Development and characterization of cell cultures from a few tissues of Mud Crab *Scylla serrata*

A Thesis submitted to GOA UNIVERSITY for the Award of the Degree of

DOCTOR OF PHILOSOPHY

In

Zoology

By

Ms. ANUMOL. G. SHASHIKUMAR, M.Sc, M.Phil

Research Guide

Prof (Dr) P.V.Desai

DEPARTMENT OF ZOOLOGY

Goa University,

Taleigao, Goa

AUGUST 2012

Dedicated

To My

Loving Mother

CERTIFICATE

*This is to certify that the thesis entitled "**Development and characterization of cell cultures from a few tissues of mud crab Scylla serrata**" is a bonified research work carried out by **Ms Anumol G Shashikumar** under my guidance and supervision and that no part thereof has been presented for the award of any other Degree.*

Prof.P. V. Desai

Ex-Prof& Head,

Dept. of Zoology,

Goa University

August, 2012

DECLARATION

*In accordance with the university ordinance, I hereby declare that the thesis entitled "**Development and characterization of cell cultures from a few tissues of mud crab Scylla serrata**" is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text, and a list of reference is included.*

Goa University ***Ms Anumol G Shashikumar***

August, 2012

ACKNOWLEDGEMENTS

From the depth of my heart I express my deep sincere gratitude to the Almighty for the blessing he had bestowed upon me to do this work.

I am immensely pleased to place on record my profound gratitude and heartfelt thanks to my supervisor, ***Dr P.V. Desai****, Ex Professor, Ex. Head, Department of Zoology, Goa University for his valuable and precise guidance throughout the present study. His constant interest, attention, constructive criticism, deep vision, advice, encouragement and help throughout the period of research have enabled me to complete this work.*

I am thankful to my Doctoral committee members V.C's, Nominee ***Dr Baban Ingole****, NIO for his valuable suggestion on my work* ***Dr G.N. Nayak,*** *Dean of Life Science and Professor of Department of Marine Science for his constant support and encouragement.*

I am thankful to ***Dr A. B. Shanbhag, Dr R. Roy, Dr S. K. Shyama, Dr I. K. Pai*** *Department of Zoology for their kind encouragement during the execution of work.*

I do take pleasure in thanking the non-teaching staff of Dept of Zoology, ***Mrs Gulabi Desai., Mr Dasharath., Mr Rama., Mr Tanu., Mr Yoganand., Mrs Helen., Mrs Sangeeta., Mr Domingo*** *for all the help they rendered to carry out my work without any hurdle.*

I extend my heart felt thanks to my colleagues, ***Mrs Aruna Clemente,*** *M.Sc,* ***Mrs Beena Sony****, M.Sc, M.Phil., and* ***Ms Shamshad Shaikh****, M.Sc for helping me get through*

the difficult times and for all the emotional support, camaraderies, and caring they provided in time of need.

I extend my sincere thanks and gratitude to ***Mr Renosh P.R****, M.Sc.,* ***Ms Ratna Siraswar****, M.Sc,* ***Mr Aditya, Sangelkar,*** *M.Sc.,* ***Mrs Radhika Nair****, PhD.,* ***Ms Jyoti Vaingankar****, M.Sc.,* ***Ms Socchorina D'Costa****, M.Sc.,* ***Ms Manju Surlekar****, M.Sc.,* ***Mr Shivraj Bhoite****, M.Sc for their pleasant association and help in various form.*

I feel a deep sense of gratitude for my late mother ***Mrs Retnamma Shashikumar*** *who formed part of my vision and taught the good things that really matter in life. The happy memory of my mother still provides a persistent inspiration for my journey in this life.*

Words do not suffice the gratitude I owe to my father ***Mr G. Shashikumar****, for his undaunted support, love, constant help and encouragement to carry out this research. I am indebted to my brother* ***Er Anish Shashikumar****, sister* ***Adv Asha Shashikumar*** *and Cousin brother,* ***Er Arun Prasad*** *for helping me in various capacities to complete this work.*

Last but not the least my thanks are due to all those who have contributed directly or indirectly in the successful completion of this thesis.

ANUMOL G SHAHSIKUMAR

PREFACE

The global demand for the production of more aquatic food has increased so as to meet the increasing population needs; this has led to the development of aquaculture industries. Crustacean fisheries have gained significant attention of the world aquaculturists owing to its economic value. Like other aquatic animals, crustaceans are susceptible to a wide variety of pathogens, including viruses, bacteria, fungi and protozoans. During the last decades, disease was considered to be the most limiting factor for expansion of crustacean aquaculture industries. This has led to the realization that we need to develop an *in vitro* cell culture system, as it offers opportunity for studying and understanding the mode of viral infection, its propagation as well as to develop reagents and probes. Though, primary cell cultures from crustacea have been initiated since 1960s, no established cell line of marine crustacean is reported except a transformed shrimp lymphoid cell line using pSV-3 neo vector and hybridized *Penaeus monodon* cell line by cellular fusion. However, these cell lines failed to support viral growth and its replication. These failures were attributed to the absence of crustacean genes and proteins in the hybrid cells.

