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In vitro culture of mantle tissue of freshwater mussel (*Lamellidens marginalis*) for bio-mineralization process

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ABSTRACT

Lamellides marginalis, a species of freshwater mussel, is primarily cultivated for its application in pearl farming and the production of animal feed. In recent times, the population of this species has experienced a significant decline due to environmental pollution and harmful harvesting practices. This decline has had a detrimental impact on the provision of ecosystem services. With a view to conserve this mussel species and to introduce in vitro pearl culture technique using nacre-secreting mantle tissue the present study was conducted from September 2020 to February 2021. Mantle tissue was excised from both the shell valves of the mussels. Processed mantle tissue strip of 2×2 mm^2 were preserved in a petri-dish containing nutrient rich medium. 3-5 ml sterilized tissue culture media; Dulbecco's Modified Eagle Medium, Gibco BRL (pH adjusted to 7.4) was aseptically dispensed in each petri dish with the tissue explants and incubated in a CO_2 incubator at 5% CO_2 maintaining the temperature 28°, 30° and 32°C in triplicates. During the experiment, migrating epithelial cells, granular hemocytes and hyalinocytes were not evident for 10 days from the commencement of the experiment might be due to the contamination of explant tissue and incomplete shell proliferation process. After 10 days, all the explant tissue becomes dark and shrink which considered as the death of the cells. The findings of the current study will enhance the knowledge of in vitro pearl culture technique using *L. marginalis* and will be beneficial for further research work.

Introduction

In vitro pearl culture technique or process is defined as the maintaining or cultivating cells or tissues derived from living organisms in a culture medium. Collins English Dictionary defined in vitro culture as the growth of small pieces of animal or plant tissue in a sterile controlled medium. An essential part of the method for creating high-quality pearls in the lab is the in vitro cultivation of nacre-secreting pallial mantle tissue from freshwater pearl mussels (Phuc et al., 2011). Implanting a piece of donor mantle tissue epithelium and a tiny inorganic bead into the recipient's gonad is what's known as in vivo pearl cultivation (Suja et al., 2017). As for example, Samata et al. (1994) observed nacre crystals after 15-30 days of mantle epithelial cell cultured in their experiment. Tissue culture of *Pinctada radiata* mantle showed conchiolin deposition and nacre crystal formation, as shown by Bevelander and Martin (1949). Furthermore, calcium crystals were found to secrete from mantle tissue explant cultures of *P. fucata* (Machii and Wada, 1989).

Culturing pearls in a lab (in vitro culture) and skipping the farming phase altogether is an appealing prospect in the pearling business as of late. Mantle cells can be cultured in vitro to create pearl, as has been shown in a numerous particles. Successful long-term culture of epithelial cells from Akoya pearl oysters was reported by Wang et al. (2002); however, cellular secretion started to diminish after 31 days of culture and the cells began to die by 41 days. Scientists are currently investigating this topic (Chen et al., 2005). Although, many scientists have attempted lab-grown nacre production in different mussel species (Suzuki and Mori, 1991; Awaji and Suzuki, 1995; Dharmaraj and Suja, 2001; Barik et al., 2004b) the freshwater mussel *L. marginalis* has not been documented yet for using in vitro pearl culture in laboratory scale.

It is necessary to develop methods for production of pearls by in vitro tissue culture due to the challenges faced in conventional pearl culture, such as the uncertainty of survival of the recipient mussel in its natural environment, difficulties in management of color and quality of the cultured pearls, and the cost of production (Suja et al., 2017). Moreover, in vitro culture techniques facilitate research on pearl sac and pearl formation processes. Because of the high stakes involved in developing this pearl culture method, mantle tissue culture has emerged as a

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frontrunner in the field of molluscan science. Scallop (Endoh and Hasegawa, 2006), Abalone (Suja et al., 2007), oyster (Gong et al., 2008), mussel (Quinn et al., 2009), and clam (Dessai, 2012), mantle cell primary cultures have been obtained.

Bangladesh has taken some initiatives for the sustainable freshwater pearl culture and successfully manufactured pearl in pearl producing native mussel such as *L. marginalis*, *L. corrianus*, *L. phenchooganjensis* and *L. jenkinsianus*. Generally, two culture methods of freshwater mussels are used in Bangladesh i.e., grazing method, and hanging net bag method. Despite the growing popularity of pearl culture, the production has not yet reached upto the mark due to low survival rate and quality of produced pearls. Therefore, the present study has been taken to investigate the suitability of in vivo pearl culture using explant tissue of native freshwater mussel *L. marginalis*.

