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Development and partial characterization of primary cell culture from liver tissue of *Pangasianodon hypophthalmus*

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Abstract
The present study was undertaken to optimize the conditions for development of primary hepatocyte cultures from Pangasionodon hypophthalmus. Liver tissue explants were used as cell seeding source and a protocol for liver cell culture was optimized considering four parameters i.e. incubation temperature, culture media, pH of the media and serum concentration. Among growth media, L-15, Hepatocyte Basal Medium, DMEM and RPMI-1640 with varying concentration of Foetal Bovine Serum (FBS) were tested for their ability to support the growth of cells. Later, the effect on cell confluence at different pH and at different incubation temperature was monitored. The cells were primarily characterized by Best's carmine staining solution, which was later partially characterized by amplification of mt 16S rRNA gene fragment of 546 bp size that confirmed its origin from the liver explants of P. hypophthalmus at 20th passage. This study proved that the L-15 medium with pH 7.6 and 20% of serum concentration at incubation temperature of 28 ^{0}C yielded the best result in terms of attachment and proliferation of cells which can be used to develop the liver cell line or as a screening tool for further cytological studies.

Keywords: *Pangasionodon hypophthalmus,* hepatocytes, cell culture, partial characterization, mt 16s rRNA

Introduction

The first report of fish tissue culture was made by Osowski (1914) who maintained fry and embryonal trout explants for 24 hours in both Ringer's solution and in frog lymph whereas the first permanent cell line RTG-2 from gonad origin of rainbow trout (*Salmo gairdneri*) developed by Wolf and Quimby (1962) and since then several cell cultures and cell lines from a variety of fishes have been developed. Teleost cells are the 2nd most numerous among the animal cell lines (Wolf and Quimby, 1969) and presently, about 283 cell lines have been established from finfish around the world (Lakra *et al*., 2010). In India, only a few freshwater fish cell lines and primary cell cultures have been developed so far such as a cell line from caudal fin of rohu, *Labeo rohita* (Lakra and Bhonde, 1996), heart tissue of major carp (Rao *et al.,* 1997), gill of mrigal, *Cirrhinus mrigala* (Sathe *et al.,* 1995), kidney tissue of *Lates calcarifer* (Sahul hameed *et al.,* 2006), fin tissue of *Clarias batrachus* (Babu *et al.,* 2011) primary cell culture from kidney of *Heteropneustus fossilis,* larvae of *Poecilia reticulata* (Kumar *et al.,* 1998), liver and kidney tissue of *Labeo rohita* (Lakra *et al.,* 2005) heart tissue of *Chitala chitala* (Kapoor *et al.,* 2012) and gill tissue of Tor tor (Kamlendra *et al.,* 2012), but no reports were available about their commercial availability.

Pangasionodon hypophthalmus, known as Tra catfish, is now an important species of aquaculture in India. However, it has not yet been explored for cell culture applications. Since long, the liver has been playing an important role to understand physiology of finfish both in anabolism (protein, lipids and carbohydrates) and catabolism (glycogenolysis, detoxification). It also acts as a storage centre for many substances, mainly glycogen[1, 2]. In this regard, fish liver can be an interesting model to study the interactions between environmental factors and the function of hepatic tissue with respect to xenogeneic materials. Therefore, establishment of liver cell line is extremely important to study the effects of external stimuli on cellular functions *in vitro,* especially in aquaculture where there are problems induced by anthropogenic factors. Thus, the present work has aimed to develop a cell culture protocol from the liver tissue of *Pangasianodon hypophthalmus*.

Materials and Methods

Development of primary cell culture

Healthy Tra cat fish juveniles weighing 15 ± 5 g were collected from a local fish farm in Kolkata (West Bengal) and were maintained in sterile aerated freshwater containing 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin for 24 hours at room temperature (25 – 30 ^{0}C) prior to sacrifice. After anaesthesia, fish liver was taken aseptically and washed in Leibovitz's-15 medium containing 500 I.U. Penicillin/ml, 500 µg Streptomycin/ml and 1.25 µg/ml of Amphotericin B. The tissue fragments were transferred to 25 cm^2 (T25) cell culture flasks with 5 ml of growth medium with respective serum concentration, pH and temperature. The monolayer formed were trypsinized using 1x trypsin versene solution. Primary cell culture was kept in the ratio of 1:2 to different experimental medium and conditions. For the optimization of culture criteria of the liver tissue explant, four sets of experimental trials were conducted.

