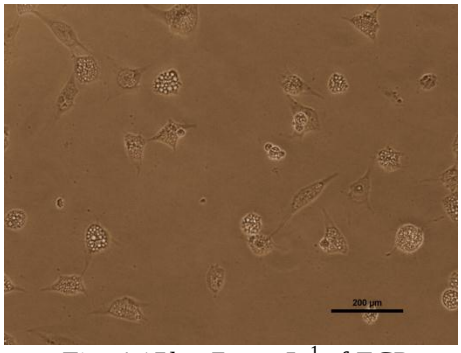

Fig. 4.15*b v* 7µg mL^{-1} of ECP

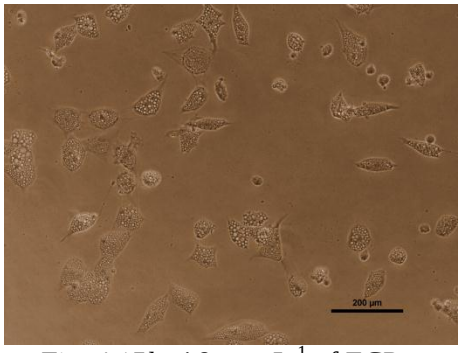

Fig. 4.15*b vi* 9µg mL^{-1} of ECP

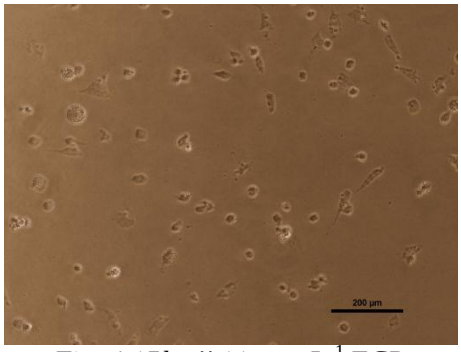

Fig. 4.15*b vii* 11µg mL^{-1} ECP

Fig. 4.15b) PC1CpTr cells at passage 48 were plated at 10,000 cells per well in 96 well TC plates using L-15 containing 5% FBS. PC1CpTr cells were exposed to six different concentrations of ECP: 1µg ml^{-1}, 3 µg ml^{-1}, 5 µg ml^{-1}, 7 µg ml^{-1}, 9 µg ml^{-1}, 11 µg ml^{-1} and observed at time intervals. Phase contrast micrographs were taken after 48 h and the morphologies were compared to the control cells. A decrease in cell viability correlated to an increase in concentrations of ECP. (i) control cells maintained in L-15 containing 5% FBS displaying an intact fully confluent monolayer and vacuolation in a few cells; (ii) cells exposed to 1µg ml^{-1} showing a discernible decrease in the density of the monolayer; (iii) cells exposed to 3 µg ml^{-1} displaying vacuolation; (iv) cells exposed to 5 µg ml^{-1} showed vacuolation and shrinking of cells; (v) cells exposed to 7 µg ml^{-1} displaying vacuolation, shrinking and detaching of cells; (vi) cells exposed to 9 µg ml^{-1} exhibited rounding, vacuolation, detaching of cells and finally change in cell shape and cell death; (vii) cells exposed to 11 µg ml^{-1}presented complete cell death. *Scale bar =200 µm.*

In PC1L1Tr, the cells were comparatively least sensitive to increasing doses of ECP, with complete monolayer destruction observed at 11µg mL^{-1} observed on the 2nd day (Fig. 4.15*c i-vii*).

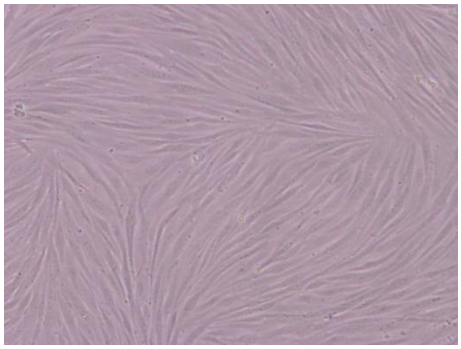

Fig. 4.15*c i* CONTROL

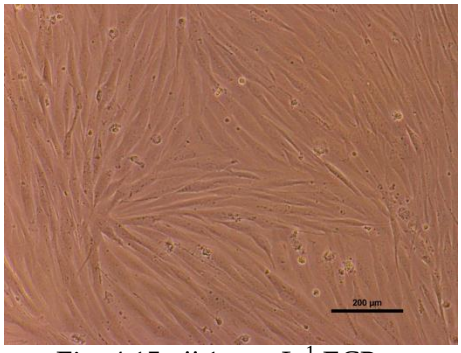

Fig. 4.15*c ii* 1µg mL^{-1} ECP

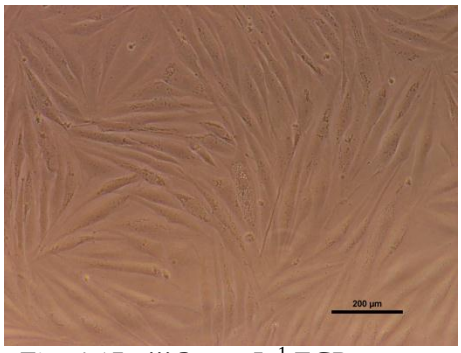

Fig. 4.15*c iii* 3µg mL^{-1} ECP

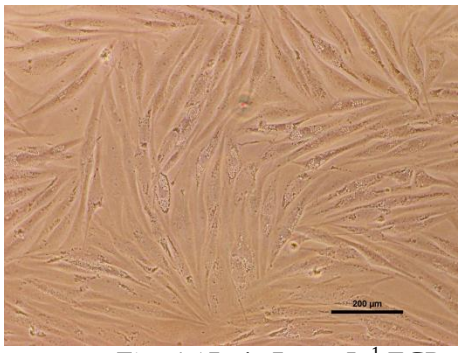

Fig. 4.15*c iv* 5µg mL^{-1} ECP

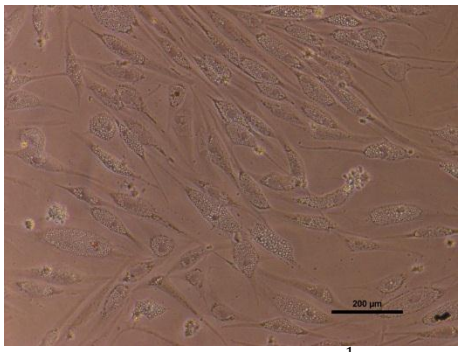

Fig. 4.15*c v* 7µg mL^{-1}

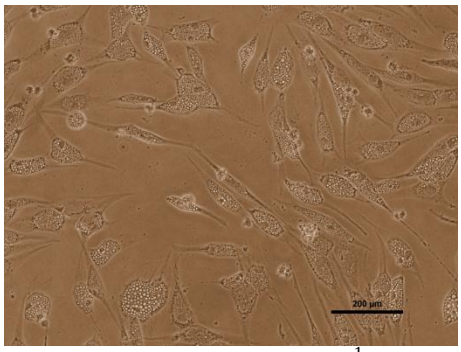

Fig. 4.15*c vi* 9µg mL^{-1}

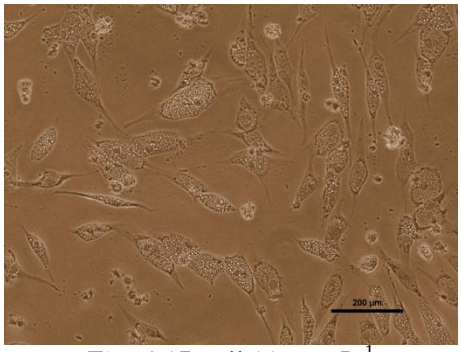

Fig. 4.15*c vii* 11µg mL^{-1}

Fig. 4.15c) Dose dependent toxicity of ECP from *Vibrio alginolyticus* as observed by morphological changes in PC1L1Tr cells. PC1L1Tr cells at passage 50 were plated at 10,000 cells per well in 96 well TC plates using L-15 containing 5% FBS. PC1L1Tr cells were exposed to six different concentrations of ECP: 1 µg ml^{-1}, 3 µg ml^{-1}, 5 µg ml^{-1}, 7 µg ml^{-1}, 9 µg ml^{-1}, 11 µg ml^{-1} and observed at time intervals. Phase contrast micrographs were taken after 48 h and the morphologies were compared to the control cells. A decrease in cell viability correlated to an increase in concentrations of ECP. (i) control cells maintained in L-15 containing 5% FBS displaying an intact fully confluent monolayer; (ii) cells exposed to 1 µg ml^{-1} showing normal cells with no effect; (iii) cells exposed to 3 µg ml^{-1} displaying minimal vacuolation and almost a confluent monolayer; (iv) cells exposed to 5 µg ml^{-1} showed vacuolation and shrinking of cells and a discernible decrease in the density of the monolayer; (v) cells exposed to 7 µg ml^{-1} displaying vacuolation, shrinking and detaching of cells; (vi) cells exposed to 9 µg ml^{-1} exhibited rounding, vacuolation, detaching of cells and finally a majority of the cells were dead; (vii) cells exposed to 11 µg ml^{-1} presented vacuolation and almost complete cell death. *Scale bar =200 µm*.