Materials and methods

Location and duration of the experiment

The experiment was conducted at the Pearl Research Laboratory (PRL) at Bangladesh Fisheries Research Institute (BFRI), Mymensingh, Bangladesh (24°43'49.6"N, 90°25'27.4"E) during September 2020 to February 2021.

Experimental design

The experiment was designed with three different temperatures (28, 30 and 32°C) considering three different treatments. Treatment 1 (T_1) was maintained at 28°C, treatment 2 (T_2) was set at 30°C and treatment 3 (T_3) was controlled at 32°C. All the treatments were run in triplicates.

Collection and pre-operative treatment of mussel

Freshwater mussels with a yellow shell margin on their shell line were collected from the ponds of BFRI by hand-picking which was previously collected from natural environment and stocked in the ponds for relaxation. After the collection, the mussels were cleaned to remove attached algae and other waste materials. Mussels were then transferred to a cemented cistern ($2.42\times1.88\times1$ m^3) of 3000 L water holding capacity. The cistern was also facilitated with water exchange and aeration facilities. Before the stocking in the cistern, disinfection was done with 10 ppm $KMnO_4$. The mussels were reared for four weeks in the cistern. During this period, the mussels were provided with sufficient planktonic food materials. Regular siphoning was done to remove the feces and other waste materials. Before harvesting for further experiment, the mussels were kept starved for seven days (Tanu et al., 2022) to harden the mussels for mantle operation. After the pre-operative treatments, the mussels were cleaned with tap water, transferred to the laboratory, and held in a downward position (hinge upward) in a porous basket for 2 h, which facilitated the draining of waste materials from the internal organ of the mussel.

Preparation of mantle tissue block

The outer shell surface of the mussels was wiped with 70% Ethanol and opened by a sterile knife. Mantle tissue was excised from both the shell valves. The selected mantle strip was washed thoroughly in sterile natural salt water (5 ppt) till the mucus and other adhering particles were completely removed. The strip was then treated in antibiotic solution containing streptomycin according to Dharmaraj and Cheruvathoor (2010) (0.5 to 1.5 mg/ml). Further washing in sterile water was continued for the removal of the effect of antibiotic solution. The processed strip was cut into pieces of (2×2 mm^2) on cleaned wooden surface. Each piece of the mantle tissue block along with a sterile shell bead was placed in a culture petri-dish containing nutrient rich medium.

Nutrient media and placement of mantle tissue block

3-5 ml sterilized tissue culture media; Dulbecco's Modified Eagle Medium, Gibco BRL (pH adjusted to 7.4) was aseptically dispensed in each petri dish with the tissue explants. The media used here was enriched with filter sterilized fetal bovine serum (10%; Gibco BRL), and supplemented with antibiotics viz., Penicillin (0.2 mg/ml, Sigma) and Streptomycin (0.2 mg/ml, Sigma). The mantle tissue pieces were attached to one side of the sterilized (70% Ethanol) shell bead (3mm diameter) and the other side was submersed into the culture medium. The tissue culture plates were then incubated in a CO2 incubator (BIOLAB, BCAJ-101, Canada) at 5% CO2 and 28°, 30° and 32°C (Barik et al., 2004a).

Sampling

The culture dishes were regularly observed for any cellular development under a microscope (Labomed,

LB 272) and the medium was exchanged at every 3-4 days intervals.

Results

Cell viability and survival

During the study period, it was only possible to maintain the explant mantle tissues in the culture condition for 10 days. The experiment also reported no notable effect of temperature on the explant tissue culture.

