Cell culture from fin explant of endangered golden mahseer, *Tor putitora* (Hamilton)

Isswar Prasanna*, W. S. Lakra*[†], S. N. Ogale and R. R. Bhonde[‡]**

*Division of Fish Genetics and Biotechnology, Central Institute of Fisheries Education, Versova, Mumbai 400 061, India
**Tata Electric Company Ltd, Lonavala 410 401, India
[‡]Department of Biotechnology, National Centre for Cell Science, Ganeshkhind, Pune 411 007, India

A simple and reproducible short-term fish cell culture technique has been described for *Tor putitora*. A fin tissue was used to develop primary cultures without sacrificing the fish. It yielded successful culture on the fourth day employing L-15 medium supplemented with 20% foetal calf serum (FCS) and 10% fish muscle extract (FME), at 28°C. The technique provided consistent epithelial cell monolayer from the mother explants. On subculture, the cell morphology changed to fibroblast-like cells. The technique used offers an elegant way to develop primary cultures without sacrificing the fish, thus making it a far more practical way of obtaining cell cultures from endangered species. In addition, the influence of *in vivo* regeneration on *in vitro* cell growth of the same tissue was studied. Regenerated fin tissue showed early and rapid cell growth when compared to normal fin tissue, indicating the presence of growth factors.

SHORT-TERM and continuous cell cultures from a variety of fish species have been reported in the past due to their potential use in virus isolation, toxicological and cytogenetic studies[1–4]. Most of the established cell lines are derived from cold or cool water fish of European origin[5]. Very few cell lines have been developed from the tissue of tropical fish species. The Indian scenario is changing fast towards developing cell lines and short-term cultures from species of aquaculture and commercial importance. Recently, cell lines from the gill tissue of mrigal, *Cirrhinus mrigala*[6], and rohu, *Labeo rohita*[7], have been reported. Successful primary cultures from a variety of tissues including heart tissue of Indian major carp[8], kidney of stinging catfish, *Heteropneustes fossilis*[9], and caudal fin of rohu[10] have also been developed.

Several workers have studied the regenerative ability of piscine tissue in the past. Teleost fin rays are able to regenerate when they are cut, restoring the whole structure in a few weeks[11]. The present investigation deals with primary cultures from caudal fin and the influence of *in vivo* regeneration on *in vitro* cell growth of the fin tissue of golden mahseer.

The live fish (fry and fingerlings) were obtained from the Tata Electric Company Fish Farm, Lonavala and were maintained at the National Centre for Cell Science, Pune. The caudal fin was collected from live specimens and the cut wound was treated locally with Betadine. For regenerated fin explant, culture tissues were collected from the same fish after 2, 4, 8, 12, 16, 20, 22, 24 and 45 days of initial tissue collection.

Four different media (Gibco, BRL, USA) with foetal calf serum (FCS) and fish muscle extract (FME) were tried. Initially Minimum Essential Media (MEM), Grace Insect Media (GIS), Rosewell Park Memorial Institute (RPMI) 1640 and Leibovitz L-15 with 10% FCS and 10% FME were used. Amongst these, L-15 was found to yield the best results. Therefore, the rest were discontinued for further study. Four different proportions of L-5 with FCS and FME – 8 : 1 : 1, 6 : 2 : 2, 7 : 2 : 1 and 7 : 1 : 2 were tried. To prepare FME, 40 g of fish muscle was macerated in 200 ml 1X PBS and centrifuged at 500 rot/min to remove the debris. The supernatant was inactivated in a water bath at 56°C for 30 min. It was again centrifuged to remove coagulated protein and filtered through 0.45 μm and 0.22 μm filters for sterilization.