The susceptibility of the three cell lines could be arranged in the following ascending order: PC1L1Tr (least sensitive) < PC1CpTr < PC1F1Ex (most sensitive). The mean EC_{50} values estimated for the cell lines by carrying out XTT assay were 4.042 µg mL^{-1} for PC1F1Ex, 5.819 µg mL^{-1} for PC1CpTr and 8.2 µg mL^{-1} for PC1L1Tr (Fig. 4.14d).

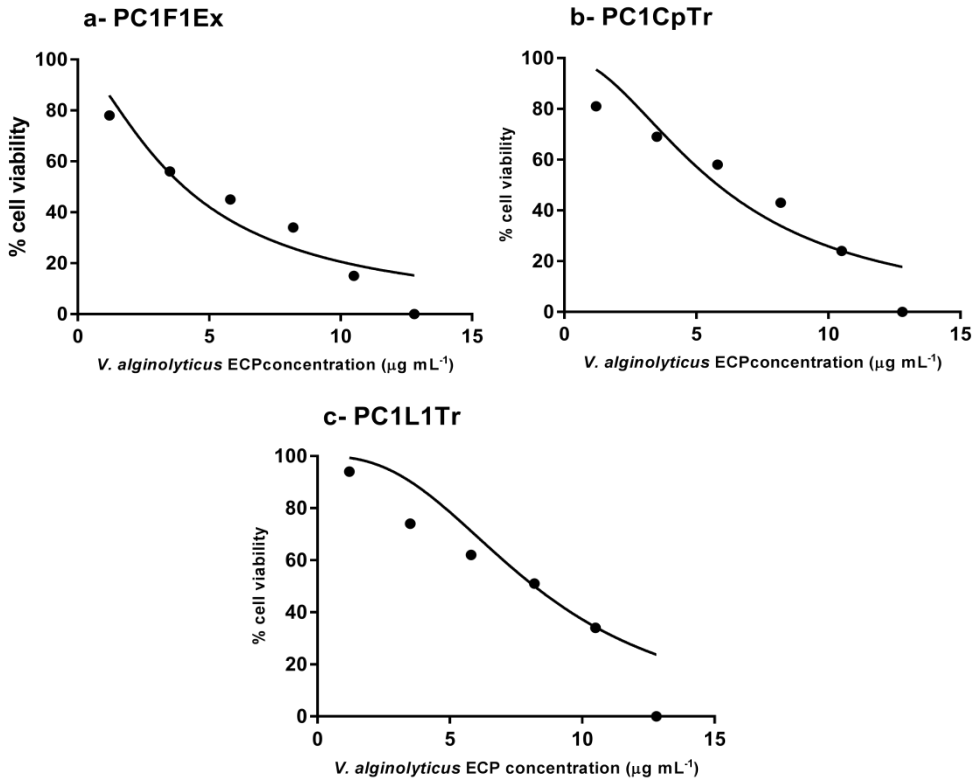

Fig. 4.15d) Concentration-response curves of ECP from *V. alginolyticus*. Metabolic activity was measured using XTT. a) PC1F1Ex; b) PC1CpTr and c) PC1L1Tr

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Chapter

5

Discussion

Contents

Development of established lines from normal cell populations has in most cases been considered a rather infrequent and unpredictable occurrence in higher vertebrates but continuous cell lines appear to arise rather spontaneously from fish (Nicholson, 1989; Bols and Lee, 1991). To confirm the use of authenticated cell lines, full cell line documentation, including the source used during experiments, should be submitted for scientific publications. Cell lines are critical components of experiments and should be considered as standard research reagents and given the same care and quality control measures that surround the use of kits, enzymes, and other laboratory products commercially obtained (Lakra *et al.*, 2010c).

5.1 Preparation of tissues to initiate primary cultures

Disinfection prior to developing primary cultures is critical for obtaining successful lines. Cell cultures can be contaminated by the following means: firstly, contaminated primary cultures (when source of the cells are infected), secondly, contamination due to raw materials, and thirdly, *via* an

animal passage (Merten, 2002). The frequency of contaminants at source for fishes caught in the wild are higher as there is no control of the disease risks with these species.

To reduce stress caused due to transport, the fishes were acclimatised to the wet laboratory conditions at ICAR-CMFRI, Kochi. The formation of foam and scum in transporting fish is common due to stress. The foam interferes with oxygen exposure at the water surface and also makes it difficult to observe the fish being carried. To prevent contamination from faeces or regurgitated stomach contents, the donor fish was not given food for several days prior to primary culture (Wolf and Quimby, 1969). Sodium hypochlorite functions both as an anti-bacterial as well as an anti-fungal reagent (Akimoto *et al.*, 2000). The Safety Division of Fort Detrick found 500 to 5,000 ppm (active chlorine) of sodium hypochlorite solution with an exposure time of five minutes was adequate to kill spores of microorganisms. Vegetative forms of bacteria such as *Salmonella*, *Brucella* and *Pastuerella* were killed by a concentration of 200 to 1,000 ppm with an exposure time of one minute (Phillips *et al.*, 1962).

Jordan *et al.* (1970) stated that 1000 ppm sodium hypochlorite can easily be employed to disinfect equipment or materials used in tissue culture work. When caudal trunk tissues were used for cell culture, external tissues were killed by sterilisation, these tissues comprised only a small percentage of the biomass and therefore the loss was negligible. In obtaining tissues for establishing the FHM cell line, Gravell and Malsberger (1965) immersed the donor minnows for 1 min in a filtered solution of 10% calcium hypochlorite and followed that with a rinse in 70% ethanol. The tissues were then washed in BSS containing penicillin and streptomycin for 10 min thrice (Wolf and Quimby, 1969).

5.2 Development of primary cultures

Primary cultures were initiated form various tissues of the caerulean damsel fish employing explantation as well as trypsinisation protocols. Cultures from fin, gill, caudal peduncle, liver, spleen, kidney, heart and brain were initiated. Teleost fish cell lines have been developed from a broad range of tissues namely, ovary, fin, swim-bladder, heart, spleen, liver, eye muscle, vertebrae, brain and skin (Bol and Lee, 1991; Lakra *et al*., 2010c) and most fish cell lines originated from normal tissues.

An optimised method for isolation of cells must be developed for each fish species and specifically for each tissue type. Assessing the viability of yield as well as the resulting cell recovery and attachment would indicate the best isolation method. Comparing explantation and trypsinisation techniques, the former technique was found more efficient to develop confluent monolayer cultures from heart, brain, kidney and spleen tissues of the caerulean damsel. In case of liver tissue, trypsinisation was found to yield comparatively better cell attachment and proliferation. For tissues such as fin, gill and caudal peduncle, both techniques yielded an adequate number of cells for seeding.

5.2.1 Explant method

Attachment of explants to the culture flasks is the primary requirement for successful cell culture systems. Explants exhibited variation in the rate of attachment to the culture flasks. The different tissue explants exhibited variation in the degree of attachment to the culture flasks on incubation. Tissues from fin, caudal peduncle, gill, heart and liver exhibited appreciable attachment to the flask surface, attributed to several cell attachment factors that are released by these tissues. Attachment was comparatively deficient in the case of brain and kidney tissues. Various factors such as nature of the

tissue, size of the explants, duration of semi-drying, growth surface and presence of coating materials on the growth surface to enhance attachment can influence the degree of attachment (Part and Bergstrom, 1995).

Lakra and Goswami (2011) developed PSCF cell line from the caudal fin of *Puntius sophore* using explantation technique, cultures were developed in Leibovitz's L-15 which contained 20 % FBS. Similarly, PDF cell line from *Puntius denisonii* was developed from the caudal fin and cultured at 26˚C in L-15 with 10% FBS. The presence of both epithelial-like and fibroblast-like cells was observed in primary cultures (Lakra *et al*., 2011). Fin cell lines have also been developed from *Epinephelus fuscoguttatus via* trypsinisation technique by digesting fin tissues with hyaluronidase and collagenase II. Primary cultures were initiated at 24˚C using 20% FBS-DMEM/F12 medium, which was further supplemented with carboxymethyl-chitooligosaccharide, basic fibroblast growth factor and insulin-like growth factor-I. The fibroblastic fin cells grew at a steady rate during subsequent subculture and had a population doubling time of 50.6h at passage 60 (Wei *et al.*, 2009). However, Cheng *et al*. (2010) digested fin tissues of *Rachycentron canadum* (L.) in trypsin for 15 min and initiated primary cultures by incubating at 28˚C with 10% FBS in L-15 medium. These fin cells were predominantly epithelial-like.

Alvarez *et al.* (1991) reported 100% success rate with mechanical methods of initiating cell cultures compared to only 10% with trypsin digestion for marine and freshwater fish. Explant method is comparatively easier with fewer stages, thereby reducing chances of contamination. In addition, several studies have shown success in initiating successful fish cell culture systems employing the explant method (Noga *et al.*, 1981; Lakra and Bhonde, 1996; Rao *et al.*, 1997; Kumar *et al*., 2001; Sahul Hameed *et al.*, 2006).

