


Awaji, M. 1997. Primary culture techniques for the outer epithelial cells of pearl oyster mantle. In: Maramorosch, K., Mitsuhashi, J. (Eds.), Invertebrate cell culture, Novel Directions and Biotechnology Applications, Science Publisheres, NH, USA, pp. 239-244.


Chen, J and H.Y. Yang. 1996. The effects of Cu super (++) on cellular immunity in


Jodrey, L.H. 1953. Studies on shell formation III. Measurement of calcium


Lebel, J.M, W. Giard, P. Favrel and E. Boucaud-Camou. 1996. Effects of different vertebrate growth factors on primary cultures of hemocytes from the


Mcfadzen, I.R.B. 1995. Cryopreservation of the sperm of the pacific oyster,


841-842.


Tsujii, T. 1976. An electron microscopic study of the mantle epithelial cells of 
Anodonta sp. during shell regeneration. In: The Mechanisms of Mineralization 
in the Invertebrates and Plants" (N. Watabe and K.M. Wilbur, eds.) pp. 339-

Technol., 1: 3-15.

92-95.

Uozumi, S and S. Suzuki. 1979. ‘Organic membrane shell’ and initial calcification in 

pacific oyster Crassostrea gigas, sperm, Bull. Nansei Natl. Fish. Res. Inst., 30:
115-123.

Vago, C. 1972. Invertebrate cell and organ culture in invertebrate pathology. In: 

250: 2751-2753.

pearls from tropical abalone Haliotis varia (Linn.) at Mandapam. Mar. Fish. 


Wada, K. 1960. The relation between the crystalline structure of the cultured pearls 
and the elongation of the transplanted mantle tissue in the process of pearl-sac 

7:703-828.

Wada, K. 1964a. Studies on the mineralization of the calcified tissue in molluscs-IV. 
Selective fixation of $^{45}$Ca into or onto the metachromatic matter in the process 


* _Original not referred._

145
LIST OF PUBLICATIONS

Published papers


Papers submitted for publication


TISSUE CULTURE IN PEARL OYSTER

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Application of Tissue Culture Techniques
ISSUE CULTURE IN PEARL OYSTER

S. DHARMARAJ AND C.P. SUJA

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Tuticorin Research Centre, Tuticorin - 628 002

INTRODUCTION

The first work on cell culture in marine molluscs started since 1960s. Many researchers attempted to improve the culture media composition by adding vertebrate sera as growth factor. In primary cultures, the tissue dissociation methods and medium composition were frequently complemented with homologous and heterologous substance. Inspite of such improvements, establishments of marine molluscan cell line or long-term primary cultures are rather limited. This is mainly because of some contaminant micro-organisms and protozoans and lack of data on molluscan cell physiology and biology. The chief difficulty for the cultivation of new medium components was related to available methods for cell viability analysis. In *in vitro* culture, cell survival appeared greatly variable according to its nature and degree of differentiation.

Works on marine invertebrate tissue culture has been initiated at the Tuticorin Research Centre of CMFRI, Tuticorin since 1995. A well established tissue culture laboratory has been set for the purpose of culturing mantle tissues from the pearl oyster. The laboratory consists of office-cum-record room, preparation room, dressing room and clean (operation) room. The rooms are arranged in different modules to avoid contamination from one room to another. A strict hygienic condition is kept up at all times. A pass box is constructed to store sterilised materials. It has one UV lamp on its top which keeps the materials in the chamber sterile. It has three doors; one in the preparation room, the second in the dressing room and the third in the clean room.

PREPARATION OF CULTURE MEDIUM

The culture media are developed based on the analysis of inorganic ion and free amino acids concentration of the body fluid of an animal. Vitamins and the other minor constituents of the medium were supplemented to the culture medium.

The medium Pf35 was developed exclusively for pearl oyster cell culture. The other culture media used for marine invertebrate tissue culture are Medium 199, L-15 and Ham’s F-12. These culture media may be prepared as per the available formula or may be procured as ready made items from scientific companies.

