Tissue and Cell 46 (2014) 206–212

Contents lists available at ScienceDirect

Tissue and Cell

journal homepage: www.elsevier.com/locate/tice

Establishment and characterization of a piscean PCF cell line for toxicity and gene expression studies as *in vitro* model

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ARTICLE INFO

Article history:
Received 22 January 2014
Received in revised form 15 April 2014
Accepted 17 April 2014
Available online 26 April 2014

Keywords:
Cell line
Characterization
Genotoxicity
PCF
Transfection

ABSTRACT

A new piscean fibroblastic cell line termed as PCF derived from the caudal fin tissue of dark mahseer, *Puntius (Tor) chelynoides* was established and characterized in the present study which was found to be suitable for toxicity and gene expression studies as *in vitro* model. The cell line grew well in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum (FBS). The cells were able to grow at a temperature ranging from 20 to 28 °C with an optimal growth at 24 °C and the cell line have been expanded in culture for more than 70 passages. Authentication of the cell line was carried out using mitochondrial DNA markers (Cytochrome Oxidase subunit I and 16S ribosomal RNA). Presence of vimentin in the cells confirmed the fibroblastic origin of cell line. Significant cytopathic effects were observed upon exposure of PCF cell line to bacterial extracellular products and the study also validated the suitability of cell line in transgenic applications as well as in genotoxicity assessment as an *in vitro* model.

1. Introduction

Animal cell culture has grown substantially as an essential *in vitro* biological system in cell and molecular biology in recent years. The potential use of fish cell lines is in virus isolation, toxicological and cytogenetic studies (Wolf and Mann, 1980). Many cell lines from freshwater and marine fish species have been reported since the first fish cell line RTG-2 (Wolf and Quimby, 1962; Hightower and Renfro, 1988; Fryer and Lannan, 1994), but relatively few cell lines were developed from coldwater fishes (Lakra and Bhonde, 1996; Lakra et al., 2006a; Yadav et al., 2012).

Puntius (Tor) chelynoides commonly called as Dark mahseer, inhabits fast-flowing hill streams of India and Nepal. *P. chelynoides* is a commercially important fish and also considered as a game fish under the group mahseer. In IUCN Red List, 2010 *P. chelynoides*is categorized as Vulnerable, due to threats from non-native species and pressures from local fisheries. Therefore, establishing new cell lines from *P. chelynoides* is indispensible for carrying out research in pathogenesis and germplasm conservation of this important coldwater fish species. We previously have reported cell line from eye tissue of *P. chelynoides* (Goswami et al., 2012) but since some pathogenic viruses are known to be organ or tissue-specific, the development of new additional cell lines will be worth their weight in gold at the cellular level for studying species-specific responses to viral pathogens. Further, these novel cell lines carry their own importance in toxicological and gene expression studies.

In the present study we have established and characterized a novel fibroblastic cell line termed as PCF from fin tissue of *P. chelynoides* which grows on conventional cell culture flasks and dishes with classical culture media and low serum concentration. These cells retain their potential to differentiate for more than 70 passages. The established cell line is a suitable tool for use in different biotechnological applications as an *in vitro* biological system.

2. Materials and methods

2.1. Cell isolation and culture

Live fingerlings of *P. chelynoides* weighing 10–20 g were collected from the River Alaknanda, Garhwal, Uttarakhand, India and were maintained in the wet lab of National Bureau of Fish Genetic Resources (NBFGR), Lucknow, India. Before explant preparation the fish were maintained in aerated, sterile water

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http://dx.doi.org/10.1016/j.tice.2014.04.004
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containing 1000 IU/ml penicillin and 1000 μg/ml streptomycin for 24 h at room temperature. The fish were then anaesthetized in ice-cold water, and wiped with 70% alcohol. The fin tissue was excised under aseptic conditions and minced into small pieces (1 mm^3) in phosphate-buffered saline (PBS, Ca^{2+} and Mg^{2+} free). The tissue fragments from fin were then washed several times in an antibiotic solution (PBS containing 500 IU/ml penicillin and 500 μg/ml streptomycin) and then planted into 25 cm^2 standard cell culture flasks with approximate 50 μL volume of fetal bovine serum (FBS) and incubated at 24 °C. After 24 h of attachment, L-15 medium supplemented with 20% FBS was carefully added to the flasks without unsettling the explants. Every 4–6 days, half of the medium was removed and replaced with fresh medium until passaging.