5.2.2 Trypsinisation method

Trypsinisation protocol was utilised to develop liver primary cultures from the caerulean damsel. Similarly, liver fragments from *Leiostomus xanthurus* were digested for 30 min with 0.25% trypsin-0.02% EDTA solution to develop a continuous liver cell line (Faisal *et al.*, 1995). *Sardinops sagax neopilchardus* liver cell line was developed by slowly agitating liver tissue in 5 mL of 0.25 % trypsin solution on a magnetic stirrer at room temperature (20-24 °C). After initial trypsinisation for 4 min, supernatant was removed and second trypsinisation treatment was carried out on the remaining tissues. The supernatants were pooled to obtain the primary liver culture (Williams *et al.*, 2003). Lai *et al.* (2000) carried out the disaggregation of the liver tissues using cold trypsin (0.25% trypsin 0.2% EDTA in PBS) for 1 h at 4°C for *Epinephelus awora.*

At 24 ˚C with 0.25 % trypsin solution, primary cultures of the liver tissues from the seaperch *Lateolabrax japonicus* could be obtained (Ye *et al*., 2006). Another proteolytic enzyme used was dispase (0.6 U mL^{-1}) for rainbow trout liver fragments to initiate the development of a liver epithelial line, RTL-Wl (Lee *et al.*, 1993). However, liver primary cultures have also been obtained by explantation technique, for instance, from *L. rohita* (Lakra *et al*., 2005).

Cell cultures from caudal peduncle have been developed from *P. caeruleus* by trypsinisation technique. Similarly, caudal peduncle cell line from *Puntius schwanenfeldi* was derived using the same technique. The fragments were subjected to a series of 0.5- 1 h digestions at 20˚C in 0.25% trypsin. The harvested cells were grown in L-15 supplemented with 20% fetal calf serum (FCS). Confluent primary cell monolayers were observed from

14th day (Karunasagar *et al*., 1995). Caudal peduncle cell cultures were developed by Joseph *et al*. (1998) from the tissues of catla (*C. catla*), mrigal (*Cirrhinus mrigala*) and rohu (*L. rohita*) using explant technique.

Trypsin is a pancreatic serine protease and is most commonly used in solution with EDTA to dissociate monolayers of cultured cells. Its activity is directed towards transmembrane glycoproteins, integrins, which attach cells to extracellular matrix proteins and to ligands on other cells *in vivo*, and to culture surfaces *in vitro*. The cytotoxicity of trypsin under certain conditions is well known (Freshney, 2010), and the cell surface receptors facilitating attachment may get destroyed on extensive treatment with trypsin (Hashimoto *et al*., 1997); but is used widely in the production of fish primary cultures (Huang *et al*., 2009; Wei *et al*., 2009; Zheng *et al*., 2012) albeit with inconsistent outcomes. Comparatively, trypsinisation technique has worked favourably for the development of primary cultures from liver and caudal peduncle of the caerulean damsel. Similarly, Sobhana *et al.* (2008) derived heart cultures from *E. tauvina* using trypsin solution at room temperature (24˚C -26˚C).

An optimised method for isolation of cells must be developed for each fish species and specifically for each tissue type. Assessing the viability of yield as well as the resulting cell recovery and attachment would indicate the best isolation method. By using both explantation and trypsinisation techniques, it was found that explantation technique was the most efficient to develop confluent cultures from heart, brain, kidney and spleen. In case of liver tissues, trypsinisation was found to yield comparatively better cell attachment and proliferation. For tissues such as fin, gill and caudal peduncle, both techniques yielded an adequate number of cells for seeding.

5.3 Development of continuous cell lines

Emergence of cells was relatively faster from fin, caudal peduncle and liver as compared to other tissues. The cells were found to migrate away from the explants, then attach and multiply. Cell to cell communication is an inherent property of growing cells in culture (Trinkaus, 1984) and explains the migration of cells away from the explants. Besides, the second most common tissue used for cultivation is fin next to ovary, due to its high regenerative ability (Fryer and Lannan, 1994). Moreover, non-lethal sampling can be undertaken with fin tissues, without sacrificing the fish. Fin cells were subcultured initially at 1:2 but gradually 1:4 was also possible. In this study, fin tissues were found to be ideal in terms of attachment, growth, subcultivation and cryostorage with revival.

Similarly, Swaminathan *et al*. (2012b) developed PDF cell line from the ornamental fish, *Puntius denisonii* fin cells in L-15 medium supplemented with 20% FBS. The cells were subcultured every 4-5 days for the initial 10 passages. The rest 15 passages, 50% culture medium was replaced with fresh medium at 4-day intervals. After 15^{th} subculture, cells were subcultured at a 1:3 ratio every 3-4 days, and FBS was reduced to 15% in the culture medium.

Monolayer formation was faster in the case of fin and caudal peduncle as compared to liver, perhaps due to the nature of these tissues. Liver cells showed a higher requirement of serum and the cells developed gradually into confluent monolayers. This may be explained by a feature commonly seen in hepatic cells in some species which have a reluctance to attach and spread onto growth substrates (Bols *et al*., 1991). The liver cells could have spread and multiplied with the factors that were present in the serum. Liver cell cultures were subcultured at a ratio of 1:2. The serum requirement steadily lessened to

5% FBS in L-15. Similarly, *Epinephelus awora* liver cell line was subcultured using 0.25% trypsin; with serum concentration gradually reduced to 10% till the 80th passage and further reduced to 5% (Lai *et al.*, 2000).

Cells from caudal peduncle tissues of *P. caeruleus* in the present study showed quick attachment and growth. By the 10th passage, the cells were homogenous and the requirement for serum reduced to 5%. The cells could be subcultured at a ratio of 1:4 by the 20th passage. Similarly, caudal peduncle cell line from *P. schwanenfeldi* cells were grown in L-15 supplemented with 20% FCS at 30˚C. The cells grew at an optimum concentration of 10% FBS (Karunasagar *et al*., 1995). On the contrary, PC1CpTr cell line requires only 5% serum in L-15 at 28˚C.

5.3.1 Culture medium

The comparative suitability of different media showed DMEM was suitable for supporting fish cell lines, which was confirmed by Fernandez *et al.* (1993a) using 28 fish cell lines. Currently, more than 80% of the cell lines established since 1994 have used Leibovitz's L-15 medium (Lakra *et al*., 2010c). The tissues in this study seemed best suited for growth in Leibovitz's L-15 with 20% FBS for primary cultures, 10% FBS after 20 passages, while only 2-5% FBS was found adequate for subculture and maintenance after passage 40. Bradford *et al.* (1994) described that primary cell cultures need higher serum concentrations and seeding densities than cells in later passages. Fan *et al*. (2010) changed the media finding better growth in MEM than in L-15. Turbot fin cell line (TF) was subcultured on reaching confluency using 0.25% trypsin. From passage 30, the culture medium was changed from L-15 medium containing 20% FBS to minimum essential medium (MEM)

containing 20% bovine calf serum (BCS). On the contrary, caerulean damsel cultures adapted well in L-15 medium at all passage levels.

L-15 medium supplemented with 20% FBS supported the development of the cell lines in the present study. Faster growth and better proliferation was noticed with cells cultured in L-15 medium at pH 7.4. The Leibovitz's L-15 medium merits particular credit for it was designed to maintain pH in the physiological range under normal atmosphere without added CO_2. Part and Bergstrom (1995) reported that L-15 medium which has galactose as the energy source was the best for their studies on fish gill tissues.

The response of the ovarian tissue of the African catfish *C. gariepinus* to different media was tested by Kumar *et al*. (2001). Among the three basic media analysed, the Leibovitz's L-15 when supplemented with FBS and fish muscle extract (FME) supported the highest percentage of attachment of explant tissues leading to cell proliferation than DMEM and M199 with the same supplements.

5.3.1.1 Evaluation of the role of supplements on growth of cells

Serum contains growth factors, which promote cell proliferation and adhesion factors as well as antitrypsin activity, which in-turn promote cell attachment. It is also a source of minerals, lipids, and hormones, many of which may be bound to proteins. The sera used frequently in tissue culture are fetal bovine serum (FBS) and calf (CS) serum (Freshney, 2010). When used with synthetic media, the usual level of serum was 10-15% (Wolf and Quimby, 1969). Some fish cells have been observed to grow in 2% serum (Jensen *et al*., 2012), in contrast to others which required 20 or even 30% serum levels (Bryson *et al*., 2006; Lakra *et al.*, 2006b).

Addition of homologous fish muscle extract (FME) or inactivated fish serum (FS; homologous/ heterologous) did not contribute to any improvement in growth/ formation of the monolayer in the present study, neither was differential growth recorded. Similarly, Part *et al.* (1993) observed that growth factors did not enhance or negate the development of primary cultures of epithelial cells from rainbow trout gills. This precisely indicates that there is no fixed rule as far as growth factors are concerned and the required growth factors have to be investigated for every fish species studied, and for every organ used for cell culture development (Kumar *et al*., 2001).

Contrary to the current study which found neither FME or FS essential, Lakra *et al.* (2006a) employed [3H] thymidine uptake assay to investigate the effect of different concentrations of fetal calf serum (FCS) and fish muscle extract (FME) on the growth of TP-1 cells developed from *T. putitora* and to study the cell proliferation rate at different time intervals. It was found that L-15 supplemented with 20% FCS and 10% FME at an incubation temperature of 28˚C resulted in optimal growth. Two cell culture systems namely epithelioid cells of *L. calcarifer* (LCE) and fibroblastic cells of *L. calcarifer* (LCF) were developed from the fry and fingerlings of the Asian seabass, *L. calcarifer* (Lakra *et al.*, 2006b), using Leibovitz's L-15 medium supplemented with 20% FBS and 1% fish serum (FS). Dulbecco's modified Eagles medium (DMEM) supplemented with FBS, marine fish serum, seaperch embryo extract, selenium, basic fibroblast growth factor (bFGF) and leukaemia inhibitory factor (LIF) was used to establish the LJES1 cell line from embryos of the seaperch, *Lateolabrax japonicus* (Chen *et al*., 2003). The TEC (turbot embryonic cell line), established from embryos of turbot (*Scophthalmus maximus*), were cultured in DMEM supplemented with FBS, seaperch serum (SPS) (Chen *et al*., 2005).