Depuration of pearl oysters

The oysters to be taken for the experiment are utilised within 24 hr. of harvesting. They are kept in UV treated running seawater for a minimum period of 3 days. Everyday the oysters and the tank are cleaned with detergent powder.
Preparation of oysters
1. Depurated oysters immersed in 70% ethanol for 15 seconds.
2. The shell is allowed to dry and the oyster is cut into two halves by a sterile knife.
3. The mantle tissue is removed and the pallial organs are cut and discarded.
4. The mantle tissue is cut into 1mm explants with sterile scalpel.

Preparation of tissues
1. The cut pieces of the mantle (explants) are washed six times in 10 ml sterile seawater (SSW) or balanced salt solution (BSS) in petridishes.
2. Explants are treated in 10% ethanol for 15 seconds.
3. Washed four times in 10 ml SSW.
4. Explants are again treated four times in antibiotic solution (Table 1) each 30 minutes duration for 2 hr.
5. Three washings in 10 ml SSW; now the explants are ready for culture.

Table 1. Combination of antibiotics

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamycin</td>
<td>125μg/ml</td>
</tr>
<tr>
<td>Polymixin</td>
<td>100μg/ml</td>
</tr>
<tr>
<td>Neomycin sulphate</td>
<td>100μg/ml</td>
</tr>
<tr>
<td>Kanamycin sulphate</td>
<td>100μg/ml</td>
</tr>
<tr>
<td>Mycostatin</td>
<td>200μ/ml</td>
</tr>
<tr>
<td>Fungizone</td>
<td>5μg/ml</td>
</tr>
<tr>
<td>Pencilllin</td>
<td>200μ/ml</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>200μg/kmL</td>
</tr>
</tbody>
</table>

After Stephens and Hetrick

Organisation of explant cultures

Explants are inoculated in sterile petridishes and TD flasks. 3 ml of culture medium with 20% foetal calf serum (FCS) is added. The cultures are placed in CO₂ incubator at 30°C. Cells migrate from the explant after 24 hr. Fibroblast like cells are seen in the cultures from day 9 onwards (Fig. 1).

Fig. 1. Mass of cells dissociating from the explant of pearl oyster mantle tissue.

**Organisation of Cell Culture**

**Trypsinisation**

The mantle tissues are subjected to trypsinisation. Cut pieces of tissues are transferred to trypsinisation flask containing 30 ml of Marine Mollusc Calcium Magnesium Free Phosphate Buffer Solution (MM CMF PBS) with 0.05% trypsin. A teflon stirrer is placed in the flask for proper dissociation of cells from the tissues. Stirring is done for 10-15 minutes at 1200 rpm. The cell suspension was first filtered through 150μ and 60μ sieve. The filtrate is centrifuged at 4°C for 5 minutes at 800 rpm and the supernatant solution is removed gently without disturbing the precipitate. A drop of medium is added to the precipitate and mixed well. The mixture containing free cells is distributed to culture dishes (Fig. 2). 3 ml medium is added to each flask and incubated in CO₂ incubator at 30°C.
If there is any contamination in the culture, the medium is removed and the cells are washed with SSW. After ensuring that no organism is there, the cells are removed from the flask and inoculated into fresh flasks or cell well.

**Cell well culture**

The cell well is also called as microplate. The microplate with 24 wells and 96 wells is available. It is disposable after use. It is provided with a lid. The cell well is used to culture single cell for cloning purposes. 3 or 4 drops of medium is added to each well. The cell well is placed in CO₂ incubator.

**Medium change**

Medium change is done on alternative days. The periodicity of medium change is decided on the basis of the condition of cells. Half the medium is changed for the first and second time and the whole medium is changed subsequently. At times cell suspension is centrifuged and fresh inoculation is done. In some of the established cell lines, the cell will be active and hence the entire medium is changed. When the cells are weak in a cell line, only half of the medium change is done. Frequent medium change is needed to establish cell line.

**Cell counting**

Cell counting is carried out with haemocytometer. It is very essential to fix the optimum cell density in each type of culture vessels. Variation in cell density may affect the growth of cells. The rate of proliferative cells in a cell population is calculated by counting the number of colonies formed by the cells.