Upon attaining 90% confluency, the standard trypsinization method using trypsin (0.1% w/v, in PBS) containing 0.2% EDTA was followed to subculture the cells at a ratio of 1:2. After the first passage, cells were passaged at an interval of 5–7 days. Initially, subcultured cells were maintained in L-15 medium with 20% FBS and after 10th passage, the concentration of FBS in L-15 medium was reduced to 10%.

2.2. Growth characteristics

Growth characteristics of the PCF cell line were assessed at selected temperatures, FBS and bFGF concentrations. To determine the growth at optimum temperature, 1×10^5 cells were seeded and incubated at 24 °C for 24 h then incubated at selected temperatures of 16 °C, 20 °C, 24 °C and 28 °C in triplicates. Every alternate day, triplicate flasks at each temperature were trypsinized and cell density was measured with haemocytometer for a total of 7 days. The growth response to various concentrations of FBS (5, 10, 15, and 20%) and bFGF (0, 5 and 10 ng/ml) on cell growth was carried out using the same procedure as for temperature.

2.3. Cell plating efficiency

Plating efficiency of the cell line was determined at seeding concentrations of 200, 500 and 1000 cells per flask (z) (25 cm^2 tissue culture flask) in duplicate at passage 12. Following 14 days of incubation, the medium was removed and cell colonies were fixed with formalin (25%) and stained with crystal violet stain (1%). Colonies were then counted (x) in each flask under the microscope and plating efficiency (y) was calculated using the formula $y = 100xz^{-1}$ (Freshney, 1994).

2.4. Molecular characterization

2.4.1. DNA isolation

DNA was extracted from *P. chelynoides* tissue and PCF cells at passage 20 according to the method followed by Lo et al. (1996). Briefly, the samples were trypsinized and homogenized in lysis buffer (100 mm NaCl,10 mm Tris–HCl pH 8.0, 25 mm EDTA pH 8.0, 0.5% sodium dodecyl sulphate and 0.1 mg ml^{-1} proteinase K) then incubated at 65 °C for 1 h, 5 M NaCl was added to a final concentration of 0.7 M followed by a slow addition of 1/10th volume of N-cetyl N,N,N-trimethyl ammonium bromide (CTAB)/NaCl solution (10% CTAB in 0.7 M NaCl). After incubation at 65 °C for 2 h, the digested samples were deproteinized by successive phenol/chloroform/iso-amyl alcohol extraction. Finally the DNA was precipitated with absolute ethanol, washed with 70% ethanol and dissolved in TE buffer. The amount of isolated DNA was quantified by using a UV spectrophotometer at wavelength of 260 nm and DNA was diluted to get a final concentration of 100 ng μl^{-1}.

2.4.2. Amplification and sequencing

Two genes cytochrome oxidase subunit I (COI) and 16S rRNA of mitochondrial DNA were amplified. The COI fragment was amplified using the following primers: forward FISHF1 (5′-TCAACCAACCACAAAGACATTGGCAC-3′) and reverse FISHR1 (5′-TAGACTTCTGGGTGGCCAAAGAATCA-3′) (Ward et al., 2005). The 16S rRNA fragment was amplified using the following primers: forward 16SAR (5′-CGCCTGTTTATCAAAAACAT-3′) and reverse 16SBR (5′-CCGGTCTGAACTCAGATCACGT-3′) (Palumbi et al., 1991).

COI gene was amplified in a 50 μl reaction volume contained 5 μl of 10X Taq polymerase buffer, 0.25 mM of each dNTP, 0.4 μM of each primer, 2.5 U of Taq polymerase and 100 ng genomic DNA using the C-1000 thermal cycler (Bio-Rad). The PCR cycling conditions involved an initial denaturation step at 95 °C for 2 min followed by 35 cycles of 94 °C for 40 s, 55 °C for 40 s and 72 °C for 1 min 10 s with a final extension phase at 72 °C for 10 min and a holding temperature of 4 °C after completion. 16S rRNA gene was also amplified in a 50 μl reaction volume contained 5 μl of 10X Taq polymerase buffer, 0.2 mM of each dNTP, 0.4 μM of each primer, 2.5 U of Taq polymerase and 100 ng genomic DNA using the C-1000 thermal cycler (Bio-Rad). The PCR cycling conditions involved an initial denaturation step at 94 °C for 4 min followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min with a final extension phase at 72 °C for 10 min and a holding temperature of 4 °C after completion.