Trial 1: To select the ideal incubation temperature initially, the Tra catfish liver tissue explants were cultured in tissue culture flask (25 cm^2) containing L-15 growth culture media supplemented with 10% FBS and incubated at five different temperatures *viz*. 24, 26, 28, 30, and 32 ^{0}C respectively.

Trial 2: To select the ideal culture media, the liver tissue explants were cultured in tissue culture flask (25cm^2) containing four different cell culture growth medium *viz.* Leibovitz-15 medium (L-15), RPMI-1640, Delbecco's modified Eagle's medium and Hepatocyte Basal Medium, each supplemented with 10% FBS and incubated at 28 ^{0}C.

Trial 3: To select the ideal pH condition of the culture medium, the liver tissue explants were cultured in tissue culture flask (25 cm^2) containing L-15 culture media having pH of 7.2, 7.4, 7.6 and 7.8 supplemented with 10% FBS and incubated at 28 ^{0}C.

Trial 4: To select the ideal concentration of FBS, the liver tissue explants were cultured in tissue culture flask (25 cm^2) containing L-15 growth culture media supplemented with different concentrations of FBS viz. 5%, 10%, 15%, 20% and 25% at 28 ^{0}C incubation temperature.

The cells were cultured in triplicate in T-25 flasks and maintained by replacing 50% of the spent medium after 24h of culture with fresh medium. Cell culture was observed under the Zeiss digital inverted microscope and evaluated for cell attachment, spreading, degree of confluence, and general appearance on regular basis. Images were taken by Axiocam-HRM camera and analyzed with cell analyzer software. Cultured hepatocytes were harvested by centrifugation at 400xg for 3 min and viability tested by 0.4% trypan blue dye exclusion method. After optimization of culture conditions, cultured hepatocytes were harvested and partially characterised using Best's carmine staining method and RT-PCR amplification of cultured hepatocytes.

Statistical analysis

The data was statistically analyzed by statistical package SPSS version 16 in which data was subjected to one-way ANOVA and Duncans' multiple range tests used to determine the significant differences between the means. Comparisons were made at the 5% level of significance ($P \leq 0.05$).

Partial characterization of hepatocytes

Characterisation of hepatocytes by Best's carmine staining

The cells had grown in a 6-well plate for further characterisation of hepatocytes. Media was aspirated off from the wells and cells were washed with 1 ml D-PBS/well. Cells were fixed by incubation with 4% Paraformaldehyde solution prepared in D-PBS for 20 min at room temperature (RT). Cells were permeabilized by addition of 0.5% TX-100 solution in D-PBS and incubated at RT for 10 mins. 1ml of Best's carmine solution was added and cells were incubated in the stain for 10 min at RT. The washing process was repeated with D-PBS after each step i.e. after fixation, permeabilization and staining. Later, cells were examined under phase contrast with a 40X objective on an Axiovert 40CFL inverted microscope.

Characterisation of hepatocytes by mitochondrial- 16S rRNA (mt 16S rRNA) gene amplification

Primer designing for mt 16S rRNA gene fragment: Sequence of mt 16S rRNA of *Pangasianodon hypophthalmus* was retrieved from NCBI (GENBANK) and after similarity search using multiple sequence alignment by CLUSTALW programme, primers were designed from highly conserved area from partial sequence of mt 16S rRNA gene. The primers used for mt16s rRNA analysis were –

Forward primer - CGCCTCCTGCAAAAATCAA
Reverse primer - CGAACCCTTAATAGCGGC

RT-PCR amplification of hepatocytes: The total RNA was extracted from cultured hepatocytes after 21st passage and liver tissue of *P. hypophthalmus* was taken as positive control by using Trizol-RNA extraction method as directed by manufacturer. cDNA was synthesized using the First strand cDNA synthesis kit (Fermentas, India) as per the manufacturer's protocol using random hexamer primer (100 mM). The PCR reaction was performed with the gene-specific primers consisting of initial denaturation at 94 ^{0}C for 2 min followed by 30 cycles of 94 ^{0}C for 30 sec and at annealing temperature of 52 ^{0}C for 40 seconds followed by the elongation and final extension at 72 ^{0}C for 1 min and 72 ^{0}C for 7 min respectively.