**APPLICATION OF TISSUE CULTURE TECHNIQUES**

There is an increasing trend in the case of tissue culture in various fields of biological research. Tissue culture techniques are being developed in marine invertebrate animals only in the recent years. Valuable information is being gathered on the aspects of cell structure, cell division, cytogenetics, cell physiology and cell viability. Tissue culture techniques are used in studying the structural and functional aspects of cells, tissues, organs, etc. by *in vitro* studies. The study on the effect of chemicals and radio elements on normal tissues and cancer cells are being taken up in tissue culture. Study on pathological organisms in culture techniques has led to curing of several diseases and production of vaccines. Careful studies in tissue culture would be useful in transplantation of tissues/cells among members of a species or from species to species.
Biotechnological approach in in-vitro pearl production

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Tuticorin Research Centre of Central Marine Fisheries Research Institute, Tuticorin

ABSTRACT

Explant culture of mantle tissue of the pearl oyster Pinctada fucata (Gould) was undertaken at the tissue culture laboratory of CMFRI, Tuticorin. Mantle tissues were routinely cultured in Medium 199 and Pf 35 individually and combindly supplemented with 10% foetal calf serum at 28°C, pH 7.8. Explants released numerous epitheloid-like and fibroblast-like cells. Cells were in assorted sizes and formed colonies. Nacreous secretion by the cells was observed on culture plates. The study represents a new tool for cellular approach in in-vitro pearl production.

Introduction

The Indian pearl oyster Pinctada fucata is used for the production of cultured pearls. The pearl production technology was perfected and the techniques involved were standardised. Manipulation of colour and quality of cultured pearls was rather difficult in in-vivo culture. Hence an attempt on in-vitro pearl production was made.
Perspectives in Mariculture

through tissue culture technique. The work on pearl oyster mantle tissue was limited. Bavelander and Martin (1949) organised organ culture of mantle of the marine mollusc *Pinctada radiata* and obtained the deposition of conchiolin crystals typical to those found in normal regenerating shells. Machii and Wada (1989) reported the secretion of organic substance by the cells dissociated from an explant culture of pearl oyster mantle tissue. A similar approach was undertaken at the tissue culture laboratory of the Central Marine Fisheries Research Institute, Tuticorin. The results obtained in the present study are presented here.

Materials and methods

A. Depuration of oysters: The pearl oyster *Pinctada fucata* brought from the farm was cleaned thoroughly and placed in U.V. treated running seawater in a fibreglass tank for depuration for 3-7 days. Everyday the oysters and the tank were cleaned to remove the organic waste.

B. Preparation of tissues: The depurated oysters were dipped in 70% ethanol for 15 seconds and taken to clean room for further processing. They were cut open by a sterile knife and the mantle tissue was removed. The pallial organs at the free end and the connective tissue at the distal end were cut and removed. The mantle strip thus obtained was cut into pieces of 1 sq.mm.

The mantle pieces were washed six times in 10 ml of sterile seawater (SSW) or in a balanced salt solution (BSS) in petri dishes. The composition of BSS is shown in Table 1.
### Table 1. Balanced salt solution for marine mollusc (MM BSS)

<table>
<thead>
<tr>
<th>Components</th>
<th>MM BSS (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>26.22</td>
</tr>
<tr>
<td>KCl</td>
<td>1.08</td>
</tr>
<tr>
<td>Mg SO₄</td>
<td>3.18</td>
</tr>
<tr>
<td>Mg Cl₂</td>
<td>2.20</td>
</tr>
<tr>
<td>Ca Cl₂</td>
<td>1.12</td>
</tr>
<tr>
<td>Na HCO₃</td>
<td>0.30</td>
</tr>
<tr>
<td>Na H₂PO₄</td>
<td>0.044</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.30</td>
</tr>
</tbody>
</table>

(After Machii and Wada, 1989)

### Table 2. Composition of antibiotics in a sterile seawater

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamycin</td>
<td>125 ug/ml</td>
</tr>
<tr>
<td>Polymyxin B sulfate</td>
<td>100 ug/ml</td>
</tr>
<tr>
<td>Neomycin sulfate</td>
<td>100 ug/ml</td>
</tr>
<tr>
<td>Kamamycin sulfate</td>
<td>100 ug/ml</td>
</tr>
<tr>
<td>Mycostatin</td>
<td>200 ug/ml</td>
</tr>
<tr>
<td>Fungizone</td>
<td>5 ug/ml</td>
</tr>
<tr>
<td>Penicillin</td>
<td>200 ug/ml</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>200 ug/ml</td>
</tr>
</tbody>
</table>

(After Stephens and Hatrick, 1979)

The mantle pieces were treated in 10% ethanol for 15 seconds and again washed three times in 10 ml of SSW or BSS. They were given treatment in a mixture of antibiotic solution four times each 30 minutes for 2 hours (see Table 2). After this treatment they were washed three times in 10 ml of SSW or BSS. Now the pieces are ready for inoculation.