The amplicons were checked on 1.2% agarose gel and the products with high intense bands were selected for sequencing. Products were labeled using the BigDye Terminator V.3.1 Cycle sequencing Kit (Applied Biosystems, Inc.) and sequenced bidirectionally using an ABI 3730 capillary sequencer following manufacturer's instructions. The obtained sequences of PCR fragments were compared to known sequences in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST).

2.5. Cytotoxicity of bacterial extracellular products

PCF cells at passage 25 were treated with *Aeromonas* sp. to test the cytotoxicity of bacterial extracellular products (ECP) on the cells. The ECP from *Aeromonas* sp. was prepared according to the protocol described by Balebona (1995). The cells were seeded and grown as a monolayer in 24-well plates using L-15 medium supplemented with 10% FBS at 24 °C. For the toxicity test, the cell line was inoculated with 0.1 ml serial dilutions of ECP. For negative controls, wells were inoculated with sterile saline. Plates were incubated at 24 °C and the effects of ECP on the cells were observed after 24 h and 48 h.

2.6. Immunocytochemistry

PCF cells were examined for the expression of monoclonal antibodies directed against vimentin and cytokeratin for the morphology confirmation at selected passages. Cells (2 or 4×10^4) were planted on 12-mm diameter uncoated glass coverslips in 12-well plates. Cells were grown on coverslips at 24 °C for 1–3 days, washed with PBS and fixed in paraformaldehyde (4% in PBS, v/v) for 10 min at room temperature, again washed twice with PBS and blocked with a solution containing 5% sheep serum and 0.1% Triton X in PBS and then incubated at 37 °C for 1 h. Block was removed by washing twice with PBS, dilution of primary antibody either a 1:40 antivimentin clone V9 (V6630-CLONE 9 Sigma) or a 1:400 antipan cytokeratin clone-11 (C2931-Clone C-11 Sigma) in PBS was added and incubated for overnight at 4 °C. Next day cells were washed with PBS and were incubated with 100 μl of a 1:300 dilution of secondary antibody FITC-labeled anti-mouse IgG at 37 °C for 30 min. Nuclei were counterstained with Hoechst 33258 stain (861405

Sigma–Aldrich) for 1 min. Labeled cells were visualized under fluorescence microscope. Negative controls (omission of the primary antibody) were included in each experiment.

2.7. Cell transfection with GFP reporter gene

pEGFP-C1 plasmid (Invitrogen) was used with transfection reagents lipofectamine LTX and Plus Reagent (Invitrogen) for transfecting the sub confluent monolayers of PCF cells at passage 32. In brief, the PCF cells were seeded at a density of 1×10^5 in a 12 well plate individually and incubated for 18 h at 24 °C in normal atmospheric incubator. Before transfection, cells were washed with PBS and supplemented with 400 μl of fresh L-15 medium devoid of serum and antibiotics. The plasmid DNA (200 ng of pEGFP-C1) was dissolved in 100 μl of optimum (Gibco) and then 0.5 μl of plus reagent was added. The mixture was incubated for 5 min at room temperature. 2 μl of lipofectamine LTX was added to the mixture containing plasmid DNA and incubated for 30 min at room temperature. Finally the mixture was added dropwise on 70–80% confluent PCF cells in 12 well plate. The medium was changed with fresh medium after an incubation of 6 h at 24 °C. The green fluorescence signals were observed after 6 h under a fluorescent microscope (Olympus).

2.8. Genotoxicity assessment

PCF cells at passage 38 were treated with hydrogen peroxide (H_2O_2), a genotoxic model compound, in order to assess the efficiency of the comet assay for estimating genotoxicity on the cells. For the assessment, cells were first grown in 24 well plate in 1 ml medium for 24–48 h. The medium was then removed and replaced with 1 ml of medium containing serial dilutions of the tested genotoxic compound (H_2O_2). Exposure was carried out for 24 h. After exposure cells were washed with PBS, trypsinized and processed for Comet assay.