Results and Discussions

Growth studies

Primary cell culture of *P. hypophthalmus* hepatocytes was able to grow in all the media but the cell confluence monolayer was observed significantly higher ($P \leq 0.05$) in L-15 Media of pH 7.4 containing 20% serum at 28 ^{0}C temperature (Table No. 1 Fig. No. 1-4).

Initially, the attachment of liver explants on to the culture flasks occurred within 36 to 42 hours (Fig. 5). The cells started migrating after 72 hours of seeding and appearance of spindle shaped cells was recorded in early hepatocytes culture (Fig.6-8). The tissue explants grew within 1st week and consisted of mixed population of both epithelial-like and fibroblast-like cells (Fig. 9). However, the growth of fibroblast-like cells dominated over epithelial-like cells in the subsequent passages, which exhibiteds 70 to 75% of cell monoconfluent layer (Fig. 10-13). Similar morphologic changes were also observed and recorded by several workers in the past [3, 4, 5, 6]. The present morphologic observation were found supported by reports of Lakra and Bhonde, and Singh

et al., where they illustrated that most of the primary cultures from fish in India comprised of fibroblast-like cells [7, 8].

In the first trial the study has been done to know the effect of different temperatures on cell growth which was found significantly higher ($P \leq 0.05$) at 28 ^{0}C in L-15 culture medium (Table No.1, Fig. No.1) and supports the observation done on cells of different fish species in India by different authors in the past [9, 10, 11].

During second trial, the cell growth was assessed using different culture media *viz.* L-15, DMEM, RPMI-1640 and Hepatocytes Basal Medium and a comparative study was made to know the best suited medium for the liver cell attachment and growth *in vitro*. The liver tissue explants were supplied with different culture media of pH 7.2 supplemented with 10% serum concentration and incubated at 28 ^{0}C. The results found were in conformity with the earlier reports of Singh *et al*. on kidney of *Heteropneutes fossilis* [8], Lakra and Bhonde on *Labeo rohita* caudal fin[9], Rao *et al*. on heart tissue of *Labeo rohita* [12] and Kumar *et al*. on ovarian tissue of *Clarias gariepinus* [13].

pH is an important factor that can dramatically affect the growth and survival of cell culture *in vitro,* hence the study on optimum pH required for the cell growth and proliferation was done. In this regard the cultured cells were provided with L-15 medium of varying pH (i.e. 7.2, 7.4, 7.6 and 7.8) supplemented with 10% serum incubated at 28^{0}C showed that the optimum pH of 7.4 was significantly higher ($P \leq 0.05$) compared with the other varying pH of culture media. The results observed found were similar to the early reports[11, 14, 15].

FBS is the most essential component for survival and growth of cell culture. In the present study, the trypsinised cells seeded in T25 cm^2 flasks were supplemented with L-15 medium of pH 7.4 with different serum concentration of 5, 10, 15, 20 and 25% respectively and incubated at 28 ^{0}C. Among the varying concentrations of serum, the serum concentrations of 20% were more suitable for the cell attachment and outgrowth. The present study supported the observations made by Kumar *et al*., Lakra *et al.* and Fernandes *et al.* [7, 11, 16]. Contradictory to other reports, in present study the additional necessary component such as fish muscle extract or fish serum were not added but the cell attachment and proliferation was found satisfactory [7, 11].

Partial characterization of hepatocytes

In this present study, the authentication of hepatocyte culture was proved by appearance of stained purple colour cells by staining with Best's carmine stain (Fig.14) initially, later on after 21st passage the cells were partially characterised and analysed with mt 16s rRNA gene amplification (Fig.15).

Best carmine staining

The staining of glycogen with Best's Carmine is widely regarded as a useful but empirical procedure. Best (1906) suggested about the properties of carmine stain which is used to stain glycogen granules present in several biological entities [17]. In this regard, the cultured liver cell *in vitro* was characterized with Best's carmine stain (HiMedia, Mumbai) after the attachment and proliferation of cells after the initial passages for the authentication and validation of hepatocytes. The stained cells confirmed that the outgrowths were from liver explants which contained glycogen granules whereas fibroblast became unstained. This is probably the first report of carmine staining for characterization of cultured fish hepatocytes *in vitro*. The study found conformity with the illustrations made by Horobin and Murgatroyd in mammalian cells [18].