Primary cell cultures from several crustacean species using various culture conditions have been reported with increased frequency, though in most cases these cultures survived well, their rate of cell multiplication was low and on sub culture no cell proliferation and success beyond the primary stage had been obtained. Since there is no established long lasting proliferating crustacean cell culture as well as cell line, an attempt is made to establish long surviving cell culture from crab *Scylla serrata*, a species of a great economical importance. Such cell cultures are essential for isolation, cultivation of

crustacean viruses, besides they increase reproducibility of biological investigation of pathogenesis.

The thesis is presented in four chapters:

The first introductory chapter gives a brief account on the need and problems in crustacean cell culture, review of literature pertaining to crustacean cell culture, incorporating different media, supplements and antibiotics used in crustacean cell culture and also focuses on crustacean infective studies, the need to carry out the present work, and the objectives selected for the research work.

The second chapter deals with materials and methods. It gives the techniques developed to isolate cells from different tissues of *Scylla serrata* as well as the different media, supplements and substrates tested for maintaining the cell culture for longer period. It also shows the protocols employed for characterizing cultured cells. The last section of this chapter deals with the utility of cultured cell line for testing White spot syndrome virus infectivity.

The observations and results are compiled in the third chapter. The first half deals with the development of primary culture and cell line while the second half deals with the characterization of primary culture and cell line developed. The remaining section of this chapter deals with the results obtained by exposing the cultured testicular cells to White spot syndrome virus.

The findings are discussed in the fourth chapter. This is followed by summary, recommendation for future research, presentations and publication and bibliography.

The present work is a significant contribution towards the development of crustacean cell line and testing it for WSSV pathogenicity. Thus this work has provided a tool for those researchers who are interested in investigating the viral pathogenicity, host parasite relationships and diagnostics.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVATIONS

ASW	Artificial sea water
B	Blasenzellen
BC	Background control
BLAST	Basic Local Alignment Search Tool
CDM	Chemically defined medium
CS	Culture supernatant
DMEM	Dulbecco's Modified Medium
DNA	deoxyribonucleic acid
DPX	Di-n-butyl-Phthalate in Xylene
E	Embryonalzellen
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
F	Fibrenzellen
FACS	Fluorescence- activated cell sorting
FBS	Fetal bovine serum
FCS	Fetal calf serum
FGF	Fibroblast growth factor
G	Granular
G0 Phase	State of Dormancy
G2 Phase	Gap phase
HPV	Hepatopancreatic parvovirus
HS	Horse serum
L15	Leibovitz 15
LDH	Lactate dehydrogenase
MACS	Magnetic-activated cell sorting
MBV	*Monodon baculovirus*
MCRV	*Mud crab reovirus*
MQW	Milli Q water

MTT	Methylthiazol Tetrazolium
MWPs	Multi well plates
N	Number
NCBI	National Centre for Biotechnology Information
NDC	Non damage control
NGF	Nerve growth factor
OD	Optical density
P53	Protein 53
PBS	Phosphate buffer saline
PCN	Product code number
PCR	Polymerase chain reaction
PMSF	Phenyl methane sulfonyl fluoride
PVP	Poly Vinyl Pyrrolidone
R	Restzellen
RAPD	Random amplification polymorphic DNA
RB	Retinoblastoma
RNA	Ribonucleic acid
S phase	Synthetic phase
SEM	Scanning electron microscopy
TEB	Tris-Borate EDTA
UATP	Upper aqueous transparent phase
VS	Viral suspension
WSSV	White spot syndrome virus
XTT	(2, 3-bis [2-methoxy-4-nitro-5-sulfopheny]-2H-tetrazolium-5-carboxyanilide inner salt) Tetrazolium hydroxide

CHAPTER - 1

Introduction

INTRODUCTION

Aquatic products are in great demand all over the world. Wild-caught fisheries cannot sustain the increasing pressure of global seafood demands. Currently one billion people are dependent on aquatic animals as the principle source of animal protein (FAO, 2006). By 2030, an additional 41 million tons of fish per year will be needed to maintain current levels of seafood. Thus, sustaining fish supplies won't be able to meet the growing global demands for aquatic food. Aquaculture appears to have the potential to play a significant role in meeting the dwindling demand for aquatic products. In 2006, the aquaculture industry celebrated a significant milestone. For the first time in the history, of fisheries the aquaculture industry out-produced wild caught fisheries. Among the various marine species, crustaceans constitute a class, which includes species of economic importance with a high global demand. Due to their commercial importance they are aqua-cultured and are an important source of food in many countries.