Though the present study has not reported any adverse effects of supplemented fish sera, several other workers have demonstrated that heterologous or homologous fish sera may be inhibitory (Clem *et al*., 1961) or toxic (Fryer, 1964; Fryer *et al.*, 1965; Collodi *et al.*, 1990; Part *et al.*, 1993; Avella *et al.*, 1994) and may also increase the risk of introducing latent viruses. But others have used it normally as a culture supplement (DeKoning and Kaattari, 1991; 1992; Diago *et al.*, 1993; 1995; Moritomo *et al.*, 1994). Growth of gold fish fin cells was significantly reduced when Hashimoto *et al*. (1997) used heat treated carp serum but normal serum was found to favour growth of gold fish fin cells during early passages.

5.3.2 Cryopreservation

PC1F1Ex, PC1CpTr and PC1L1Tr cell lines have been successfully cryopreserved in liquid nitrogen at different passage levels. The cryostored cells showed >80% survival rate on revival after 4 years of storage. Fetal bovine serum at a concentrations of 20% and higher were ideal for storage and revival rates were as high as 80% and above. A high concentration of serum is used in the medium which acts as a complex protective agent that may help prevent cell damage during freezing and thawing and also it presumably supplies some cryoprotection as well as additional growth factors. DMSO was used as the cryoprotectant in the present study. Similar to the present study, concentrations of 20% FBS and 10% DMSO have been used to cryopreserve spleen (EAGS) and swim bladder (EAGSB) cell lines from *Epinephelus akaara* (Huang *et al.*, 2009). Lakra *et al.* (2006b) stored LCE and LCF cell lines using similar concentrations, whereas, Ku *et al.* (2010) used higher concentrations of FBS (50%) for storing *Pagrus major* fin cell line (RSBF-2). In contrast, Swaminathan *et al*. (2012b) used only 10% for storing *P. denisonii* fin cell line (RTF).

Wang *et al.* (2003) derived and stored three cell lines (fin - WSF, head soft tissue - WSHST and body muscle WSBM) derived from *Acipenser transmontanus,* using DMSO as the cryoprotectant. WSF, WSHST and WSBM cells exhibited a viability of >90% after a 16- month storage period. Rougée *et al*. (2007) established two goldfish cell lines GFM and GFSB from muscle and swim bladder which showed 92% and 93% survival rates respectively. Following storage, no obvious alterations in morphology or growth pattern were observed for GFSB or GFM.

Periodic and judicious storage of the cultures were carried out during the course of this study to serve as a steady source of cell lines at the desired passage for further application studies such as gene transfection studies and cytotoxicity assays. Hence, these cells would be free of spontaneous genetic drift or contamination from microbial cultures as well as safe from unexpected power failures resulting in loss of experimental data or laboratory lines.

Most authors conform with the present study that the cell lines developed retained their initial characteristics; for instance, Tatner (1988) described rainbow trout thymocytes which retained their responsiveness to Con A after having been cryopreserved. Although contrary findings have also been reported, such as, cryopreservation using DMSO causes differentiation in some cultures (e.g., HL-60 promyeloblast cells).

5.3.3 Optimum serum concentration

Bols *et al.* (1994) concluded that the optimal level of serum supplementation in the cell culture is 10%. Some cells grow satisfactorily with 5% serum. Serum levels above 10% have been recommended for some primary cultures and only very few cell lines. During the initial stages of developemnt, 15% -20% FBS is required (Lannan *et al*., 1984; Follet and Schmitt, 1990; Ristow

and Avilla, 1994; Sahul Hameed *et al*., 2006; Parameswaran *et al*., 2006a, b) after which, a 5% concentration is adequate (Part *et al*., 1993). In the present study, similar conclusions were derived, 20% FBS was used in the primary cultures and gradually 10% was sufficient for the initial passages and progressively the cultures grew in 2-5% FBS.

Dependence on serum for growth of the cell cultures was vital, lack of which resulted in gradual peeling off of the monolayer. Growth rates of the cultures after establishment, progressively increased and continued to do so in Leibovitz's L-15 with 5% FBS. For routine maintenance, 2% FBS supplemented in L-15 medium was found to be adequate when split at a ratio of 1:3. A higher FBS content of 10- 20% FBS led to an increased saturation density.

Supplementing the culture medium with nutritional factors was essential to promote the growth of cells *in vitro*. Hence, FBS was constantly supplemented at various concentrations depending on the stage of development. Comparably, much of the earlier development of fish cell lines was done with FBS. Serum contains hormones, micronutrients, growth and cell adhesion factors, which are known to stimulate cell spreading in culture (Yamada and Olden, 1978; Hashimoto *et al.*, 1997). Hence when the serum level is reduced, substitutes must be provided to perform these functions (Cheng *et al*., 1993). However, high concentration of serum limits the growth of promising cultures, as they may contain undefined inhibitory factors for some cell cultures (Todaro and Green, 1963; Loo *et al*., 1987). Medium containing low serum concentration (2-3%) has also been used for some cultures (Cheng *et al*., 1993).

5.3.4 Optimum temperature

Temperature studies carried out using L-15 medium with 5% FBS showed that 28±2˚C is the most suitable temperature for optimum growth and proliferation of the cultures, which is in concurrence with other marine fish cell cultures (Sobhana *et al*., 2008, 2009). Caerulean damsel usually inhabits waters with a temperature range of 20- 28 ˚C, with optimal growth at 25 ˚C - 26 ˚C. Cells from warm water fishes generally do well at 25˚C to 30˚C but may grow well at 15˚C through 35˚C and higher (Wolf, 1973). Two carp cell lines have been reported to grow optimally at 37˚C (Ku and Chen, 1992). Furthermore, the highest growth rate of varied tropical fish cell lines were observed at 34˚C (Middlebrooks *et al*., 1979) and as low as 20- 25˚C (Grunow *et al*., 2011; Bain *et al*., 2013). Another finding that has contributed to the understanding of optimum temperatures for growth was in LCF and LCE cells, developed from fry and frylings of *L. calcarifer* which exhibited maximum growth rate at 28˚C, but were growing over a wide temperature range tested from 20˚C - 36˚C (Lakra *et al.*, 2006). On the contrary, Tong *et al.* (1997) observed that temperatures above 35 ˚C were lethal for the cell line FG-9307 from the gill of flounder, *Paralichthys olivaceus*.

Cultured fish cells will tolerate considerable drops in temperature, can survive several days at 4 ˚C, and can be frozen and cooled to -196 ˚C (Freshney, 2010).

The principal advantage of vertebrate cell culture and notably fish cell culture is their innate ability to metabolise through a wide range of temperatures. The adaptation of cells in cell culture can be aided by slight increases of incubation temperature at each subculturing or after several subcultures (Wolf and Ahne, 1982).

Fish cell culture is simpler, as room temperature is adequate for incubation however; the number of times cultures are handled can be greatly reduced by incubation at lower temperatures (4- 15˚C). Optimal growth temperature of cells depends upon the fish species and its natural environment (Gabridge, 1985). Based on physiological requirements and environmental preferences, fishes are classified into cold water or warm water species and the temperature factor applies to the *in vitro* culture conditions. Cells from warm water fishes generally do well at 25 to 30˚C but may grow well at 15˚C through 35˚C and higher (Wolf, 1973). Two carp cell lines have been reported to grow optimally at 37˚C (Ku and Chen, 1992). For cold water fishes like salmon and trout, the optimum temperature ranges from 15 to 20 ˚C. Generally, the *in vitro* growth temperatures are a few degrees above the preferred *in vivo* environmental temperature (Lakra and Bhonde, 1996).

5.3.5 Quantitation studies

Growth curves of the cell lines were plotted to understand cellular growth and determine time, temperature and the rate of seeding. The log phase is much required for further studies such as the effect of drugs and chemical agents that stimulate or inhibit cell growth. The cytostatic effects are best assessed at this phase. The high plating efficiency and cell proliferation observed in the cell lines is indicative of a genotypic change or transformed characteristic (Freshney, 2010). Seeding efficiency was observed to be the highest in PC1F1Ex and the least in PC1CpTr. The split ratio was calculated to maintain the cells at its optimum growth and viability. High split ratios are indicative of immortalised cells (Kyo *et al.*, 2003) as observed for PC1F1Ex, PC1CpTr and PC1L1Tr.

5.4 Characterisation of the cell lines

5.4.1 Determination of transformation by chromosome analysis

The dosage of colchicine was assessed based on the mitotic index, after which chromosome spreads were obtained. A lower concentration (< 0.01µg mL^{-1}) resulted in a decreased mitotic index caused by a failure to arrest the division of cells and also chromosomes that are overlapped. A higher concentration (>10 µg mL^{-1}) of colchicine resulted in poor morphology due to shrinkage of the arms. To improve the spread of metaphase chromosomes, cell suspensions were dropped from a height of 30 cm onto a clear glass slide, which was maintained in a tilted position. However, even when all conditions were standardised, a few cells showed poor quality spreads or even with disturbed shapes.