Molecular characterization

Species identification of cell lines is crucial for scientific research accuracy and reproducibility. Among the molecular characterisation of cell line chromosome analysis, mitochondrial 16S rRNA and COI gene sequence analysis are common toll for authentication and validation of cell lines nowadays. In this study, the final characterization of cultured liver cells from *P. hypophthalmus in vitro,* was done with the 16s rRNA gene fragment amplification after 21st passage. In this regard, an analysis of mitochondrial 16S rRNA was done to verify the origin of cultured cell *in vitro*. An expected 546 bp amplified PCR product of mt 16S rRNA obtained from cell line (L_3) after 21st passage as well liver tissue of *P. hypophthalmus* (L_2) was found of similar sizes. This indicated their similar origination and validated the results after partial molecular characterisation (Fig. 15). The results obtained from the study was based on the work done by past researchers where they claimed that the mt 16s rRNA gene sequence analysis can be used as a reliable molecular method to accurately identify the origin of cell line from fish [19, 20, 21, 22].

Conclusion

The methodology developed in the present study can prove to be potential for the cell culture in developing continuous cell line from liver of *P. hypophthalmus.* To the best of our knowledge till date there is no report regarding the cell culture or cell line of this species hence the study will open new vistas for researchers to study the pathological process at cellular level, to understand the cause underlying the disease and reveal the mitigation measures against disease occurrence of this emerging cultured species.

Acknowledgement

The authors are extremely grateful to the Director, ICAR-Central Institute of Fisheries Education, Mumbai, India for his support and facilitation for carrying out the research work successfully.

Tables and Figures

Table 1: Effect of different parameters on cell growth as observed by cell confluence monolayer formation

Sl. No.	Temperature (^{0}C)	Cell confluence (in %)	Media	Cell confluence (in %)	pH	Cell confluence (in %)	Serum (in %)	Cell confluence (in %)
1.	24	35.00 ± 5.00	L-15	73.33 ± 6.00	7.2	55.00 ± 2.88	5	45.00 ± 2.88
2.	26	50.00 ± 2.88	HBM	66.66 ± 3.33	7.4	73.33 ± 6.00	10	60.00 ± 2.88
3.	28	60.00 ± 2.88	RPMI-1640	46.66 ± 4.40	7.6	71.66 ± 6.00	15	73.33 ± 3.33
4.	30	55.00 ± 2.88	DMEM	60.00 ± 5.00	7.8	56.66 ± 4.40	20	80.00 ± 2.88
5.	32	38.33 ± 4.40	-	-	-	-	25	70.00 ± 2.88

Figures (Fig.)