The Comet assay (single cell gel electrophoresis assay) was performed following previously described protocol (Singh et al., 1988) with minor modifications. In brief, the cell suspension was mixed with 0.6% low melting agarose and placed on a slide pre-coated with 1.0% normal melting agarose. Third layer of 0.5% low melting agarose was added and left to solidify. Subsequently, the cells, not the nuclei, were lysed in a lysis buffer (100 mM EDTA, 2.5 M NaCl, 10 mM Tris base, 1% Triton X-100, pH adjusted to 10) at 4 °C for at least 1 h. Single-strand DNA was prepared by unwinding the DNA in electrophoresis buffer (1 mM EDTA, 10% DMSO, 300 mM NaOH, pH 13) for 30 min and then subjected to micro-electrophoresis in the same electrophoresis buffer (25 V, 300 mA) for 15 min. After electrophoresis, the alkalis in the gels were neutralized by rinsing the slides in a neutralization buffer (0.1 M Tris, pH 7.5) for 5 min followed by drying and fixing of slides in methanol. After staining with ethidium bromide (20 μg/ml) for 10 min, the slides were viewed under a fluorescent microscope (Olympus).

2.9. Cryopreservation

The viability of PCF cells after freezing in liquid nitrogen (LN_2) was evaluated. The slow freezing method was used to cryopreserve cells. For cryopreservation, 72 h old cultures of PCF cells were used at passage 35 and 45. In brief, cells were removed from the flask surface by trypsinization, concentrated by centrifugation, and resuspended in a freezing medium consisting of L-15 medium containing 20% FBS and 10% dimethyl sulphoxide (DMSO) at a density of 10^6 cells per ml. The cell suspensions were poured into 2 ml sterile cryovials and tubes were placed in an insulated low-thermal mass freezing container (CoolCell) and kept in −80 °C freezer for 6 h. The cryovials were then removed from the container and transferred to a liquid nitrogen tank (−196 °C) for storage. After 3 months of post-storage the cells were recovered. For recovery the vials were thawed in running water at 24 °C. Following removal of the freezing medium by centrifugation, the cells were suspended in L-15 with 10% FBS and tested for viability by haemocytometer counting after trypan blue staining. The viable cells were seeded into 25 cm^2 cell culture flask and observed.

3. Results

3.1. Establishment of a long term culture and cell line

Cell culture was developed from caudal fin explants of *P. chelynoides*. Cells started showing radiation from the explants within 5–7 days and confluent monolayer of cells was observed after 9–11 days (Fig. 1a). Cells grew continuously and were subcultured

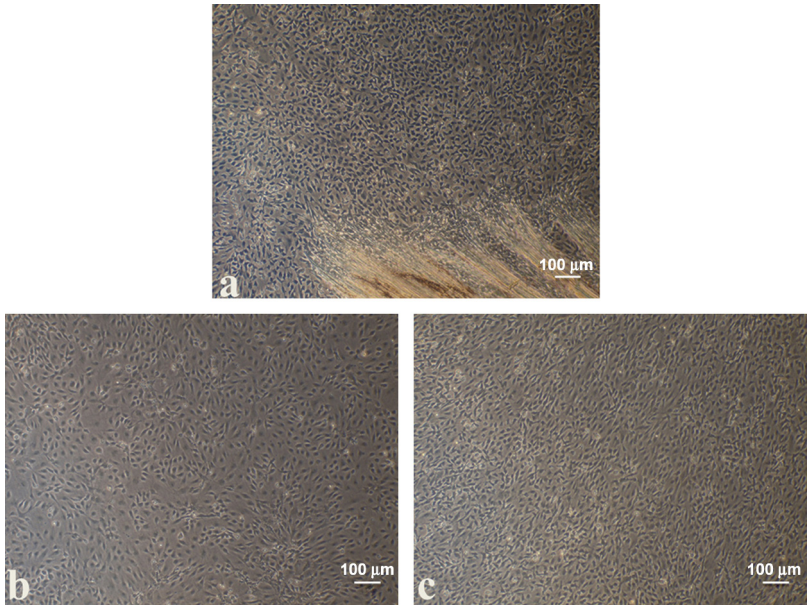

Fig. 1. Derivation of PCF cell line from caudal fin of *P. chelynoides* (100×). (a) PCF cells primary culture at day 7. (b) Cells at passage 15. (c) Fibroblastic cells at passage 30.