The results from chromosome analysis clearly indicated that the cells are aneuploid. Immortalisation occurs spontaneously in fish cell lines at early stages before ploidy levels may be determined (Bols *et al*., 2005). Similar findings were published by Cheng *et al.* (2010) who reported heteroploidy with CB and CF cells (brain and fin cells from *Rachycentron canadum*) having widely distributed number of chromosomes distributed from 50 to 88 and 48 to 89 in CB and CF cell lines, respectively. However, the predominant numbers were 73, 78 and 79 for CB cells and 57, 66 and 74 for CF cells. Bloch *et al.* (2016) developed eelB cell line from the American eel and reported that after 19 passages, the modal chromosome number was 38 and after 45 passages, no clear modal number was observed.

The variation in chromosome number may reflect aneuploidy or chromosome rearrangement during the establishment of the lines. *In vitro* changes in chromosome morphology and ploidy levels have been documented

in various long term fish cell culture lines (Regan *et al.*, 1968; Chen and Ebeling, 1975). The adaptation of ectothermic vertebrates cell lines to *in vitro* conditions is often associated with dynamic chromosomal variations as reported by Wolf and Mann (1980). Similarly, mouse fibroblasts and cell cultures from a variety of human and animal tumours often become aneuploid in culture and frequently give rise to continuous cultures. Possibly the condition that predisposes most to the development of a continuous cell line is inherent genetic variation, and therefore find genetic instability perpetuated in continuous cell lines (Freshney, 2010).

5.4.2 Determination of cell type by immunotyping

Monoclonal antibodies directed against three cell type specific protein markers *viz.*,vimentin, cytokeratin and fibroblast surface antigen were used for characterisation of the cell type of the three cell lines developed from *P. caeruleus*. Vimentin is an intermediate filament indicating mesenchymal origin such as endothelial cells, smooth muscle cells and fibroblasts. Therefore it is a typical and widely applied fibroblast marker (Sappino *et al*., 1990; Richards *et al*., 1995). Cytokeratin is an intermediate cytoskeleton filament produced by cells of epithelial origin. In the present study, epithelial-like cells and fibroblast-like cells coexisted in the initial stages. However, as the culture progressed fibroblast-like cells were predominant. The cell lines also stained negative for the fibroblast surface antigen indicating all the three cell lines are transformed continuous cell lines. Fibroblast surface (SF) antigen is a major cell surface glycoprotein located in discreet cell surface ridges and cytoplasmic extensions of cultured chick fibroblasts (Linder *et al*., 1975). SF has been found to be absent from cell surface after malignant transformation of human cells by Rous sarcoma virus (Vaheri and Ruoslahti, 1974). Lakra *et al.* (2011) reported the presence of both epithelial cells and fibroblast-like

cells in the caudal fin primary culture of the ornamental fish, *Puntius denisonii*. However, beyond 15 subcultures, fibroblast-like cells proliferated more rapidly than the epithelial cells and were ultimately predominant. Many serum factors derived from platelets have a strong mitogenic effect on fibroblasts with a tendency to inhibit epithelial proliferation, subsequently causing fibroblasts to overgrow in subcultures (Freshney, 2010). Predominance of fibroblastic cells over epithelioid cells in cell cultures from fish has been extensively reported (Béjar *et al*., 1997; Lai *et al.*, 2003; Lakra *et al*., 2006a). Ye *et al.* (2006) developed a fibroblast-like cell line (LJH-2) from *Lateolabrax japonicus*. In contrast, seaperch heart (SPH) cells have been reported to be of epithelioid morphology even after successive passaging (Tong *et al*., 1998).

The predominance of fibroblast cultures could be due to platelet-derived growth factor (PDGF) (Antoniades *et al*., 1979; Heldin *et al*., 1979), a family of polypeptides with mitogenic activity and probably the major growth factor in serum, which is known to stimulate growth in fibroblast cells (Freshney, 2010).

Parameswaran *et al.* (2007) showed that the fibroblastic heart cell line derived from milkfish, *Chanos chanos*, was strongly positive for desmin and fibronectin. Brain cell line, ASBB, derived from Asian seabass had a mixture of epithelial and fibroblastic cells up to the 10^{th} subculture, after which fibroblastic cells predominated in the culture (Hasoon *et al*., 2011). From the ornamental fish, *Puntius sophore*, caudal fin cell line, PSCF, was developed which was found to be fibroblastic (Lakra *et al*., 2011a). Similarly, a fibroblastic cell line was derived from the tissues of the caudal fin of *Puntius denisonii* (Swaminathan *et al*., 2012).Yan *et al.* (2011) developed a tail fin cell

line, GFTF from *Carassius auratus* (L.), and found majority of the cells to be fibroblastic in nature.

Wen *et al*. (2008) showed that epithelial brain cell lines (GBC1 and GBC4) derived from *Epinephelus coioides* was strongly positive for cytoplasmic cytokeratin. Cell line from newly hatched Atlantic cod (*Gadus morhua*) larvae (ACL) cells had a polygonal shape and the morphology appeared homogenous with epithelial-like cells (Jensen *et al*., 2012).

5.4.3 Authentication of species of origin

The mitochondrial DNA (mtDNA) of vertebrates is a common molecular marker, used in phylogenetics, population genetics, and species identification. One essential property this DNA exhibits is its rapid rate of evolution estimated to be 5 to 10 times higher in comparison to nuclear genomes. Maternally inherited, it reflects the female-specific part in the evolution and can point to the history of a certain taxon; besides it also lacks homologous recombination system, avoiding the effect of intermolecular recombination on the rate of mtDNA mutations and thus has a significant impact on the interpretation of mtDNA diversity. Another feature is its high copy number which is estimated to be greater than 1000 copies, playing a key role in all fundamental questions of mitochondrial genetics *e.g*., the recombination and segregation of mtDNA sequences (Zischler, 1999).

The partial sequencing of cytochrome oxidase subunit I (COI) of mitochondrial DNA (mtDNA) genes covers the 640-bp segment of the 5’ region of the COI gene that continues to be used to form the library of primary barcodes for the animal kingdom (Hebert *et al*., 2003) and has been successfully used to identify many other cell lines (Lorenz *et al*., 2005; Cooper *et al*., 2007). A BLAST search indicated greater than 99% sequence identity

among the COI genes from PC1F1Ex, PC1L1Tr and PC1CpTr and the muscle tissue with the known caerulean damsel mitochondrial DNA sequences in NCBI Genbank.

Origin of the PSCF cell line, derived from the ornamental fish *Puntius sophore*, was confirmed by the amplification of 655 bp fragments of cytochrome oxidase subunit I (COI) of mitochondrial DNA (mtDNA) genes (Lakra and Goswami, 2011). Similarly, the ornamental fish, *Puntius denisonii*, was also authenticated using the same gene sequence (Swaminathan *et al*., 2012b).

Authentication using COI has a range of applications such as phylogenetic studies, spatial differentiation and deep divergence within species as well as species discovery and validation (Ward *et al.*, 2009).

5.4.4 Transmission electron microscopy and mycoplasma screening

TEM analysis was performed to rule out the presence of any inherent viruses or contamination due to the presence of mycoplasma in these cell lines. Freshney (2010) reported that potential sources of viral contamination are bovine serum and cross contamination. The cells were free of contaminants and on observation were found to be fibroblastic in nature and the cell organelles were also clearly visible. Other ornamental fish cell line studied using TEM is the goldfish macrophage cell line (Wang *et al.*, 1995). Electron microscopy may also be used for ultrastructure studies in cell lines to determine their viral susceptibility (Dannevig *et al*., 1995; Karunasagar *et al.*, 1995; Qin *et al*., 2006). Virus may be inoculated in cell lines to obtain *in vitro* purified virus in higher titre, which may be for studies such as viral structure (Lai *et al*., 2001), for pathogenic studies (Qin *et al*., 2006) and also for study of substrate material to annul the presence of viruses that would harm

downstream processing of biotechnological products and also performance studies which assess virus inactivation and removal (EMEA, 2006).

Presence of mycoplasma was negated using MycoFluor™ Mycoplasma Detection Kit (M-7006, Sigma, USA). The fluorescence technique usually involves staining the cells with DNA specific fluorescent dye, 4, 6 diamidino-2 phenylindole (DAPI). In mycoplasma free cultures only the fluorescent cell nuclei can be seen. Infected cultures show typical starry sky appearance due to mycoplasma cells. All the three cell lines were routinely checked for mycoplasma to maintain integrity of the cell lines.

Mycoplasma contamination of sera and cell cultures has been studied by several authors at the ultrastructural level (Boatman *et al.*, 1976), by its growth in cell cultures, broth or solid media (Hayflick, 1965; Barile *et al.*, 1973; Hopps *et al.*, 1973) and by enzymatic detection (Bonissol *et al.*, 1984). Barth and Majerowicz (1988) employed negative staining of electron microscope preparations of sera and cell cultures for the detection of mycoplasma, concluded that it is possible to distinguish mycoplasma particles from other debris, such as lipid droplets or cell membrane fragments. It is also possible to recognise two types of mycoplasmas: a visible form with smooth and white bordered particles including the "elementary bodies" and a form which also lacks this border, non-viable particles (Robertson *et al.*, 1975).