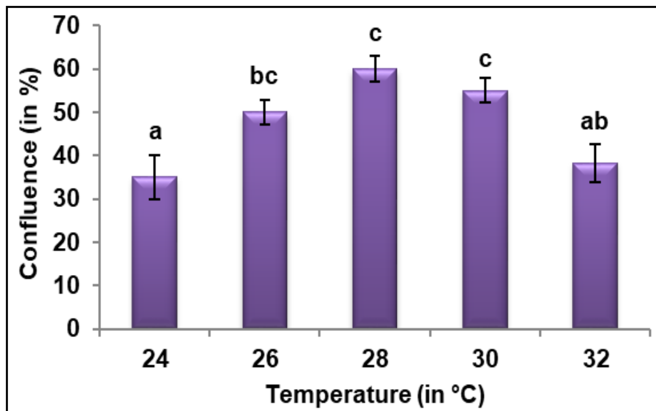

Fig 1: Effect of different incubation temperature on cell confluence

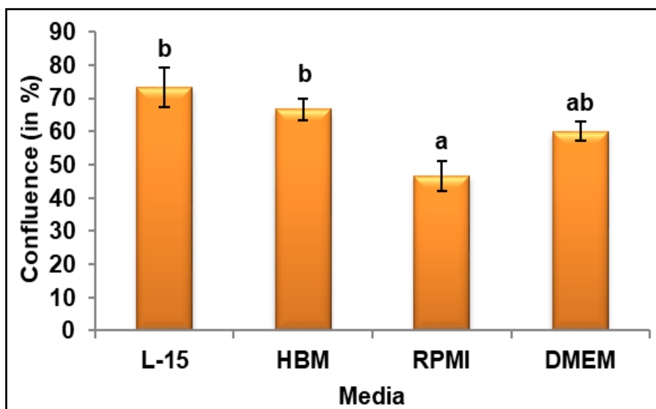

Fig 2: Effect of different culture media on cell confluence

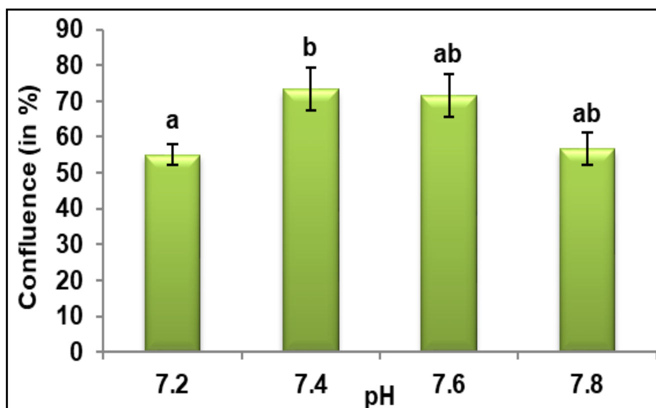

Fig 3: Effect of different pH of L-15 media on cell confluence

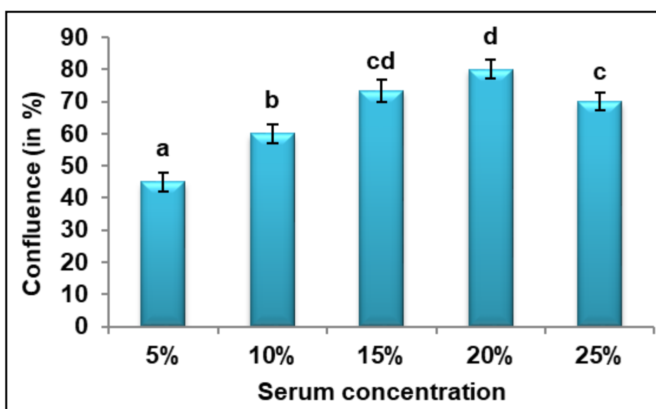

Fig 4: Effect of FBS concentration in L-15 medium on cell confluence

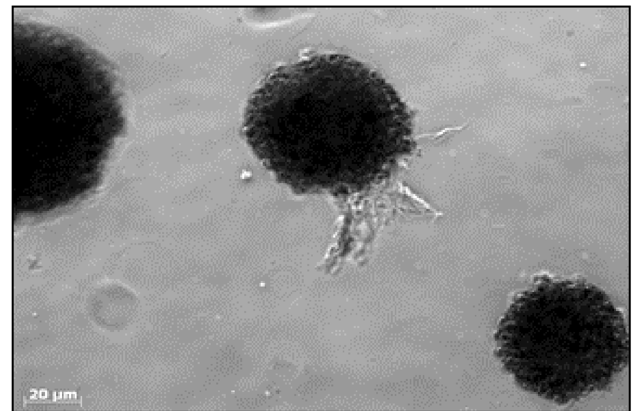

Fig 5: Attachment of liver tissue explants within 36-42 hrs.

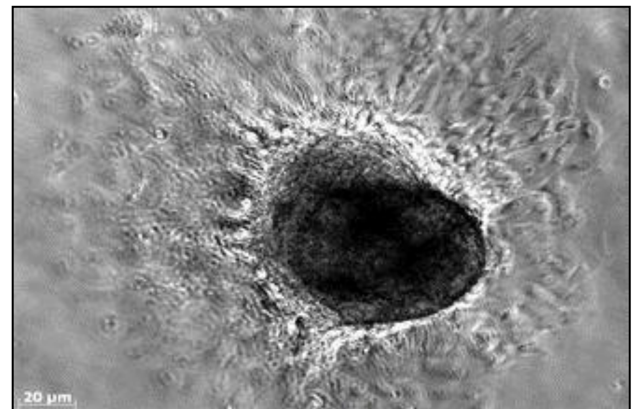

Fig 6: Cell outgrowth from liver tissue explants of *P. hypophthalmus* after 72 hrs.