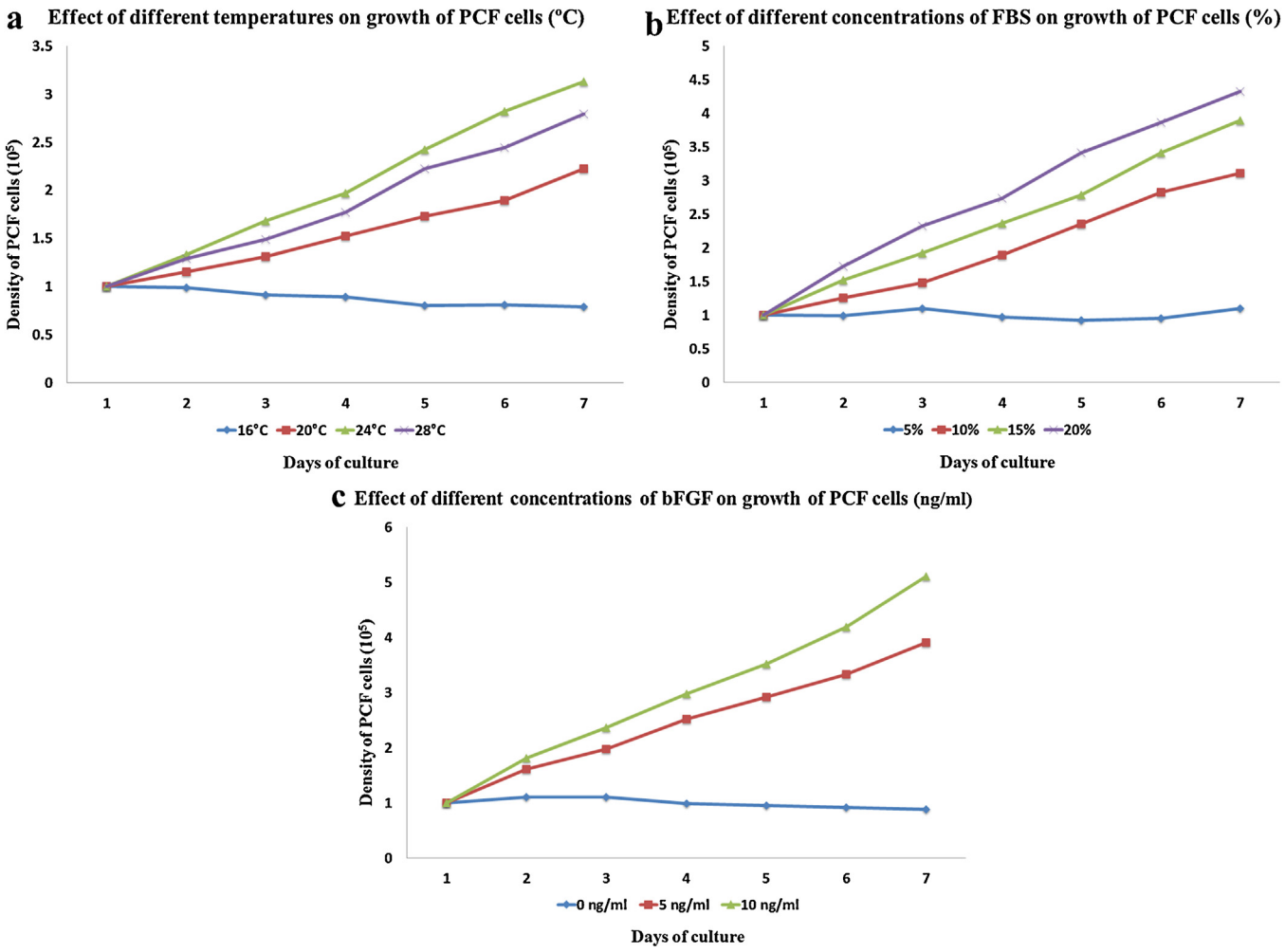

Fig. 2. Growth curves of PCF cell line. (a) Growth at different temperatures. (b) Growth at different FBS concentrations. (c) Growth at different bFGF concentration.

successfully at an interval of 5–7 days. Cells were repeatedly passaged to establish a cell line. Primary cell culture consisted of both epithelial and fibroblastic cells (Fig. 1b) whereas only fibroblastic cells were observed after passage 15 (Fig. 1c). We termed this piscean caudal fin tissue derived fibroblastic cell line PCF, and we have expanded it in culture for more than 70 passages.