Diago *et al.* (1995) detailed the morphology of the pronephric stromal cell line, TPS derived from the rainbow trout using electron microscopic technique (TEM). Sathe *et al*. (1997) carried out electron microscopic studies on RG-1 cell line derived from *L. rohita*, which revealed a fibroblast-like morphology. Similarly, the ultrastructure of RTL-W1 (rainbow trout liver cell line) developed from the liver tissue of rainbow trout was examined in

confluent cultures by TEM (Lee *et al*., 1993). In addition, ultrastructural examination of RTgill-Wl (rainbow trout gill cell line) revealed standard cellular features and absence of specialised structures (Bols *et al.,* 1994).

5.5 Application: gene expression studies and *in vitro* cytotoxicity testing using bacterial ECPs

5.5.1 Gene expression studies using GFP reporter gene

Fish cells stably expressing exogenous genes have potential applications in the production of fish recombinant proteins, gene-function studies, gene-trapping, and the production of transgenic fish. Transfection of cells with plasmids encoding a gene of interest coupled to a reporter gene, *e.g.* green fluorescent protein (GFP) has become a pivotal technique for the study of gene expression, protein trafficking and localisation (Tsien, 1998).

While there is a need to improve transfection efficiency, results obtained from the present study indicate that caerulean damsel cells are susceptible to vector transfection and could serve as an *in vitro* system for the evaluation of promoter efficiency in various gene constructs, intracellular cell signalling and expression studies.

In this study, the cell lines were transfected separately with Lipofectamine 3000 and JetPrime™ reagent to evaluate the transfection efficiency of the commercially available transfection reagents. Lipofectamine 3000 produced higher fluorescence signal and more cells were found to be transfected when compared to JetPrime™ after 48h of incubation.

Similar to the present study, Ku *et al.* (2009) transfected continuous cell lines derived from *Epinephelus quoyanus* brain, gill and heart tissues and designated RGB, RGG and RGH respectively with the vector pEGFP-C3

under the control of the human cytomegalovirus (CMV) promoter, using Lipofectamine 2000 for transfection. The expression of GFP in these cell lines could be detected as early as 30 h after transfection. Similar results were obtained in the RSBF-2 cells (obtained from fin tissues of *Pagrus major*) which were transfected successfully with pEGFP-C3 by means of Lipofectamine 2000. Expression of GFP in RSBF-2 cells could be detected as early as 30 h after transfection (Ku *et al*., 2010). *Epinephelus coioides* spleen cell line (GS) when transfected with pEGFP expression vector exhibited clear and strong green fluorescence signals after 48 h (Qin *et al*., 2006). Turbot kidney cell line (TK) was transfected with pEGFP-N3 plasmid DNA; this cell line expressed the reporter gene and produced signals after duration of 36 h since transfection (Wang *et al*., 2010). The snout cell line from *Epinephelus akaara* (GSC) expressed pEGFP vector on transfection using lipofectamine 2000 as early as 24 h after transfection (Zhou *et al*., 2007).

Contrary to this study, Sandbichler *et al.* (2013) evaluated four transfection reagents and found that certain variations of JetPrime™ reagent and X-tremeGene™ HP reagent produced the highest fluorescence signals per cell after 24- and 48-h incubation, respectively with transfection efficiency of 25-30%.

5.5.2 *In vitro* cytotoxicity testing using bacterial extracellular products (ECPs)

Vibrio alginolyticus is a Gram-negative halophilic bacterium that is commonly found as part of the normal microbial flora in marine environments. It has been recognised as an opportunistic pathogen to both humans and marine animals (Balebona *et al*., 1998; Campanelli *et al*., 2008). As global warming increases ocean temperatures, incidences of out-of-season infections caused by *V. alginolyticus* are escalating and drawing more attention (Sganga *et al*., 2009).

Susceptibility studies were carried out in PC1F1Ex, PC1CpTr and PC1L1Tr at a range of concentrations to determine EC_{50}. As evident in earlier studies, disruption of the monolayer was observed when the cells were inoculated with high doses of *V. alginolyticus* ECP (12 µg mL^{-1}). Further, XTT assay was carried out to determine sensitivity of each cell line to ECP. The susceptibility of the three cell lines could be arranged in the following ascending order: PC1L1Tr (least sensitive) <PC1CpTr < PC1F1Ex (most sensitive). By carrying out XTT, the average EC_{50} values obtained were 4.042 µg mL^{-1} for PC1F1Ex, 5.819 µg mL^{-1} for PC1CpTr and 8.2 µg mL^{-1} for PC1L1Tr. These results proved that the caerulean damsel cell lines are more sensitive as compared to PC12 cell line with required 16.3 µg mL^{-1} when inoculated with *V. alginolyticus* AO35 strain (Balebona *et al*., 1998).

V. alginolyticus caused apoptosis, cell rounding and osmotic lysis in Epithelioma papulosum cyprini (EPC) cells (a representative carp fish cell line) which led to the cell death of fish cells within hours of infection (Zhao *et al.*, 2010). The bacterium requires its type III secretion system (T3SS) to cause rapid death of infected fish cells. Dying cells exhibited some features of apoptotic cells, such as membrane blebbing, nuclear condensation and DNA fragmentation (Zhao *et al*., 2010). Lee (1995) and Balebona *et al*. (1995) suggested that extracellular products such as proteases are involved in the pathogenicity of *V. alginolyticus* to cultured grouper and seabream. Bejar *et al*. (1997) and Sahul Hameed *et al.* (2006) reported similar observations that ECP from *Vibrio* strains tested produced rounding, shrinking, detaching and finally monolayer destruction. Numerous vesicles and dendritic formations were also observed in the SAF-1 from fin fibroblasts of the gilthead seabream (*Sparus aurata*). Environmental monitoring of pathogenic bacteria have also been tested using extracellular products (ECP) from *Vibrio anguillarum* and *Vibrio alginolyticus* on TOGB cells lines from the brain of *Trachinotus ovatus* which caused a dose-dependent morphological

changes (Li *et al*., 2016). Ku *et al.* (2009) used RGB, RGG and RGH cells, developed from rockfish grouper brain, gill and heart respectively, to detect the cytotoxic factors responsible for fish pasteurellosis.

The metabolic capability of fish cell cultures has been monitored by measuring their ATP content or their ability to reduce 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2*H*- tetrazolium hydroxide (XTT). NADH present in the mitochondria reduces XTT *via* trans-plasma membrane electron transport and an electron mediator (Berridge *et al*., 2005). XTT is metabolically reduced in viable cells to a water soluble formazan product which can be directly used to obtain OD readings (Scudiero *et al.*, 1988). The amount of water-soluble product generated from XTT is proportional to the number of living cells in the sample and can be quantified by measuring absorbance at wavelength of 475 nm. Shuilleabhain *et al*. (2004) employed NR assay to determine the effect of zinc chloride, zinc sulphate heptahydrate and zinc nitrate hexahydrate on EPC, CHSE and RTG-2 cell lines.

Twelve adherent fish cell lines derived from six species (rainbow trout, fathead minnow, zebrafish, goldfish, haddock, and American eel) were used to comparatively evaluate viability of cells by measuring metabolic impairment using Alamar Blue (Vo *et al.*, 2003).

Three cell lines, eye (IEE), gill (IEG) and kidney (IEK) were developed from *Etroplus suratensis* which was tested for susceptibility to tannery effluents and three end points were studied, 3-(4, 5-dimethylthizol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), NR and alamar blue (Babu *et al*. 2012). In this study, XTT was found to react with the cells and produce a water soluble product that was easily read on a spectrophotometer. XTT assay is easier than MTT assay, since it does not require a solubilisation step.

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SUMMARY

Cell lines and primary cell cultures from fish tissues have been used for research in disease diagnosis and cytotoxicity evaluation of environmental pollutants. The principal aim of this work was the development of continuous cell lines from the caerulean damsel, *Pomacentus caeruleus*. To achieve this, tissues were first sourced from donor fishes, devoid of contaminants using standardised disinfection protocols. Primary cultures were then initiated and suitable media, additives, dissociation reagents as well as optimum incubation temperature were determined. Successful primary cultures were subcultured and passaged to derive continuous cell lines which were cryopreserved for long term storage. The continuous cell lines established were characterised by immunotyping using cell type markers and authenticated to confirm the species of origin using previously established barcoding techniques. Preliminary applications such as gene transfection studies and cytotoxicity assays using bacterial extracellular products were done in addition to ensuring that the cultures and cryostocks were contamination-free.

- Various tissues of *P. caeruleaus viz*., fin, gill, heart, liver, caudal peduncle, brain, kidney and spleen were evaluated for initiation of primary cultures employing explantation and trypsinisation techniques.
- The technique involved explantation of 1-2 mm^3 fragments of tissues to the cell culture flasks. Cells proliferated and multiplied from the explant eventually forming confluent monolayers. Fin tissue explant cultures were successfully developed in to continuous cell lines.

- In trypsinisation method, tissues were minced and cells were dispersed by treating with trypsin-EDTA solution. Cells were harvested by neutralising the enzyme with media containing serum (FBS) followed by centrifugation to harvest the cells. The cells were then resuspended in fresh complete growth media and seeded into flasks, to multiply and form confluent monolayers. Successful cell lines have been developed from liver and caudal peduncle tissues using this method.