Crustacean fisheries and shrimp aquaculture production are undergoing a significant global expansion. Crustacean aquaculture accounts for 6.3 % of world production in terms of quantity, it corresponds to 20 % in terms of value. Twenty-six percent of the output from this type of aquaculture is from fresh water. This activity started in 1980s, had slow development during 1990s but now its increasing by more than 8 % per year. However, the development of aquaculture faces a number of problems of which diseases of diverse etiologies, is of particular importance and represents serious risk not only for the health of aquatic animals, but also to farmers and the consumers of aquaculture products, leading to severe impact on the economic and socio-economic development in many countries. Any disease outbreak and progression depends on the host susceptibility,

its genetic background as well as on the nature of the pathogen, its toxicants / toxins and several environmental factors (Snieszko, 1974., Bly et al 1997). During 1960s and till today, more emphasis is given on diseases of marine crustaceans. This is mainly due to the frequent occurrences of gill and shell infections leading to extensive mortalities of economically valuable species of shrimps, lobsters, and crabs harvested from commercial ponds. Furthermore, global interest in the field of crustacean mariculture has drawn attention to the possibility of imported animals carrying infectious agents. Several state and government agencies, fisheries institutions, and aquaculture laboratories have recognized the impact of imported diseases particularly of viral, fungal, and bacterial origin. These diseases serve as major deterrents to breeding, hatchery, and growout operations as well as to husbanding aquaculture species. Prevention and control of such diseases are absolute priority for the durability of crustacean aquaculture industry. Currently no treatments are available for viruses in crustaceans so when even one animal becomes infected, the virus can spread rapidly, causing devastation to the industry. Hence, the need is compelling for early detection and disease control strategies.

During past ten years major efforts have gone into identification, prevention and treatment of diseases, since aquaculture is beleaguered by diseases for the want of sensitive investigative methods that can assist in diagnosis and pathogen control. The methods available for diagnosis of diseases include: the traditional methods of morphological pathology by direct light microscopy, histopathology, electron microscopy (Lightner, 1993., Takahashi et al 1994., Wang et al 1995., Catap et al 2003), immunological methods (Nadala et al 1997., Hameed et al 1998., Hsu et al 2000), in situ hybridization method using DNA probes (Lu et al 1993., Lu et al 1995., Durand et al 1996., Chang et al 1998., Pantoja and Lightner, 2001., Phromjai et al 2002) and polymerase chain reaction (PCR)

technique (Nunan and Lightner, 1997., Otta et al 1999., Tapay et al 1999., Hsu et al 2000., Phromjai et al 2002). Among the various diagnostic methods, polymerase chain reaction (PCR) technique is considered to be the most sensitive method for detecting crustacean viruses (Lightner and Redman, 1998., Phromjai et al 2002).

However, understanding of host-parasite interactions is important for the prevention, control and treatment of diseases in aquaculture. The complex interactions underlying disease outbreak and progression are better studied using *in vitro* models that use cell / tissue culture methods and experimental systems, in which the interactions between aggressors and the host can be detected (Villena, 2003). *In vivo* models have limitation for studying diseases and isolation of pathogens. The standardized *in vitro* system provides a means to study the effects and mechanisms of pathogen invasion and provides a medium for isolation of infectious pathogens. The availability of such cellular tool (*in vitro*) is thus especially important to aqualture industries which experience disease problems that are exaggerated by intensive aquaculture methods (Ellender et al 1992., Toullec et al 1996).

Cell culture is a complex process by which cells are grown in a controlled condition outside their natural environment. Primary cell culture is the first step for the establishment of a cell culture which eventually leads to a cell line. Primary cells are more likely to reflect the true activity and functions that they display in their natural environment (Morgan and Darling, 1993). Primary culture is obtained either by allowing cells to migrate out of fragments (explant) of a tissue adhering to a suitable substrate or by disaggregating the tissue mechanically or enzymatically to produce a suspension of cells, some of which will ultimately attach to the substrate (Freshney, 2000). Explant culture

results in migration of mixed population of cell types. Frequently some of the cells may survive without proliferating and will therefore be lost in the increasing population of those which are able to multiply in the conditions supplied *in vitro*. Cells from explants may sometimes be converted to cell lines by passages (sub-culturing). These may continue to proliferate for a number of cell generations. In some instances the primary cells are fused with so-called immortal (cancer) cells to produce a hybridoma line. Many of the migrated cells of the explant survive only for one or a few passages before dying.

However, when tissue samples are disaggregated mechanically or enzymatically, the cell suspensions thus obtained along with the small aggregates contain a proportion of cells capable of attaching to a solid substratum to form a monolayer. Those cells from the monolayer that are capable of proliferation are naturally selected at the first subculture and eventually may give rise to a cell line. Generally, tissue disaggregation generate larger cultures more rapidly than explant cultures, but explant culture may still be preferred when only small fragments of tissue are available or the fragility of the cells precludes survival after disaggregation. Since disaggregated tissues give rise to variety of cell types, it becomes necessary to employ separation techniques such as density gradient separation, immunosorting by magnetizable beads (MACS) or by using fluorescence-activated cell sorting (FACS) (Pretlow and Pretlow, 1989., Carr et al 1999., Saalbach et al 1997) so as to obtain the desired cell types. The cell population can then be further enriched by selecting an appropriate medium and supplements. Survival and enrichment of cells can be enhanced in some cases by coating the substratum with collagen, laminin, Poly-D-Lysine or fibronectin (Freshney, 2010).