3.2. Growth characteristics

PCF cells were exposed to different temperatures to determine optimum temperature for maximum growth of the cells. The cell line exhibited satisfactory growth at 20 °C with optimal growth at 24 °C, while no significant growth was observed at 16 °C (Fig. 2a). The cells were able to grow at 28 °C. Cells exhibited poor growth at 5% concentration of FBS, relatively good growth at 10%, but higher growth occurred with FBS concentrations of 15% and 20% (Fig. 2b). In the presence of bFGF (5 or 10 ng/ml), the cell proliferation was more rapid than the normal one (Fig. 2c). In the absence of bFGF, the proliferation decreased significantly.

3.3. Cell plating efficiency

The plating efficiency of PCF cells seeded at a density of 200 cells per flask was 35% whereas the plating efficiency increased to 63% and 85% at 500 and 1000 cells per flask respectively with no significant differences between replicates.

3.4. Molecular characterization

An analysis of mitochondrial COI and 16S rRNA genes was performed to verify the origin of the PCF cell line. Amplification from the COI gene for PCF cells revealed the expected PCR product of 655 bp. Subsequent comparative analysis of the identified sequences demonstrated a 99% to 100% match for COI to known *P. chelynoides* mitochondrial DNA sequence (GenBank Acc No. EU714105). Similar comparative analysis was performed for amplified mitochondrial 16S rRNA sequence. The tissue and cell line sequences were submitted to GenBank and accession numbers were JQ435843–JQ435844 and JQ435841–JQ435842 for COI and 16S rRNA genes, respectively.

3.5. Cytotoxicity of bacterial extracellular products

The ECP from *Aeromonas* sp. was cytotoxic for PCF cells. Cytotoxic effects could be observed within 24 h after inoculation of ECP. Cells showed rounding, shrinking, detachment and finally obliteration of the monolayer (Fig. 3).

3.6. Immunocytochemistry

Immunocytochemistry showed that PCF cells were strongly positive for vimentin (Fig. 4) and were negative for cytokeratin.

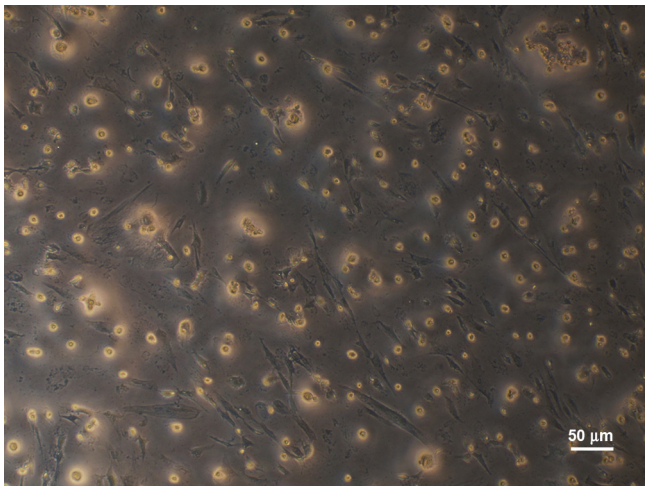

Fig. 3. Cytotoxic effect of extracellular products of *Aeromonas* sp. on the PCF cell line (200×).

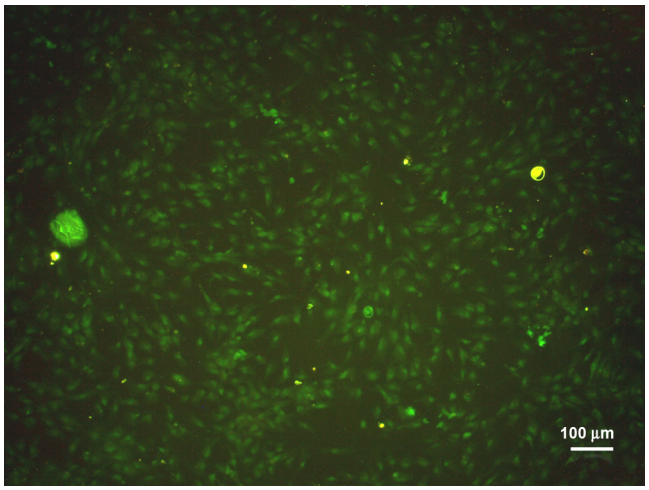

Fig. 4. Expression of fibroblastic protein in PCF cells labeled with antivimentin and FITC-conjugated secondary antibody (100×).