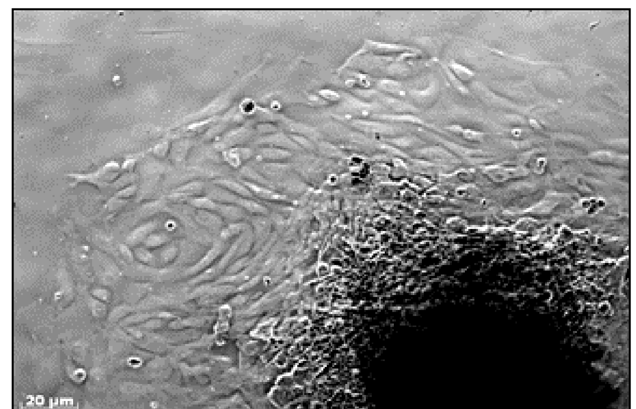

Fig 7: Appearance of spindle shaped cells in early culture of liver cells

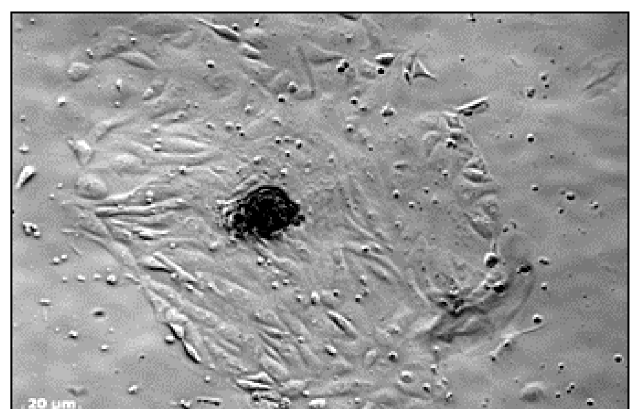

Fig 8: Migration of cells from liver tissue explants

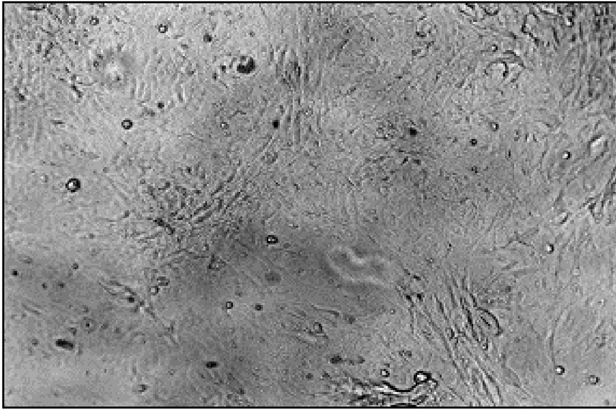

Fig 9: Hepatocytes after 1st passaging

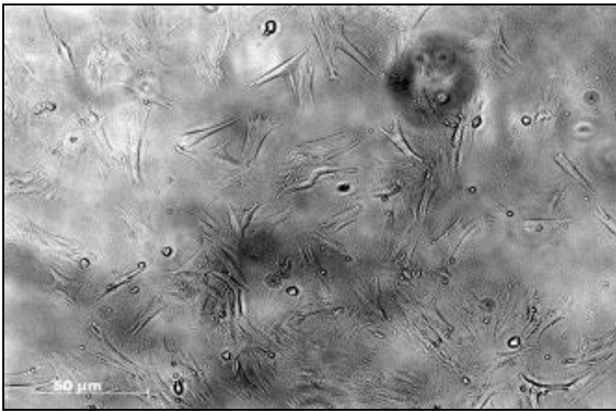

Fig 10: Cells forming monoconfluent layer of hepatocytes (200X)

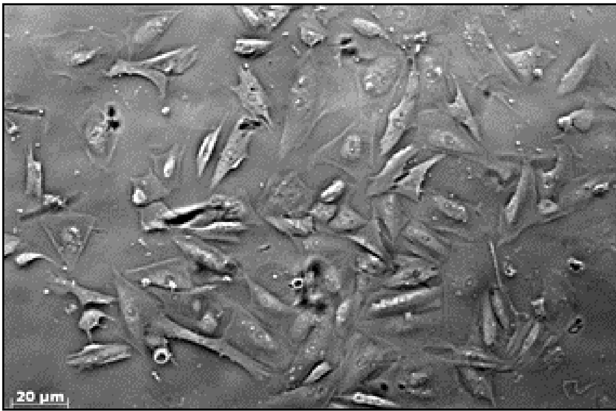

Fig 11: Dominance of stellate shaped cells after subsequent passage (200X)