Development of cell line provides many advantages; the major aim of cell line development is to improve knowledge of disease-causing agents. Once, cell lines is established, it reduces the need for animal experimentation, which precludes legal, moral and ethical problems, as cells can be continuously cultivated without the need to sacrifice whole organisms (Kerry, 2009).

1.1 Need of crustacean cell culture

Crustacean cell culture has gained interest over the last two decades, especially when disease problems began to affect the commercially valuable species (Chen et al 1986., Leudeman and Lightner, 1992., Mulford and Austin, 1998). Crustaceans are particularly vulnerable to viral diseases, as their immune system lacks the antigen/antibody system of vertebrates, and consequently classic vaccination against lethal viruses is not possible (Claydon and Owens, 2008., Sánchez-Paz, 2010). There are currently no treatments available for crustacean viruses, therefore, even one virally infected animal can spread virus rapidly to the entire population leading to devastating losses to the aquaculture industry. The lack of standardized system for the isolation, identification and purification of viruses is a major obstacle to control crustacean viruses in aquaculture (Claydon et al 2010).

To understand and ultimately control the viral diseases of crustacea, the tools to investigate these pathogens must be developed. Most of the breakthroughs in the study of viruses have been made using cell culture system as this technique offers opportunity to study the effects of pathogens in *in vitro* state; in understanding the mechanism of host pathogens interactions (Chen et al 1995), to produce large amount of viral material to their characterization and to improve tools for diagnosis and cure of diseases (Loh et al

1990., Jiravanichpaisal et al 2006a). Thus, *in vitro* system provides a potentially limitless source to grow viruses in the culture and thus facilitate their characterization.

Besides it's applicability for studying the effects of pathogens, cell culture system is an important tool for toxicological studies and for testing the effects of pharmaceutical agents. Further more, during preparation of primary cultures the cells are released from the three-dimensional framework of tissue architecture, therefore the influence of intracellular contact is greatly decreased or completely abolished. Hence, the *in vitro* system offers the possibility of observing the effects of toxicants, drugs on cells in an artificial environment, without interference by nervous and humoral factors present in the intact organism. Furthermore as the cells in culture are isolated from each other, the individual cell behavior can be studied in greater detail than when they are in animal. *In vitro* systems also allow the study of cellular cytochemistry and can facilitate studies of ecotoxicology (Freshney, 2010).

1.2 Problems in crustacean cell culture

Although primary cell cultures from crustacea have been initiated since 1960s, no established cell line of marine crustacean is reported (Claydon and Owens, 2008) except a transformed shrimp lymphoid cell line using pSV-3 neo vector containing the tumor antigen gene from Simian virus-40 (Tapay et al 1995) and hybridized *Penaeus monodon* cell line by cellular fusion (Claydon et al 2010). However, primary cell cultures from several crustacean species using various culture conditions have been reported with increased frequency (Chen et al 1986., Al-Mohanna and Nott, 1987., Nadala et al 1993a, 1993b., Chen et al 1995., Hsu et al 1995a, 1995b., Toullec et al 1996., Mulford and Austin, 1998., Mulford et al 2001., Uma et al 2002). Though in most cases the primary cultures

survived well, their rate of cell multiplication was low and when sub cultured no cell proliferation and success beyond the primary stage had been obtained (Toullec et al 1996).

Crustacean primary cell cultures have limited proliferative capacity in culture due to induction of cell senescence. This finite life span is regulated by a group of dominant senescence genes, the products of which negatively regulate cell cycle progression (Sasaki et al 1994). The cell cycle consists of three major interphases such as initial gap phase (G1 Phase), a DNA synthesis stage (S Phase), a second gap phase (G2 Phase) and finally mitosis, the actual cell division. A cell may enter into a state of dormancy (G0 Phase) by taking a pause in the G1 phase before entering the S phase. However, entry into S phase is tightly regulated by signals emanating from the environment. Normally the cycle duration depends upon the cell type, as well as on species variation. Due to cellular stress and DNA damage the cell regulatory genes such as P53 and the retinoblastoma (Rb) get activated by phosphorylation and often result in either growth arrest at the G1/S interphase or apoptosis (Sager, 1992., Levine, 1997., Kerry, 2009). Since, in the normal cells P53 and Rb genes are inactive, cells proceed to the normal cell cycle.

For developing a cell line or a suitable cell culture, as a first step, one has to understand the need of developing appropriate culture conditions such as suitable medium for culture and the disaggregation of cells. Therefore, choice of growth media is an important factor in cell culture as each medium has different characteristics with respect to pH, salinity, osmolality and composition. The osmolality is a very crucial factor for crustacean cell culture as the crustaceans unlike mammals, live in an environment with high osmolality (Tangkrock and Ketpadung, 2010). Much research pertaining to cultures involved the adjustment of the culture media via nutritional supplementation in an effort to

enhance continual mitosis (Toullec, 1999). Regarding the disaggregation of tissues for cells separation, mechanical and enzymatic (using Trypsin, Pronase, Collagenase, Dispase) separation techniques are available but all of them are not suitable to each type of tissue.