- The cell cultures developed were designated PC1F1Ex (derived from fin explant tissues), PC1L1Tr (from trypsinised liver tissue) and PC1CpTr (trypsinised caudal peduncle tissue). The media, incubation temperature and serum requirements (fetal bovine serum along with fish muscle extract plus heterologous or homologous fish serum) were evaluated for optimal growth and proliferation of the cells. The cultures grew well in Leibovitz's L-15 media with a pH 7.2 at 28˚C. Primary cultures and initial passages required 20% FBS to develop confluent monolayers. As the cultures progressed, the requirement gradually decreased to 10% and then to 5% and 2% FBS. The use of heterologous or homologous fish serum/fish muscle extract did not enhance attachment or growth; hence its use was discontinued from the culture media. Dissociation of cells to obtain primary cultures was done using 0.5% trypsin-EDTA but for further subcultures 0.05% was found to be sufficient.

- The cells were cryopreserved, at every 20 passages using the cryoprotectant dimethylsulphoxide (DMSO), and were found to show 80% survival on revival even after 4 years of cryostorage. The cryopreservation media comprised Leibovitz's L-15 media with 20% FBS and 10% DMSO.

- Chromosome analysis of all the three cell lines showed aneuploidy which is a property of transformed lines. The chromosome number of the fin cells counted from the PC1F1Ex cell line varied from 41 to 60. From the PC1CpTr caudal peduncle cell line a range from 36 to 65 chromosomes was observed. For PC1L1Tr liver cell line, chromosomal count ranged from 43 to 62.
- The cells present in the monolayers of all the three cell lines showed a certain degree of staining against the fibroblast marker, vimentin which indicated that the cell lines PC1F1Ex, PC1L1Tr and PC1CpTr are fibroblastic in nature.
- DNA barcode analysis of *mitochondrial cytochrome c oxidase subunit 1* (*CO1) gene* was performed in order to verify the species of origin of the cell lines. Amplification of the COI gene from the cell lines and caerulean damsel muscle tissue gave a product of ~640-bp. PCR amplification of CO1 based DNA barcoding from the cell lines yielded 99% match when aligned with CO1 gene of the caerulean damsel in the NCBI database (Accession No. KJ129032.1).The GenBank accession numbers of the partial *cytochrome c oxidase subunit 1* (*CO1) gene* of the caerulean damsel muscle tissue, PC1CpTr, PC1F1Ex and PC1L1Tr are KY982625, KY982626, KY982627 and KY982628 respectively.
- Cells were transfected using two techniques which have been earlier studied in fish cell lines: a lipid based reagent (Lipofectamine 3000) and a cationic polymer (jetPRIME). PC1F1Ex, PC1CpTr and PC1L1Tr cell lines were successfully transfected with pcDNA3-EGFP plasmid. The estimated transfection efficiency was 10% and 12.5% respectively

for JetPRIME and Lipofectamine 3000 respectively which indicates the proneness of these cells to be transfected using cytomegalovirus (CMV) as promoter to drive the expression of EGFP gene in *P. caeruleus* cell lines, PC1F1Ex, PC1L1Tr and PC1CpTr.

- The PC1F1Ex, PC1CpTr and PC1L1Tr cell lines were inoculated with various dilutions of extracellular products (ECPs) from a fish pathogenic strain of *V. alginolyticus*. The cells were rounded, cytolysed or both with the consequence of monolayer disruption. The susceptibility of the three cell lines could be arranged in the following ascending order: PC1L1Tr (least sensitive) < PC1CpTr < PC1F1Ex (most sensitive). The mean EC_{50} values recorded employing XTT assay were: 4.042 µg mL^{-1}, 5.819 µg mL^{-1} and 8.2 µg mL^{-1} for PC1F1Ex, PC1CpTr and PC1L1Tr respectively.
- Transmission electron microscopy and screening using mycoplasma detection kit results revealed that the cell lines were not contaminated by viruses, viral related particles or mycoplasmas.
- The fin, liver and caudal peduncle cell lines designated PC1F1Ex, PC1L1Tr and PC1CpTr developed in the present study have been deposited at the ICAR-CMFRI fish cell line repository, Kochi and also at the National Repository of Fish Cell Lines (NRFC) of the ICAR-National Bureau of Fish Genetic Resources, Lucknow.

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Appendix A

List of NCBI accessions* (relevant to the present study)

S. No.	Accession No.	Details
1.	KY982625	Githa Ann G., Sobhana, K. S. and Sheethal Mary, S. *Pomacentrus caeruleus* isolate PC1 cytochrome oxidase subunit I (CO1) gene, partial cds; mitochondrion
2.	KY982626	Githa Ann G., Sobhana, K. S. and Sheethal Mary, S. *Pomacentrus caeruleus* cell-line PC1CpTr cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrion
3.	KY982627	Githa Ann G., Sobhana, K. S. and Sheethal Mary, S. *Pomacentrus caeruleus* cell-line PC1F1Ex cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrion
4.	KY982628	Githa Ann G., Sobhana, K. S. and Sheethal Mary, S. *Pomacentrus caeruleus* cell-line PC1L1Tr cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrion

Sequences would be released to the public domain on 7th July 2018

Appendix B

List of cell lines deposited at NRFC-National Repository of Fish Cell Lines (ICAR-NBFGR) along with the accession numbers (relevant to the present study)

S. No.	Accession No.	Details
1.	NRFC035	PC1CpTr- *Pomacentrus caeruleus* caudal peduncle cell line
2.	NRFC036	PC1F1Ex- *Pomacentrus caeruleus* fin cell line
3.	NRFC037	PC1L1Tr- *Pomacentrus caeruleus* liver cell line

Appendix C

Publications

Proc. Natl. Acad. Sci., India, Sect. B Biol. Sci.
DOI 10.1007/s40011-016-0751-x

RESEARCH ARTICLE

Evaluation of Various Tissues of the Caerulean Damsel, *Pomacentrus caeruleus* for Initiating In Vitro Cell Culture Systems

Githa Ann George[1] · K. S. Sobhana[1] · Sheethal Mary Sunny[1] · S. Sreedevi[1]

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Abstract Explantation and trypsinisation methods for tissue dissociation were attempted for the establishment of primary cell cultures from the caerulean damsel, *Pomacentrus caeruleus*. Among the tissues taken, fin, liver and caudal peduncle showed good attachment with emergence of cells. The cells were best suited to grow in Leibovitz's L-15 basal medium supplemented with foetal bovine serum (initially 20 % which was later reduced to 5–10 % during subsequent passages) at an ambient temperature of 28 ± 2 °C and pH 7.2 ± 0.2. These cultures persisted at temperatures from 17 to 32 °C, and proliferated at temperatures from 24 to 30 °C. The cells have been cryopreserved successfully with a survival rate of 80 %. Results suggest that fin, caudal peduncle and liver cell cultures have potential for development into cell lines.

Keywords Caudal peduncle · Fin · Liver · Marine ornamental fish cell lines · *Pomacentrus caeruleus*

Introduction

The global ornamental fish trade in retail is worth more than USD 8 billion [1] and the export value was approximately USD 337 million in 2008 [2]. Ornamental fish trade has enormous potential in tropical countries with unlimited avenues in India. Quality, quantity and sustainability are three pre-requisites for the ornamental fish trade to prosper [1]. Therefore, the exploitation of wild fish stocks for the aquarium trade may become restricted resulting in a trend towards conservation of natural resources world over. Hence for the expansion of trade, brood-stock development and controlled breeding need to be developed. Among the marine ornamentals, caerulean damsel, *Pomacentrus caeruleus* (Quoy and Gaimard, 1825) is popular among aquarium traders for their hardiness, beauty and interesting display behaviours. They are in general "reef safe", tolerant to wider and varying chemicals and physical water qualities, typically disease-resistant, and accepting all types of prepared commercial feeds. In order to keep with the growing trends in the ornamental fish industry, fish health management and quarantine regimes will also have to be adopted, along with the development of transgenic varieties which would add to their commercial significance. To circumvent overexploitation from the wild, hatchery technology has been developed for the caerulean damsel at the ICAR-Central Marine Fisheries Research Institute (CMFRI), India [3]. However, various infectious diseases may be anticipated in captive rearing and aquariculture. In this context, the availability of cell lines that would aid efforts to detect and characterise species specific pathogens, especially of viral etiology is felt essential.