3.7. Cell transfection with GFP reporter gene

Transfection efficiency of PCF cell line was successfully analyzed with pEGFP-C1 plasmid using lipofectamine LTX and Plus Reagent. Clear and strong green fluorescent expression of EGFP in PCF cells was detected as early as after 6 h post transfection (Fig. 5a and b). Transfection efficiency was found to be 27% as determined by counting the fluorescent protein positive cells as well as total number of viable cells.

3.8. Genotoxicity assessment

The Comet derived upon exposure of the PCF cells with H_2O_2 is shown in Fig. 6a. There was a significant difference between the comet tail-length of PCF cells exposed to H_2O_2 in comparison to the control (Fig. 6b). The toxicity percentage was 73% as determined by counting the cells with comet as well as total number of cells.

3.9. Cryopreservation

The cryopreserved PCF cells following dimethyl sulfoxide (DMSO) slow freezing procedure showed significant viability of cells after recovery. The revived cells recovered well and grew to confluency within 7 days. No appreciable morphological alterations or change in growth rate observed after freezing and thawing. Average estimated recovery percentage was 83% to 85% of the initial cell population.

4. Discussion

Fish cell lines are indispensable for virus isolation, toxicological studies, carcinogenesis, genetic regulation and expression, as well as DNA replication and repair (Bols and Lee, 1982; Hightower and Renfro, 1988; Babich and Borenfreund, 1991). So far, most of the fish derived cell lines are from freshwater or anadromous fish species. An increasing interest in the cultivation of coldwater fish species have been seen in India; however, very few coldwater fish cell lines and primary cell cultures have been reported. This article describes the establishment and characterization of PCF cell line from caudal fin of coldwater fish *P. chelynoides*. An attempt was also made to make use of the established cell line for genotoxicological and gene expression studies.

The cell line exhibited characteristics of continuous cell line which coincides with the previously established eye cell line from *P. chelynoides* (Goswami et al., 2012) and other published fish cell lines (Bejar et al., 1997; Lakra and Goswami, 2011). The PCF cell line adapted well to grow in Leibovitz's L-15 medium supplemented with 10% FBS. The major advantage of maintaining the PCF cell line was that no additional antibiotic used except during explant preparation, whereas the use of antibiotics have been previously reported in maintenance of many fish cell lines (Cheng et al., 2010; Ou-Yang et al., 2010). This indicates that antibiotics will not become a variable factor for bacterial pathogenesis studies using this cell line.

The PCF cell line exhibited ideal growth at 24 °C which shows conformity with previously reported coldwater fish lines (Lannan et al., 1984). The cells were also able to grow at 28 °C, several reported fish cell lines were optimally grown at this temperature (Tong et al., 1997; Lakra et al., 2006b). If a cell line can be grown at high growth temperature, then this becomes advantageous for virus isolation studies (Nicholson et al., 1987). PCF cell growth increased as FBS concentration increased from 5 to 20%. Although

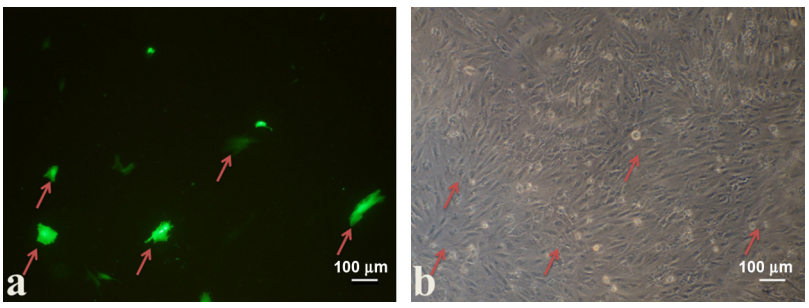

Fig. 5. Expression of green fluorescent protein in PCF cells transfected with pEGFP-C1 vector (100×). (a) Fluorescent view. (b) Phase contrast view.