1.3 REVIEW OF LITERATURE

Attempts to maintain cells and grow them in *in vitro* state started one hundred and twenty years ago as a method of biological research in which fragments of tissues from an animal are transferred to an artificial environment where they could survive and function. Harrison (1907) made first attempt to culture animal cells, and cultivated embryonic nerve cells of a frog by hanging drop method. Thereafter, this method was extended and wide range of mammalians cells were cultured *in vitro*. Harrison (1907) technique initiated a wave of interest in the cultivation of animal tissues *in vitro* and he is considered as the father of cell culture (Mothersill and Austin, 2000). Burrows (1910) further developed a technique for culturing chick embryonic cells with better cell proliferation and was first to describe the process of cell mitosis in detail. A few years later, Carrel (1913) developed a complicated methodology for maintaining cultures free of contaminations, especially bacterial. Moreover, cell culture method was quite improved after the discovery of antibiotics in 1940s. Carrel and Rivers (1927) developed the first viral vaccine from a cell culture. The significance of animal cell culture further increased when viruses were used to produce vaccines using animal cell cultures in late 1940s.

Marine invertebrates were used initially to attempt invertebrate cell cultures. Harrison (1907), known as the founder of vertebrate tissue culture because of his famous

experiments on the *in vitro* development of nerve fiber, had already tried to culture *Limulus* (Crustacea) nerve fiber *in vitro* before his work on the neuroblast culture of amphibians. In the same year Wilson (1907) reported *in vitro* maintenance of dissociated sponge cells. Goldschmidt (1915) made a first attempt to grow insect tissue *in vitro* in the hemolymph using follicle cells from the pupae of Cecropia moth. Since then, invertebrate tissue culture has progressed by following the techniques developed for the culture of vertebrate tissues.

During the early stages of culture attempts, limited success was achieved in the primary culture of invertebrate tissues. For a long time, sub-culturing of invertebrate cells was not possible and the idea that invertebrate cells, unlike the vertebrate cells, cannot grow for long period *in vitro* was generally accepted around the time of the 1950's. A break through in this pessimism was made by Grace (1962), he designed a culture medium through a technique described as "organized neglect" and was successful in obtaining the first continuous growing cells from an insect, *Antheraea eucalypti* (Lepidoptera, Saturniidae). His success encouraged workers in the field of invertebrate cell culture, mostly insect cell culturist and thereafter, many continuous cell lines were established from various insects. The cell culture of invertebrates other than insects seemed to be more difficult. There are many economically important marine invertebrates, and establishment of cell line form these invertebrates is eagerly awaited, especially in view of their pathology, economic importance as their cells or tissues produce metabolites of pharmacological significance or because the host serves as a vector for pathogens (Gong et al 1997). Recently, various cell culture techniques have been developed for invertebrates other than insects which resulted in developing cell cultures of invertebrates such as:

Sponge – Wilson (1910) cultured *Microciona* sponge cells in filtered seawater without any supplement and maintained them for several weeks. During that period he observed that the dissociated cells fused with one another to form cell masses which eventually differentiated into individual sponge. Humphrey et al (1960) developed a procedure for obtaining complete dissociated sponge cells. Curtis (1962) and Sara et al (1966a) reported the possibility of intermingling and re-aggregation of cultured cells from different sponge species. Sara et al (1966b) promoted re-aggregation of dissociated sponge and sea anemone cells however, this heterologous cell aggregation did not last longer than 72 hrs. Klautau et al (1993) cultured *Clathrina* sponge cell, some of these cells formed small aggregates, but transformation of these cell aggregates into functional structures was not observed. Pomponi et al (1997) cultured archaeocytes from *Teichaxinella morchella* in Iscove's medium, they recorded cell growth in presence of phytohemagglutinin or bovine pituitary extracts. According to Rinckevich (1998) cells of desmospongia remain arrested in long-term primary culture. Zhao et al (2005) developed a culture medium that supported 100 % increase in viability of cell culture of Sponge *Hymeniacidon perleve*.

Coelenterates – Loomis and Lenhoff (1956) cultured hydra cells that showed growth and differentiation. Martin and Tardent (1963) grew the coenosarc of *T. laynx* in filtered sea water, in this culture cnidoblasts multiplied at a faster rate after the death of all other cell types and survived for at least 50 days. Yu-Ying et al (1963) cultured hydra cells, the initial population consisted of interstitial cells, gastrodermal cells, epitheliomuscular cells and very rarely cnidoblasts cells, but only interstitial cells remained viable after few passages. Burnett et al (1968) demonstrated growth and differentiation of *Tubularia* cells in a chemically defined physiological medium. Rannou (1968) cultured dissociated cells from *Eunicella Stricta* and could maintain the cells for several months. Frank et al (1994)

propagated cells from ten species of cnidarians, and obtained four continuously growing cell lines from *Stylophora pistillata* (Anthozoa), *Plexaura* (Anthozoa) and *Millepora dichotoma* (Hydrozoa). Muller et al (1999) reported the culture conditions required for the formation of multicellular aggregates of *Suberites domuncula* from dissociated single cell.