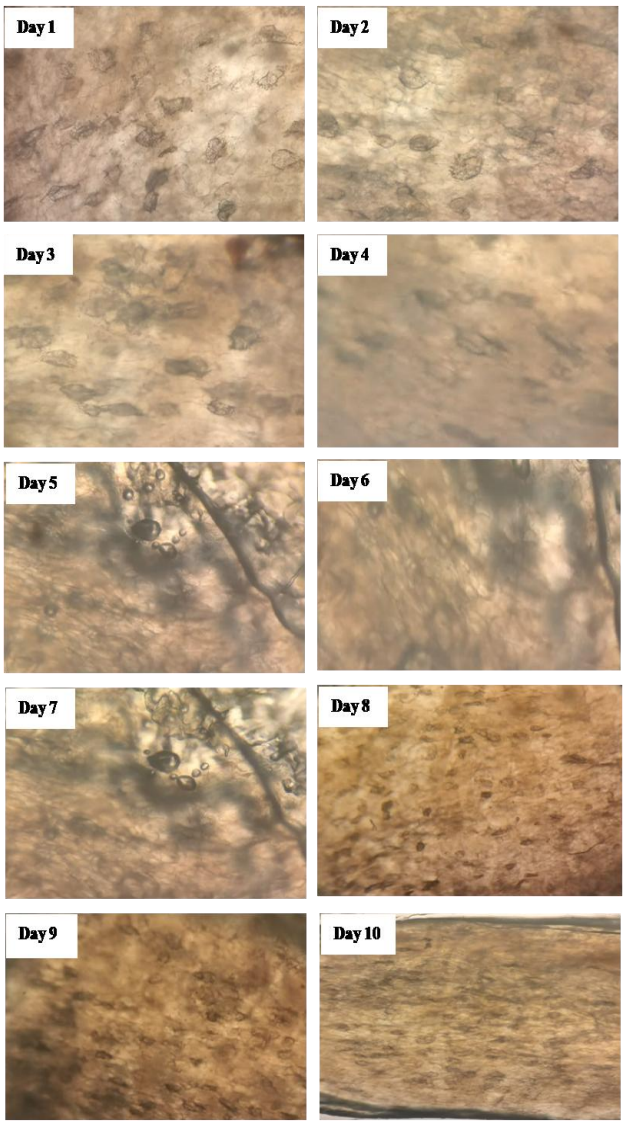

Figure 1: Microscopic observation of mantle tissue explant of *L. marginalis* during 10 days of culture period.

During the experiment, the explant tissue was remained viable for observation. However, the original morphometry of the explant tissue was changed. During the culture period, any of the cell types such as migrating epithelial cells, granular hemocytes and hyalinocytes (agranular hemocytes) were not visible. Cell debris was evident at initial stages only for 2-3 days of culturing. However, after that period, the explant tissue becomes darker and shrink at the edges which assumed as the death of the cells (Figure 1). Therefore, it was not possible to conduct further enzymatic treatment and cell proliferation processes.

Discussion

Mantle epithelia of mussels are generally studied for biological control of calcification process (Joubert et al., 2014; Herlitze et al., 2018). Several factors namely culture condition, composition of culture medium and contamination of cells and culture media are responsible for success of cell culture. Incubation temperature is one of the determining factors in the growth and proliferation of mantle epithelial cells. According to Suja et al. (2017) cell density was found to be good when the plates were incubated at 25°C. Dessai (2012) also reported that the cell density was higher at 20-25°C. Bacterial and fungal contaminations remain a severe concern in primary cell culture, especially for organs that are directly exposed to seawater such as the gills and the mantle (Van der Merwe et al., 2010). Furthermore, formulation of a suitable medium for cell culture also poses a big challenge. Because experimental failures are typically not appropriate for publication in most scientific articles, there has no detailresearch workon the in vitro cultivation of molluscs (Rinkevich, 1999). Around 200 cell lines derived from insects and ticks have been successfully established in various areas of invertebrate tissue culture (Mitsuhashi, 1989), but most attempts to create stable, proliferative cell cultures from aquatic invertebrates have failed.

In view of the above literature, the failure of mantle cell culture in the present experiment can be described either by the contamination of the explant or by the inappropriate use of culture media. Study conducted by Barik et al. (2004a) achievedsuccess in the crystallization of $CaCO_3$ in primary culture of mantle epithelial cells of freshwater pearl mussel. Although, the similar process was followed in the present experiment, failure might be due to the contamination of explant tissue. Again, in terms of culture media, use of seawater was not followed in the present experiment which also might be a reason for death of the explant tissue. Together, these results indicate that aseptic control of culture

environment and effective selection of culture media should be opted to investigate crystal formation at the cellular level.

Conclusion

In conclusion, regarding the failure of cell culture of mantle tissue explant of *L. marginalis*, the present study suggests future research toconcentrate on cell characterization and dietary needs of mussel. Potential oxidative stress occurring in vitro cell cultures should also be checked and control optimally for the survival and growth of explant tissue. In view of such negative findings in the present experiment, the best policy when controlling the culture environment and formulating a culture media for the culture of mantle tissue explants should accordance with a matter of trial-and-error process.

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References

Awaji, M. & Suzuki, T. (1995). The pattern of shell proliferation during pearl-sac formation in the pearl oyster. *Fish. Sci*., 61: 747 – 751.

Barik, S.K., Jena, J.K. & Ram, K.J. (2004b). In vitro explant culture of mantle epithelium of freshwater pearl mussel. Indian *J. Exp. Biol.*, 42: 1235–1238.

Barik, S.K., Jena, J.K. & Ram, K.J. (2004a). CaCO3 crystallization in primary culture of mantle epithelial cells of freshwater pearl mussel. *Curr. Sci.*, 86:730-734

Bevelander, G. & Martin, J. (1949). Culture of mantle tissue of marine molluscs. *Anat. Rec.*, 105: 614-614.