The tissues were dipped in 20% Betadine for 10 min and then washed several times to remove the chemical. The fin tissue was then cut into small pieces. The pieces were then distributed uniformly in a 35-mm tissue culture dish (Nunc, Denmark) and left in the laminar hood in a semi-dried condition for few minutes. After adding two drops of FCS, the tissues were left at room temperature for 3–4 h for adherence. Care was taken to avoid overdrying of the tissue. After ascertaining tissue adherence, the explants were fed with culture media. The explants were then allowed to grow at 10, 15, 20, 25, 28, 30, 35 and 37°C in the incubator. Microscopic observations were carried out everyday under an inverted microscope (Olympus, Japan). In the second phase of the experiment, primary cultures were sub-cultured. The primary cultures were treated with 0.2% trypsin in PBS (pH 7.2) twice to detach the cells from the surface and washed with PBS (pH 7.2). Pellets were re-suspended in L-15 and MEM supplemented with FCS and FME, and plated in a 35-mm tissue culture dish. The same procedure was followed for setting up explant culture from the regenerating fin.

Everyday, from the fourth to the tenth day in the case of normal fins and the first to the seventh day in the case of the regenerated fins, each culture dish was washed with PBS (pH 7.2) and cells removed using 0.2% trypsin in PBS for 5 min at room temperature. Cells were counted using a Neubour's chamber in all the three culture dishes seeded with 10 explants.

Most explants were attached to the plastic dish within 4 h of seeding. The cell spreading was observed in most of the fin tissue explants within 4 days, and the number of proliferating and migrating cells increased with time. Cells were in the growing phase for 5 to 8 days after plating and

[†]For correspondence. (e-mail: lakraws@hotmail.com)

reached the peak number after 8 days. Most of the cells seemed to spread out from the explant (Figure 1). Two types of adherent cells could be observed in culture – epithelial-like and fibroblast-like cells. Epithelial-like cells were predominant initially (about 80 to 90%) but during sub-culture, the cell phenotype changed into fibroblast-like cells. The best growth was observed in the L-15 medium with 20% FCS and 10% FME at 28°C (Figure 2). Explant fed with MEM showed cell growth (Figure 3). No growth was observed in RPMI 1640 and in Grace's Insect Media. The tissue explants obtained from different post-surgery days (regenerating fin at various time intervals) exhibited various responses in their growth pattern. The explants taken within 4 days of surgery and after the completion of total regeneration of the fin (45 days) exhibited growth patterns similar to that of normal fin explants. However, the fin explants obtained between 4 and 25 days after surgery exhibited early and rapid growth of the cells from the mother explant (Figure 4).

From the *in vivo* regeneration data it is seen that the time taken for total regeneration depends upon the amount of tissue removed. In the present study, we found that 70% removal of caudal fin took around 45 days to complete the regeneration.

Most of the primary cell cultures reported from fish species have shown the growth of fibroblast-like cell[8–10]. However, the present investigation reports the outgrowth of epithelial-like cells.

The *in vitro* profile of explant cultures obtained on different days after initial surgery showed differential growth pattern during 45 days of *in vivo* regeneration. It appears that the process of fin regeneration begins after 4 days of surgery and gets completed by 25 days of surgery due to some growth factors. There was no change in *in vivo* cell proliferation from the explants taken within 4 days of initial surgery and after 25 days of post-surgery, indicating the absence of regenerating growth factors *in vivo*. A growth factor released during this period seems to trigger on and fasten the regeneration of the fin. The rate of regeneration was noticed to be slow after 25 days of post surgery, indicating the disappearance of growth factors slowly. The similar growth pattern has been reported on regenerating pancreas in induced mice[12], where the authors have shown that 50% removal of pancreas from induced

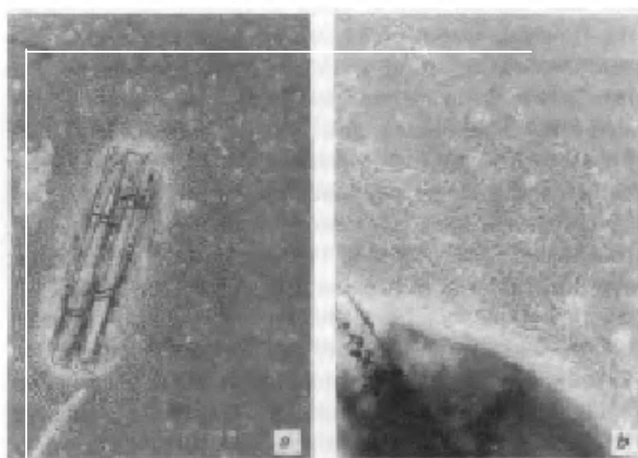

Figure 1. A primary explant culture of fin tissue (***a***, × 60; ***b***, × 600).