Annelids – According to Krontowsky and Rumianzew (1922) the cells that migrate from the explant tissues of earthworm do not exhibit mitosis and survive for 7-9 days, while the cells who don't migrate or poorly migrate survive for three weeks. Using a similar technique Battaglia and Davoli (1997) cultured minced earthworm tissues in Hansen's S-30 medium and the cultured cells survived for more than 12 months.

Mollusca – Long surviving cell cultures were obtained from various tissues of mollusk: **Heart -** Chernin (1963) reported migration of amoebocytes and epitheloids from Snail heart explants with a survivability of 1-1.2 weeks without cell division. Few researchers have attempted to culture hearts of molluscan species by explant or disaggregation method so as to obtain long surviving proliferative cells (Li and Stewart, 1966., Tripp et al 1966., Wen et al 1993., Domart et al 1994., Boulo and Cadoret, 1996). Cecil (1969) cultured proliferating cells from the cardiac tissue of Clams, these cells showed spontaneous pulsation in culture that lasted up to three months. **Gonads -** Burch and Cuadros (1965) cultured cells that survived for 60 days with cell division and growth from gonads of *Helix pomatia*. **Mantle –** Machii and Wada (1989) from mantle explant cultures of *Pinctad fucata* and *Pinctada margaritifera,* raised epithelial and fibroblast cells, along with trefoil like depositions around the explant fragments after 35 days of initiation of culture and these trefoils were like prismatic crystals observed during the *in vivo* pearl formation. Awaji and Suzuki (1998) cultured outer epithelial cells of mantle tissue of pearl oyster.

Hemocytes – Lebel et al (1996) cultured hemocytes in a medium having concanavalin A that promoted rapid cell migration from explant and enhanced the adherence of hemocytes to the substratum. According to Stephens and Hetrick (1979) migrations of amoebocytes from the explants of American Oyster start within 5 minutes of initiation of culture and the migrated cells exhibit pseudopodial extensions. **Larval tissue –** Naganuma et al (1994) reported the culture of dissociated cells of *H. rufescens* wherein the heterogeneous cell population remained attached to the substratum for 12 weeks and after 21 days majority of the cells (epithelial like and visceral cells) lost their growth potential and viability while fibroblast like cells survived for 60 days. Naganuma et al (1996) cultured *H. discus* cells and maintained them for 40 days. **Gill**s – Auzoux et al (1993) reported explant gill culture of *Hard clams* that survived for 45 days. **Neurons** – Ivic et al (1995) cultured nerve cells of Snail and observed that most nerve cells start to sprout after 48 hrs of initiation of culture and during the next three days cent percent neurons develop neurites. According to Tamse et al (1995) the neuronal cells growing in close proximity with each other frequently extend multipolar processes, forming extensive and highly complex neurite networks. **Midgut gland** – Iwanaga et al (1985) cultured explant fragments of midgut gland of *Oncomelania* to obtain migratory amoebocytes and epithelial like cells that survive for one month. **Ovotestis –** Monnier-Dadhkah et al (1991) reported culture of dissociated cells of Snail which form monolayer of polygonal cells that survive for four days

Echinodermata – Johnson and Beeson (1966) attempted culture of coelomocytes of Starfish by hanging drop method and the cells survived for 3 weeks at 4^0 C. According to Johnson (1969) the phagocytic leukocytes from *Sea urchins* survive for more than one month. Mintz et al (1981) cultured cell aggregates from Sea urchin embryos. Kaneko et al

(1995) cultured dissociated cells from esophagus, stomach, intestine and coelomic pouch of *Asterias rubens* and morphologically identified different types of cells growing in culture that survives for four days. Further Kaneko et al (1997) developed embryonic primary culture of late gastrula stage Starfish and obtained elongated cells from the aggregates of epithelial like cells distributed among the mesenchymal network after one week of culture. These elongated cells showed no proliferative activity but survived for 14 days.

Arthropods – Cory and Yunker (1971) cultured hemocytes of *Dermacentro andersoni*, which remained viable for 72-74 days and these cells were useful for growth of arbovirus. Pudney et al (1973) and Varma et al (1975) developed continuous cell lines from embryos of *Boophilus microplus* and *Rhipicephalus appendiculatus*. Rahman (1981) cultured embryonic cells of *Hyalomma rufipes* using a similar procedure and obtained fibroblast cells that survived for 2-3 weeks.