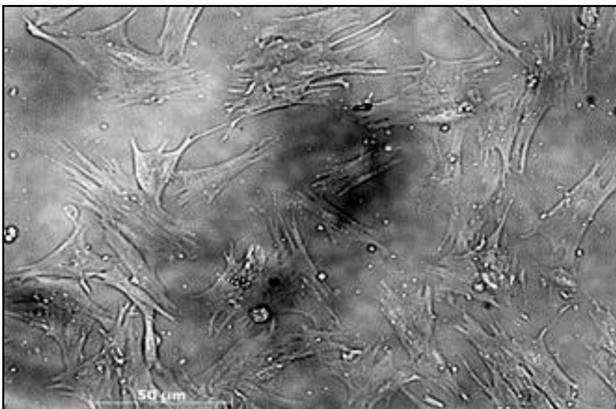

Fig 12: Dominance of fibroblastic cells after subsequent passages (200X)

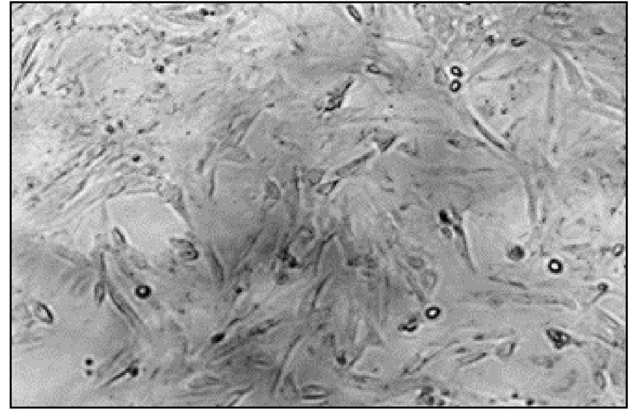

Fig 13: Cells forming monoconfluent layer of hepatocytes after 21st passage (200X)

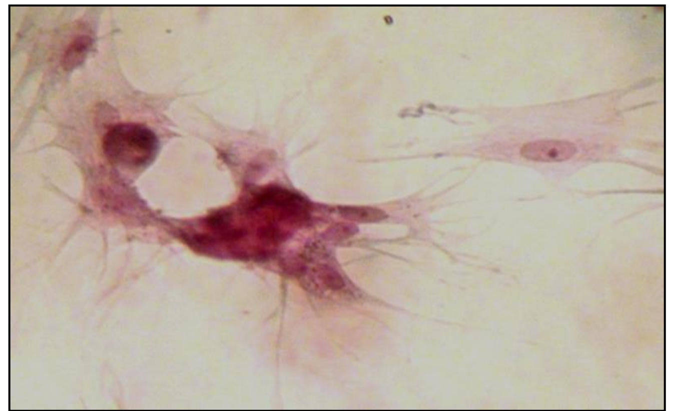

Fig 14: Partial characterization of liver cells by Best's Carmine staining (400X)

L1 L2 L3 M

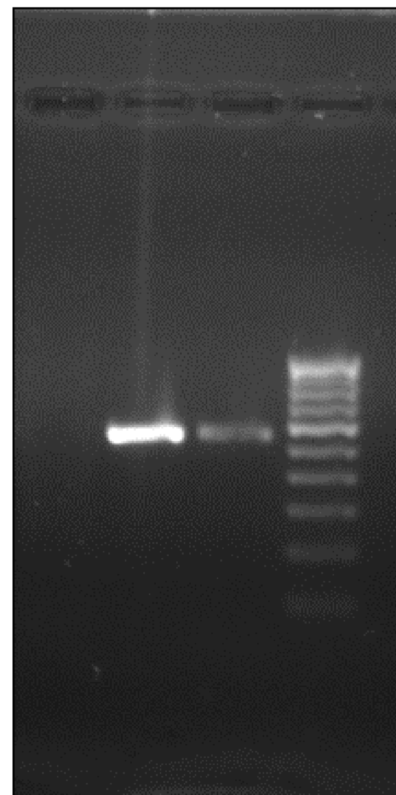

Fig 15: Gel electrophoresis PCR amplified product from 16s mtrRNA of *P. hypophthalmus* (L_1– Negative control, L_2– PCR product of liver cell, L_3 - PCR product of cell culture, M – Molecular marker (100 bp))

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