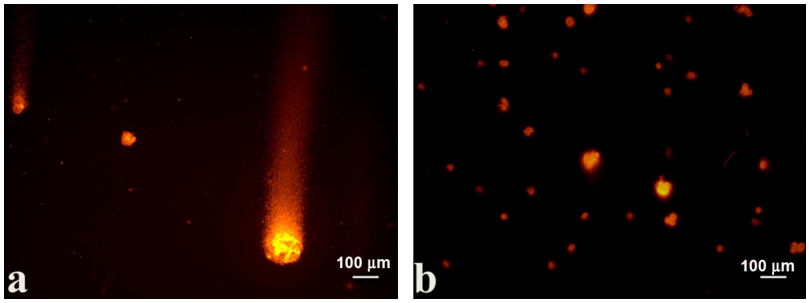

Fig. 6. Comets after single-cell gel electrophoresis of PCF cells (100×). (a) PCF cells after exposure with H_2O_2. (b) Unexposed PCF cells (control).

at 5% FBS concentration adequate cell growth occurred, the PCF cell line optimally maintained with 10% FBS instead of 15 or 20% FBS supplementation. Thus, the cell line could be maintained at lower cost. Basic fibroblast growth factor (bFGF) is a growth factor that shows important regulatory abilities in cell proliferation, migration and differentiation. Addition of bFGF in PCF cells resulted in a rapid proliferation. It probably activates tyrosine kinase by binding to tyrosine kinase receptor and speed up cell proliferation *via* various pathways (Hrzenjak and Shain, 1995).

Moderately higher plating efficiency and decreased contact inhibition was observed in PCF cell line as the passage number increased which suggests spontaneous transformation might took place during the later passages. Many properties associated with transformation *in vitro* were reported to be a consequence of cell surface modifications (Freshney, 1994). Molecular characterization of PCF cell line using COI & 16S rRNA confirmed the origin of the cell line from *P. chelynoides*. Cell line authentication using molecular marker is essential to confirm the originality of cell line. Recently, COI region has been used for identification of several cell lines (Lakra et al., 2010; Lakra and Goswami, 2011; Cooper et al., 2007). The present study validated the efficiency of COI sequence as a diagnostic marker for species identification. The PCF cell line was very sensitive to the ECP of *Aeromonas* sp. and morphological changes were seen as described by others (Bejar et al., 1997; Hameed et al., 2006). This indicates the suitability of this cell line to test the cytotoxic effects of bacterial extracellular products. Antibodies of fibroblastic and epithelial markers were applied to confirm the nature of PCF cells. The positive reaction of PCF cells to vimentin, a typical intermediate filament protein in the fibroblasts, indicated fibroblastic nature of PCF cells.

Successful transfection of PCF cells with pEGFP-C1 vector explored the utility of this cell line to serve as *in vitro* system in various constructs, gene targeting and expression studies. Gene targeting and expression applications have been reported by researchers using fish cells (Liu et al., 1990; Fernandez-Alonso and Coll, 1999). The exposure of PCF cells to H_2O_2 resulted in DNA damage which suggests the efficacy of PCF cell line to conduct Comet assay for genotoxic monitoring of toxic compounds. The application of fish cell lines has proven to be valuable, rapid and cost effective tool in ecotoxicological assessment of genotoxic compounds and aquatic pollutants due to the good correlation found between *in vitro* and *in vivo* results. Utilization of fish cell lines has been preferred over the use of *in vivo* assays due to the inherent economic and ethical constraints associated with live fish. Fish derived and other mammalian cell lines have been frequently employed in genotoxicity and cytotoxicity assays (Keddy et al., 1995; Segner, 2004; Gülden et al., 2005). The present study explored the possibility of using PCF cell line as an *in vitro* model in genotoxicity assessment. Successful revival of cryopreserved PCF cells indicates the possibility of long term storage of cell line. The result coincides with other reported fish cell lines (Chi et al., 1999; Chang et al., 2001).

Cell line designated as PCF derived from caudal fin of *P. chelynoides* was well characterized. The results of gene expression and genotoxicity studies indicate the possible suitability of this cell line in genetic manipulation studies and genotoxicological assessment of toxic compounds.

Acknowledgments

The authors thank Dr. J.K. Jena, Director, NBFGR, Lucknow, India for his support and encouragement for the research program. Department of Biotechnology, Govt. of India, New Delhi is highly acknowledged for financial support (Project ref. no. BT/PR11721/AAQ/03/452/2008).

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