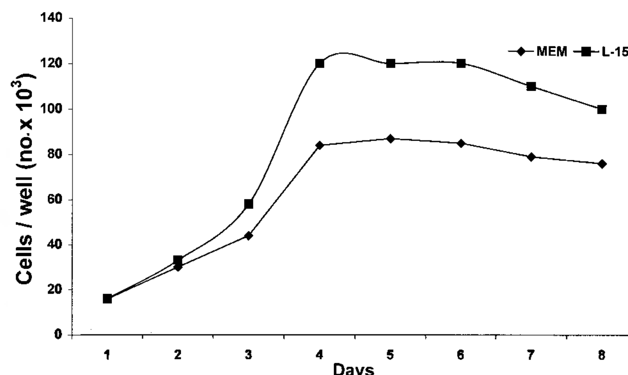

Figure 3. Growth curve for different media. Each curve represents the mean of quadruple counting in a representative experiment.

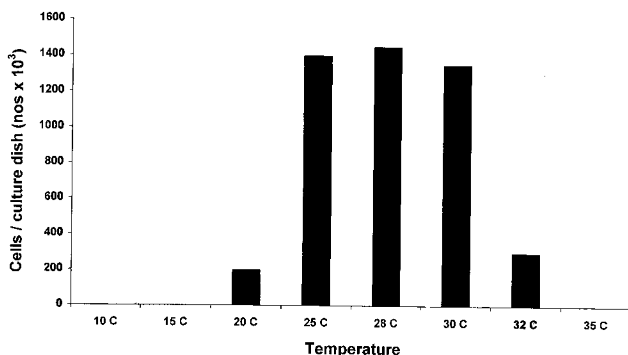

Figure 2. Effect of temperature on cell growth.

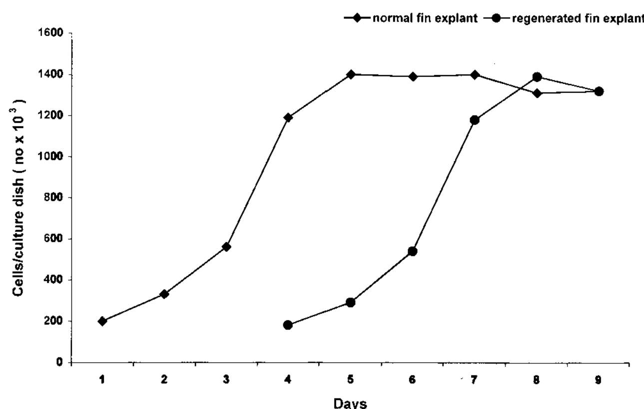

Figure 4. Growth curve for normal and regenerated fin explant cultures.

diabetic mice leads to total regeneration of the pancreas within four weeks, leading to reversal of the diabetic state. The authors have further shown that repeated injection of cytosolic extract in the diabetic mice leads to the complete reversal of diabetes[13]. In view of the above information, it is likely that regenerating mahseer fin might be harbouring several growth factors within themselves, which stimulate *in vitro* growth of explants as seen in our study. The present work also suggests that the serum from the regenerating fin at specific time might have growth-promoting activity. This feature is of great significance in developing primary cultures within a short period.