1.3.1 Crustacean cell culture

Crustacean cell culture was first attempted by Fischer- Piette (1931); he cultured hematopoietic tissue of lobster. Though numerous attempts have been made on establishing short-term crustacean cell cultures (Chang and O' Connor, 1977., Wolff, 1962) none were successful in establishing long – term cultures, and many were unable to subculture cells (Ballard et al 1993., Fyhn and Costlow, 1975). Most of the crustacean species in aquaculture come from the order decapoda, of which penaeidae (Prawns), astacidea (lobsters, crayfish) and brachyura (true crabs) are respective families. Several attempts have been made to establish cell cultures from these crustacean species (Peponnet and Quiol, 1971., Quiot and Vey, 1978., Ballard et al 1993). However, recent studies have

centred on *Penaeus* as it represents one of the largest group of crustaceans in aquaculture. Explants or dissociation techniques are often used to obtain cells from various tissues and organs of penaeid shrimp, including below mentioned tissues.

Lymphoid (Oka) organ - According to Chen and Kou (1989) active cell migration of epitheloid and hemocyte like cells that do not divide in culture occur from lymphoid tissue of shrimp within a few hours of explant culture. Itami et al (1989) cultured lymphoid cells of Kuruma shrimp (*Penaeus japonicus*) in TC-199-base medium with a survivability of 54 days. Nadala et al (1993a) cultured from lymphoid explants of *P. stylirostris* and *P. vannamei*, three week or longer surviving cells. Chen and Kou (1989) cultured confluent forming cells in double strength L15 medium from lymphoid tissues of *Penaeus penicillatus.* Hsu et al (1995a) developed an *in vitro* sub-culture system for prawn lymphoid tissues. Chen and Wang (1999) cultured cells from lymphoid tissue of penaied shrimp that could survive for 20 days with three subcultures. Kasornchandra et al (1999) reported culture of 43 days surviving migratory epithelial-like and fibroblastic-like cells from lymphoid tissue of prawn, and these cells formed 80 % confluent monolayers within three days of initial plating. The explant culture of lymphoid tissue of kuruma shrimp (*Penaeus japonicus*) developed epithelial-like and fibroblastic-like cells that survived for 54 days (Itami et al 1999). West et al (1999) cultured *Penaeus monodon* prawn lymphoid cells in defined synthetic medium. The cells divided rapidly in culture, doubling on an average once per week for 5 to 6 weeks and survived for 5 months but the rates of cell division were low after the first 5-6 weeks. Wang et al (2000) cultured lymphoid cells of *Penaeus monodon* and tested the WSSV infectivity on the cultured cells.

Heart - Peponnet and Quiol (1971) reported formation of layers of muscle fiber like cells emerging from the heart explants of marine crab. Mulford and Austin (1998) reported culture of heart fibroblasts of *Nephrops norvegicus* (Prawn). Owens and Smith (1999) grew heart cells of penaeus prawn for four weeks in 2 x L15 + 20 % FBS having osmolality 740 mOsm/kg

Nerve cord - Cooke et al (1989) reported cultures of peptidergic neurons taken from the neurosecretory system of the eyestalk of crabs (*Cardisoma carnifex*) and lobsters (*Panulirus marginatus*), these neurons survived for a week in saline supplemented with glucose and glutamine**.** Grau and Cooke (1992) reported culture of peptidergic neurons of crab in defined culture media and observed that the different types of neurons preferred to grow in crab saline supplemented with glutamine and glucose. Nadala et al (1993a, 1993b) cultured nerve cells of penaeid species. Saver et al (1999) cultured ganglionic neurons of crab, *Carcinus maenas*. Owens and Smith (1999) cultured nerve cells of penaeus prawn having two days survival.

Hepatopancreas - Liping et al (1990) reported that from explants of hepatopancreas of shrimp, the migratory cells could be sub-cultured for 17 to 28 generations over a period of more than five months. Cancre et al (1995) modified the medium used for the cell culture of hepatopancreas and improved the viability of cells. Owens and Smith (1999) maintained hepatopancreatic cells of penaeus, prawn in culture for two days.

Gonads - Chen et al (1986) cultured gonad cells of prawn which formed a confluent monolayer over a period of 7-8 days; these cells exhibited mitosis and could be sub-cultured four times consecutively with subsequent cell degeneration. Nadala et al (1993a)

cultured ovarian cells of *Penaeus stylirostris* and *Penaeus vannamei*. Toullec et al (1996) reported the use of M199 medium to enhance cell longevity for several months. Chen and Wang (1999) maintained and sub cultured (three times) ovarian cells of penaeid shrimp. Mulford and Austin (1998) attempted testicular explant culture of prawn, *Nephrops norvegicus*. Brody and Chang (1989) reported one year survival of occasionally dividing spermatogonial cells along with the mesodermal cells, that survive for one month in the testicular cell culture of *Pacifasticus leniusculus* . Leudeman and Lightner (1992) cultured ovarian epithelioid cells that form 80 % confluence within two days, from *Penaeus stylirostris* and *Penaeus vannamei*.