Fish cell lines have been developed from a wide variety of edible fish to tackle emerging viral diseases, especially the regions in Asia have contributed to over 60 % of the total fish cell lines since 1994 [4]. Cell lines are used in monitoring aquatic pollution and toxicology and in propagating viruses that are etiologic agents of diseases in economically significant species [5–7]. Genetically modified fish cells are useful tools for studying the function and regulation of genes of interest, and are a step prior to producing transgenic fish [8, 9]. However, for a reliable expression system of an exogenous gene, stable chromosomal integration that enables regular

✉ K. S. Sobhana
sobhanapradeep11@gmail.com

[1] Central Marine Fisheries Research Institute, Post Box No. 1603, Ernakulam North Post, Kochi, Kerala 682018, India

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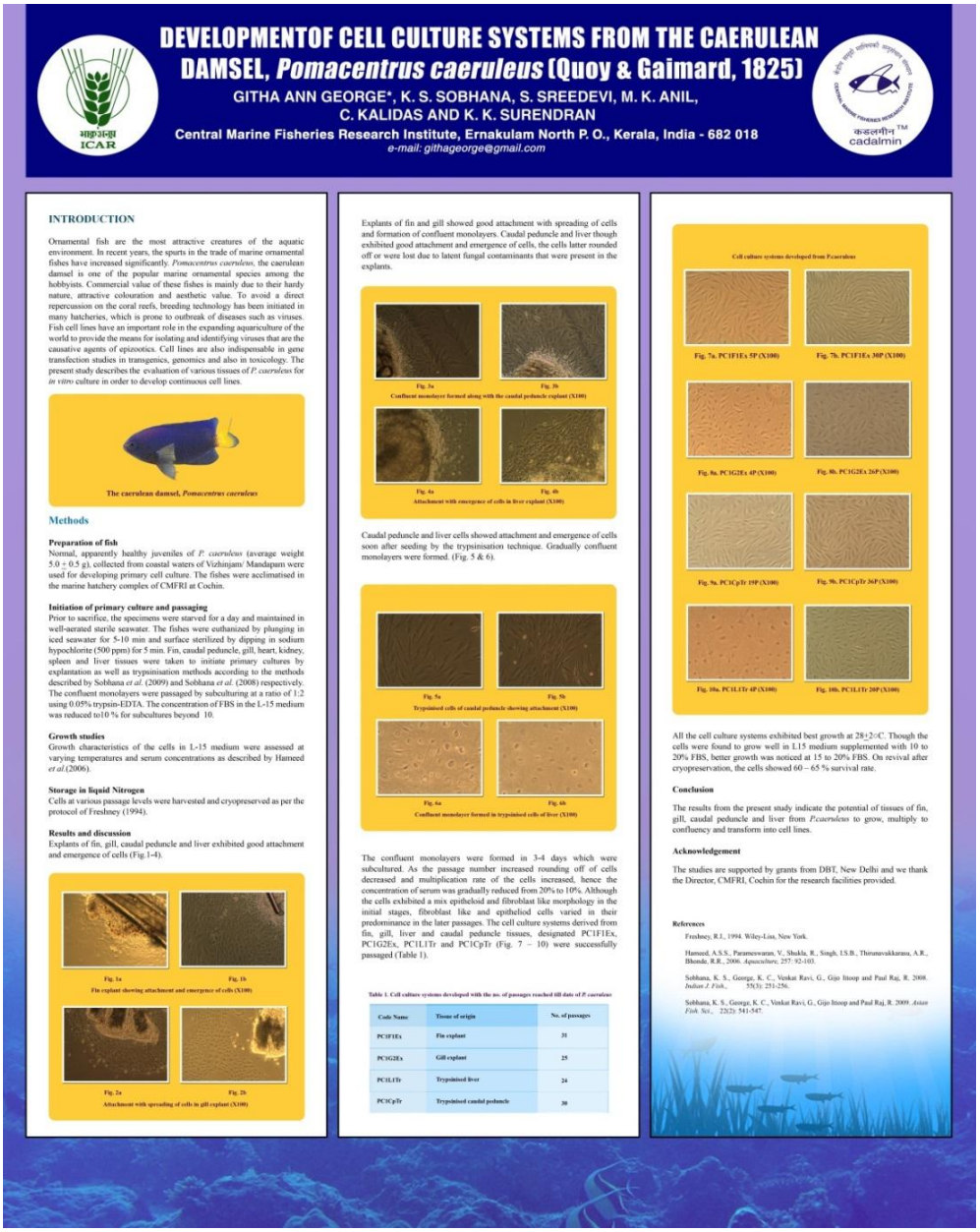

DEVELOPMENTOF CELL CULTURE SYSTEMS FROM THE CAERULEAN DAMSEL, *Pomacentrus caeruleus* (Quoy & Gaimard, 1825)

GITHA ANN GEORGE*, K. S. SOBHANA, S. SREEDEVI, M. K. ANIL, C. KALIDAS AND K. K. SURENDRAN
Central Marine Fisheries Research Institute, Ernakulam North P. O., Kerala, India - 682 018
e-mail: githageorge@gmail.com

INTRODUCTION

Ornamental fish are the most attractive creatures of the aquatic environment. In recent years, the spurts in the trade of marine ornamental fishes have increased significantly. *Pomacentrus caeruleus,* the caerulean damsel is one of the popular marine ornamental species among the hobbyists. Commercial value of these fishes is mainly due to their hardy nature, attractive colouration and aesthetic value. To avoid a direct repercussion on the coral reefs, breeding technology has been initiated in many hatcheries, which is prone to outbreak of diseases such as viruses. Fish cell lines have an important role in the expanding aquriculture of the world to provide the means for isolating and identifying viruses that are the causative agents of epizootics. Cell lines are also indispensable in gene transfection studies in transgenics, genomics and also in toxicology. The present study describes the evaluation of various tissues of *P. caeruleus* for *in vitro* culture in order to develop continuous cell lines.

The caerulean damsel, *Pomacentrus caeruleus*

Methods

Preparation of fish
Normal, apparently healthy juveniles of *P. caeruleus* (average weight 5.0 ± 0.5 g), collected from coastal waters of Vizhinjam/ Mandapam were used for developing primary cell culture. The fishes were acclimatised in the marine hatchery complex of CMFRI at Cochin.

Initiation of primary culture and passaging
Prior to sacrifice, the specimens were starved for a day and maintained in well-aerated sterile seawater. The fishes were euthanized by plunging in iced seawater for 5-10 min and surface sterilized by dipping in sodium hypochlorite (500 ppm) for 5 min. Fin, caudal peduncle, gill, heart, kidney, spleen and liver tissues were taken to initiate primary cultures by explantation as well as trypsinisation methods according to the methods described by Sobhana *et al.* (2009) and Sobhana *et al.* (2008) respectively. The confluent monolayers were passaged by subculturing at a ratio of 1:2 using 0.05% trypsin-EDTA. The concentration of FBS in the L-15 medium was reduced to10 % for subcultures beyond 10.

Growth studies
Growth characteristics of the cells in L-15 medium were assessed at varying temperatures and serum concentrations as described by Hameed *et al.*(2006).

Storage in liquid Nitrogen
Cells at various passage levels were harvested and cryopreserved as per the protocol of Freshney (1994).

Results and discussion
Explants of fin, gill, caudal peduncle and liver exhibited good attachment and emergence of cells (Fig.1-4).

Fig. 1a Fig. 1b
Fin explant showing attachment and emergence of cells (X100)

Fig. 2a Fig. 2b
Attachment with spreading of cells in gill explant (X100)

Explants of fin and gill showed good attachment with spreading of cells and formation of confluent monolayers. Caudal peduncle and liver though exhibited good attachment and emergence of cells, the cells latter rounded off or were lost due to latent fungal contaminants that were present in the explants.

Fig. 3a Fig. 3b
Confluent monolayer formed along with the caudal peduncle explant (X100)

Fig. 4a Fig. 4b
Attachment with emergence of cells in liver explant (X100)

Caudal peduncle and liver cells showed attachment and emergence of cells soon after seeding by the trypsinisation technique. Gradually confluent monolayers were formed. (Fig. 5 & 6).

Fig. 5a Fig. 5b
Trypsinised cells of caudal peduncle showing attachment (X100)

Fig. 6a Fig. 6b
Confluent monolayer formed in trypsinised cells of liver (X100)

The confluent monolayers were formed in 3-4 days which were subcultured. As the passage number increased rounding off of cells decreased and multiplication rate of the cells increased, hence the concentration of serum was gradually reduced from 20% to 10%. Although the cells exhibited a mix epitheloid and fibroblast like morphology in the initial stages, fibroblast like and epitheliod cells varied in their predominance in the later passages. The cell culture systems derived from fin, gill, liver and caudal peduncle tissues, designated PC1F1Ex, PC1G2Ex, PC1L1Tr and PC1CpTr (Fig. 7 – 10) were successfully passaged (Table 1).

Table 1. Cell culture systems developed with the no. of passages reached till date of *P. caeruleus*

Code Name	Tissue of origin	No. of passages
PC1F1Ex	Fin explant	31
PC1G2Ex	Gill explant	25
PC1L1Tr	Trypsinised liver	24
PC1CpTr	Trypsinised caudal peduncle	30

Cell culture systems developed from *P.caeruleus*

Fig. 7a. PC1F1Ex 5P (X100) Fig. 7b. PC1F1Ex 30P (X100)

Fig. 8a. PC1G2Ex 4P (X100) Fig. 8b. PC1G2Ex 26P (X100)

Fig. 9a. PC1CpTr 19P (X100) Fig. 9b. PC1CpTr 36P (X100)

Fig. 10a. PC1L1Tr 4P (X100) Fig. 10b. PC1L1Tr 20P (X100)

All the cell culture systems exhibited best growth at 28±2○C. Though the cells were found to grow well in L15 medium supplemented with 10 to 20% FBS, better growth was noticed at 15 to 20% FBS. On revival after cryopreservation, the cells showed 60 – 65 % survival rate.

Conclusion

The results from the present study indicate the potential of tissues of fin, gill, caudal peduncle and liver from *P.caeruleus* to grow, multiply to confluency and transform into cell lines.

Acknowledgement

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