Chen, H.T., Xie, L.P., Yu, Z.Y., Xu, G.R. & Zhang, R.Q. (2005). Chemical modification studies on alkaline phosphatase from pearl oyster (*Pinctada fucata*): a substrate reaction course analysis and involvement of essential arginine and lysine residues at the active site. *Int. J. Biochem. Cell Biol.*, 37: 1446-1457.

Dessai, S.N. (2012). Primary culture of mantle cells of bivalve mollusc, *Paphia malabarica*. In Vitro Cell. *Dev. Biol.*, 48: 473-477.

Dharmaraj, S. & Suja, C.P. (2001). Biotechnological approach in in vitro pearl production. Perspectives in Mariculture. The Marine Biological Association of India, Cochin, p405–412.

Endoh, M. & Hasegawa, Y. (2006). Culture of mantle epithelial cells expressing shell matrix proteins from scallop *Patinopecteny essoensis*. *Fish Sci*., 72: 1277-1285.

Gong, N., Ma, Z., Li, Q., Li, Q., Yan, Z., Xie, L. & Zhang R. (2008). Characterization of calcium deposition and shell matrix protein secretion in primary mantle tissue culture from the marine pearl oyster *Pinctada fucata*. *Mar. Biotechnol.*, 10: 457-465.

Herlitze, I., Marie, B., Marin, F. & Jackson, D.J. (2018). Molecular modularity and asymmetry of the molluscan mantle revealed by a gene expression atlas. *Giga Science*, 7: 1–15.

Joubert, C., Clémentine, L., Gilles, L.M., Claude, S., Denis, S., Teaniniuraitemoana, V., Ky, C.L. & Gueguen, Y. (2014). Temperature and food influence shell growth and mantle gene expression of shell matrix proteins in the pearl oyster *Pinctada margaritifera*. *PLOS One*, 9: 1–9.

Machii, A., Wada & K.T. (1989). Some marine invertebrates tissue culture. *Invert. Cell Syst. Appl.*, 2: 225-233.

Mitsuhashi, J. (Editor) (1989). Invertebrate cell system applications. CRC Press, Boca Raton, Florida, United States. pp. 93.

Phuc, P.V., Viet, P.Q., Hoang, N.M., Tam, N.T., Ngoc & P.K. (2011). Research on in vitro culture and inducing nacre crystal formation of freshwater pearl mussel mantle epithelial cell *Sinohyriopsis cumingii*. *Int. J. Fish. Aquac*., 3: 105-113.

Quinn, B., Costello, M.J., Dorange, G., James, G.W., Mothersill & C. (2009). Development of an in vitro culture method for cells and tissues from the zebra mussel (*Dreissenia polymorpha*). Cytotechnology, 59:121-134.

Rinkevich, B. (1999). Cell cultures from marine invertebrates: obstacles, new approaches and recent improvements. *J., Biotech*., 70: 133-153.

Samata, T., Somiya, H., Horita, C. & Akera S. (1994). SEM observation of microcrystals developed over black secretion on the cultured tissue of the pearl oyster *Pinctada fucata*. *Fish Sci*., 60: 343-344.

Suja, C.P., Raghavan, S.V., Jayasankar, V., Divipala, I., Mohamed, B.M., Mary, K.B. & Vijayan, K.K . (2017). Evaluation of different media for cell proliferation in mantle tissue culture of the green mussel, *Perna viridis* (Linnaeus, 1758). *Indian J. Fish.*, 64(Special Issue), 224-228.

Suzuki, T.& Mori, K. (1991). Immunolocalization and in vitro secretion of hemolymph lectin of the pearl oyster, *Pinctada fucatamartensii*. *Zool. Sci.*, 8: 23–29.

Tanu, M.B., Barman, A.C., Siddque, M.F., Sku, S., Hossen, M.N., Southgate, P.C. & Mahmud, Y. (2022). Impact of culture period on quality of image pearls produced by the freshwater mussel, *Lamellidens marginalis*, in Bangladesh. *J. Shellfish Res.*, 41: 75–83.

Van der Merwe, M., Auzoux-Bordenave, S., Niesler, C. & Roodt-Wilding, R. (2010). Investigating the establishment of primary cell culture from different abalone (Haliotismidae) tissues. *Cytotechnology* 62: 265-277.

Wang, Z., Guo, X., Allen, S.K. & Wang, R. (2002). Heterozygosity and body size in triploid Pacific oysters, *Crassostrea gigas* Thunberg, produced from meiosis II inhibition and tetraploids. *Aquac. Res.*, 204: 337-348.