C. **Explant culture**: The explants were inoculated in petri dishes.
Perspectives in Mariculture

2 ml Medium 199; 2 ml Pf 35; 1 ml Foetal Calf Serum (FCS) and 5 ml of SSW were combined together as medium. No antibiotic was used in the cultures. They were incubated at 28°C, pH 7.8 and the medium was changed once in two days.

Results

At this combination of culture media (Medium 199 - 2 ml; Pf 35 - 2 ml; SSW - 5 ml and FCS - 1 ml) the cells proliferated from the explant in good numbers on day 2 onwards (Fig. 1). A mass of epithelial-like and fibroblast-like cells dissociated from the explant (Fig. 2). The epithelial-like cells were spherical in shape and have short pseudopodia. As large

Fig. 1. Cells dissociating from the mantle explant on day 2

Fig. 2. Epithelial-like and fibroblast-like cells on day 8.
Explanted was found to have undergone a noticeable change from a mesh elongated, string-like and spherical cells were seen. Meanwhile, the away from the explant on day 4. On day 6 a mixture of spindle shaped, number of cells were emerging, the cells at the distal end had moved into culture.

**Fig. 4.** Secretion of crystals by the cells in culture.

**Fig. 5.** Formation of colonies by the mantle cells on day 15.

Biotechnological approach
colour to a dark brown colour on day 10. The number of cells had increased since day 13 and the colony formation had occurred on day 15 onwards (Fig.3). The colony of cells secreted an organic substance (Fig.4) and deposited on the culture plates. Prolonged culture of these cells resulted in trefoil - like crystals in the colony. The crystals were in different sizes. Apart from these crystals there were alveolar materials secreted by the colony of cells on day 20 (Fig.5). The alveolar materials showed no birefringency. They were subjected to acid testing and found to be calcareous matter. On day 14-15 the explant became fragile as large number of cells had dissociated from it.

Discussion

Although research on in-vitro culture of marine invertebrates was undertaken, establishment of cell lines from these animals was rather limited. However some studies were carried out on the culture of mantle tissue from pearl oysters. Bevelander and Martin (1949) could demonstrate the deposition of concholin and formation of crystals through an organ culture of mantle from the marine mollusc *Pinctada radiata*.
The nature of conchiolin and crystals was typical to those found in normal and regenerating shells. Subsequently Machii (1974) reported that the secretion of organic substance through an organ culture of mantle from the pearl oyster *Pinctada fucata* was nothing but calcium crystals. Later the secretion of calcium crystals was also reported from the cell line culture of pearl oyster larvae (Machii, 1985) and from an explant culture of pearl oyster mantle tissue of *P. fucata* (Machii and Wada, 1989). A similar result was obtained in the present study on the explant culture of mantle tissue of *Pinctada fucata*. In our study large number of cells had dissociated from the explant and a cell sheet formed on day 4. It was identical to the study by Machii and Wada, 1989 where the formation of cell sheet took place between day 3 and 7. On day 12 the explant underwent considerable change in colour and since then the secretion of organic substance occurred. It was quite similar to our study where the change of colour and the secretion of organic substance occurred on day 10. The deposition of alveolar material was obtained on day 30 in both the studies. The high content of calcium in the crystal as reported by Machii and Wada (1989), indicated that it must be a prism, a kind of pearl formed *in vitro*.

**Acknowledgements**

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References


Development of technology in in-vitro pearl production in India

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The technology for production of cultured pearls from the pearl oyster *Pinctada fucata* was first developed in India in 1973. Techniques related to the technology were standardized. The need for further improvement was greatly felt and hence a programme of advanced research has been taken up on pearl oyster mantle tissue culture. Earlier studies in this field had reported deposition of concholien and crystals in an organ culture of mantle of marine pearl oyster *Pinctada radiata* in 1949; secretion of organic substance from an explant culture of mantle tissue of pearl oyster *Pinctada fucata* in 1974; and deposition of trefoil-shaped material in an explant culture of pearl oyster mantle in 1989. The advances in the present investigation are a stepping stone for further research in in-vitro pearl production in India. The details of the study are presented here as a first report.