1. Wolf, K. and Mann, J. K., *In Vitro*, 1980, **16**, 168–179.
2. Blaxhall, P. C., *J. Fish Biol.*, 1983. **22**, 279–282.
3. Hightower, L. E. and Renfro, J. L., *J. Exp. Zool.*, 1988. **248**, 290–302.
4. Nicholson, B. L., in *Invertebrate and Fish Tissue Culture* (eds Kuroda, Y., Kurstak, E. and Maramoroch, K.), Japan Scientific Society Press, Springer-Verlag, 1988, pp. 191–196.
5. Chen, S. N., Chi, S. C. and Kou, G. H., *Fish Physiol.*, 1986. **18**,13–18.
6. Sathe, P. S., Mourya, D. T., Basu, A., Gogate, S. S. and Banerjee, K., *Indian J. Exp. Biol.*, 1995. **33**, 589–594.
7. Sathe, P. S., Mourya, D. T., Basu, A., Gogate, S. S. and Banerjee, K., *In Vitro*, 1997. **33**, 125–127.
8. Rao, K. S. Joseph, M. A. Shankar, K. M. and Mohan, C. V., *Curr. Sci.*, 1997, **73**, 374–376.
9. Singh, I. S. B. and Phillip R., *Indian J. Exp. Biol.*, 1995, **33**, 595–599.
10. Lakra, W. S. and Bhonde, R. R., *Asian Fish. Sci.*, 1995, **9**, 149–152.
11. Santamaria, J. A., Mari-Beffa, M. and Becerra, J., *Differentiation*, 1992, **49**, 143–150.
12. Hardikar, A. A. Karandikar, M. S. and Bhonde, R. R., *J. Endocrinol.*, 1999, **162**, 189–195.
13. Hardikar, A. A. and Bhonde R. R., *Diabetes Res. Clin. Prac.*, 1999, **46**, 203–211.

ACKNOWLEDGEMENTS. We thank Dr S. A. H. Abidi, Director, Central Institute of Fisheries Education, Mumbai, Dr G. C. Mishra, Director, National Centre for Cell Science, Pune and the authorities of the Tata Electric Company Ltd, Lonavala for their keen interest and support for the collaborative research work.

Received 15 April 2000; revised accepted 11 May 2000

Effect of synthetic zeolites on xylanase production from an alkalophilic *Bacillus* sp.

H. Balakrishnan[†], M. C. Srinivasan[†], M. V. Rele[†,*], K. Chaudhari[‡] and A. J. Chandwadkar[‡]

[†]Division of Biochemical Sciences and [‡]Catalysis Division, National Chemical Laboratory, Pune 411 008, India

The present study pertains to stimulation of enzyme secretion in a xylanolytic alkalophilic *Bacillus* in submerged culture in media supplemented with different zeolites. Among different zeolites possessing various cations, only calcium-containing zeolites exhibited two-fold enhancement of extracellular xylanase activity. 0.5% calcium zeolite enhanced the level of activity to the same extent as 0.5% Tween 80 supplementation. The possibility of supplementing zeolite as an alternative to Tween 80 in large-scale, aerated–agitated fermentations is discussed.

IN the course of our studies to evaluate the effect of zeolites on bio-processes, we had reported enhanced ethanol production by *Saccharomyces cerevisiae* in the presence of ZSM 5 (ref. 1). In the present study we report the effect of zeolite catalysts on xylanase production by an alkalophilic *Bacillus* strain isolated in our laboratory. Recent interest in xylanases, which are cellulase-free and active at high alkaline pH, has emanated from the realization that such xylanases could be extremely useful in the pulp and paper industries for biobleaching and also in the manufacture of dissolving pulp. Besides being specific in their reactions at ambient temperatures, the use of xylanase also minimizes the use of toxic chemicals such as chlorine and chlorine dioxide which are environmentally hazardous[2]. The search for cellulase-free, alkali-stable and active xylanases of microbial origin has resulted in the discovery of several xylanases from bacteria, especially the genus *Bacillus,* actinomycetes and fungi[3–9]. Xylanases are induced enzymes secreted into the medium when grown on either pure xylan or xylan-rich agricultural residues. Addition of adjuncts such as amino acids and surfactants was shown to improve enzyme yields in production[10–12]. More recently, addition of zeolites has been shown to improve yields in amylase production[13].

Zeolites are crystalline hydrated aluminosilicates of group-I and group-II elements, in particular sodium, potassium, magnesium, calcium, strontium and barium. Zeolite structures are composed of a three-dimensional network of SiO_4 and AlO_4 tetrahedra in which a unit negative charge is associated with each AlO_4 tetrahedron. This charge must be counterbalanced by a positive inorganic or organic ion. Depending on the type of connections between tetrahedral building blocks, linear or pseudolinear channels may be joined with diameters ranging from 4.2 to 7.2 Å.

*For correspondence. (e-mail: mvrele@dalton.ncl.res.in)