Mantle tissue fragments of the pearl oyster *Pinctada fucata* were used for explant cultures. Prior to excision of mantle tissue, the pearl oysters were depurated for three days in U.V. irradiated running seawater. The depurated oysters were wiped externally with 70% ethyl alcohol. The oysters were opened carefully by severing adductor muscle and the mantle tissue was removed aseptically. The strip was processed by trimming pallial organs at the free end of mantle and connective tissue at the lower portion. The middle region of the mantle was retained and washed three times in sterile seawater (SSW) or in artificially prepared balanced salt solution (BSS). It was then treated in SSW containing 1000 μg/ml streptomycin and 2000 IU/ml penicillin. The strip was again washed two times in SSW and cut into small fragments of about 2 mm square under aseptic condition. Three fragments were inoculated as explants in each T25 flask. 3 ml of Medium 199 with nutrient salt solutions and 10% foetal calf serum was added to each flask.

Organ cultures were organized in sterile petri dishes. In each petri dish a sterile shell bead nucleus of 4 mm diameter was placed over a glass ring to avoid rolling of the bead and a mantle piece was kept over it in such a way that the outer face of mantle touched the shell bead. Medium 199 was added only up to lower portion of mantle and the upper phase was kept free from the medium. Medium exchange was done every 4 days. The cultures were maintained at 28°C and pH 7.4.

Epithelial-like spherical cells emerged from the explant in large numbers on day 1. Cells having short pseudopodia formed a cell sheet around the explant (Fig. 1). The spherical cells were of two types - granulocyte and agranulocyte.
museum so preoccupied him that his stay in Cambridge was reduced to five weeks teaching each year, and, from his former pupil’s collections, he selected only two Opuntia species from Galapagos and the Keeling (Cocos) Isles flora for study. Thanks to Anne Stow’s expertise as scientific librarian at Cambridge, this biography has brought forward much previously unpublished material. The book includes genealogical tables, a chronology, brief biographies of persons mentioned, and lists of eponymous taxa, local botanical records and Henslow’s works. The authors are to be congratulated on producing a book that is a thoroughly enjoyable read, and it will be a useful addition to any library’s collection of Darwiniana.

Elizabeth Platts and Bill Bailey

NEW BOOKS

Gerhard FALKNER, Theo E. J. RIPKEN & Margrit FALKNER,

Mollusques continentaux de France –
Liste de Référence annotée et Bibliographie

with an introduction by Philippe BOUCHET


France’s extensive climatic and biogeographic diversity, at the crossroads of atlantic, continental, and Mediterranean regions, with the alpine ranges and Corsica, is reflected in the composition of its fauna. Field work in Corsica has resulted in the recognition of previously undisclosed radiations of endemic slugs (in the genera Limax and Deroceras) and Oxychilus. This checklist recognizes 660 valid species of land and freshwater molluscs, of which 180 are endemic. Sub-species are also include, boosting the total terminal taxa to 747 taxa (233 endemic). Distribution in France is briefly characterised by reference to biogeographic regions recognized by the Topic Center for Nature Conservation of the European Environmental Agency. 3000 references.

Peter HARVEY, David NELLIST & Mark TELFER

Provisional Atlas of British Spiders (Arachnida, Araneae), Vols 1 & 2
£20 incl p+p. A4 paperbacks, with 10km map for each of the 648 British species. Species accounts include status, habitat and ecology, and adult activity through year.

Order from Centre for Ecology and Hydrobiology, Publication Sales Section, Monks Wood Abbots Ripton, Huntingdon, Cambs PE28 2LS.

The authors are grateful to Dr. (Prof) Mohan Joseph Modayil, Director, CNFFRI, Cochin and to Dr. K.K.Appukuttan, Head, Molluscan Fisheries Division, Cochin for their interest. We sincerely thank the Department of Biotechnology, New Delhi for financial help in establishing the marine invertebrate tissue culture laboratory at Tuticorin and the National Agriculture Technology Project (ICAR, New Delhi) for providing funds to continue the research work.