Hemocytes - Moulton (1978) reported nine month survivability of hemocytes by means of capillary tube culture. The haemocytes of *Penaeus monodon* in a primary culture have been reported to survive for 8 days by Jose et al (2010) while Ellender et al (1992) reported their survival by one month. Li and Shields (2007a) reported survival of hemocytes of spiny lobster in primary culture for 18 days.

However, most crustacean cell culture studies concentrated on short culture periods and only a few attempted to raise long-lasting cultures (3–11 months) Brody and Chang (1989), Rosenthal and Diamant (1990), Hsu et al (1995a, 1995b) and Toullec et al (1996). Neither of the above attempts has successfully resulted into developing a cell line. However, among the various reports on penaeus cell cultures (Cory and Yunker, 1971., Fyhn and Costlow, 1975., Fyhn et al 1977., Quiot and Vay, 1978., Chen and Kou, 1989., Brody and Chang, 1989., Itami et al 1989., Liping, 1990., Nadala et al 1993a, 1993b., Le-Groumellec et al 1995., Cancre et al 1995., Ghosh et al 1995., Braasch et al 1999., West et al 1999., Shike et al 2000., Shimizu et al 2001., Lang et al 2002a., 2002b., 2004), only two reports show the development of a transformed cell line. Tapay et al (1995)

reported transformation of a primary culture of the lymphoid organ of *Penaeus stylirostris* to a continuous cell line. Claydon et al (2010) reported a hybridized *Penaeus monodon* cell line by cellular fusion. Hybrid cells were created using polyethylene glycol mediated fusion with two immortal cell line, Epithelioma papulosum cyprinid and *Spodoptera frugiperda* pupal ovarian cells fused with *Penaeus monodon* hemocytes.

1.3.2 Crab cell culture

Grau and Cooke (1992) cultured peptidergic neurons from the X-organ of crab, *Carsisoma carnifex* and examined the effects of various culture conditions on the survivability of neurons. Ballard et al (1993) cultured the cuticular epithelial cells of pre-exuvial integument of blue crabs (*Callinectes sapidus)* in T-C 199 medium and could maintain the cells for a period of 21 days retaining their columnar morphology. During the culture period, the cells seemed to deposit some additional exocuticle. Li and Shields (2007b) developed a medium term primary culture system for *in vitro* culture of hemocytes of the blue crab with high viability. The work of Li and Shields (2007b) gained importance as they could maintain the hemocytes of blue crab up to a period of 15 days in modified Graces' insect medium with more than 60% viability. Zeng et al (2010) reported cell cultures of hepatopancreas from *Scylla paramamosain* and these researchers designed a medium that can support culture for several passages. Recently Liang et al (2012) reported the survival of hemocytes cultures of crab (*Eriocheir sinensis*) for more than 35 days.

1.3.3 Most commonly used tissues in crustacean cell cultures

The selection of tissues for cell culture normally depends upon the physiological, ecological, economical (nutraceuticals and pharmaceuticals) histo-pathological, histochemical, and biochemical criteria (Kasornchandra et al 1999., Toullec, 1999., Uma et

al 2002). Besides, tissue selection is also based on the ability of its cells to proliferate actively. Therefore, embryonic and neonatal tissues are used since these possess totipotent cells that multiply rapidly *in vitro* (Quiot and Vey, 1978., Fan and Wang, 2002).) Many researchers chose germinal tissue, particularly gonads, as a source of cells for culture as they contain numerous mitotically active germ cells (Fyhn and Costlow, 1975., Chen et al, 1986, 1988., Chen and Kou, 1989., Leudeman and Lightner, 1992., Nadala et al 1993a, 1993b., Tong and Miao, 1996., Mulford and Austin, 1998., Itami et a1, 1999., Toullec, 1999., West et al, 1999., Chen and Wang, 1999., Owens and Smith, 1999., Shike et al, 2000., Shimizu et al, 2001., Lang et al, 2002b., Maeda et al, 2003, 2004). For virological studies only few tissues have remained attractive especially hepatopancreas, ovary and hematopoietic tissue (Chen and Kou, 1989., Uma et al 2002., Jiang et al 2006), However, for other applications such as cellular biology and endocrinology various tissues of different species have been used like testis, epidermis, Y-organ, nerve tissue (Toullec, 1999)

1.3.4 Different cell culture techniques used in crustacea

Crustacean cell cultures are initiated either by explant or dissociation technique. In Explant culture, cells migrate from the tissue fragments and proliferate. This technique is based on the idea that cell aggregates have better ability than single cell to adhere to the substratum (Yavin and Yavin, 1974). This technique was employed by many researchers (Chen et al, 1986., Chen and Kou, 1989., Leudeman and Lightner, 1992., Nadala et al 1993a., Lu et al 1995., Tapay et al 1995., Toullec et al, 1996., Tong and Miao, 1996., Mulford and Austin, 1998., Chen and Wang, 1999., Itami et al 1999., Wang et al 2000., Mulford et al 2001., Kumar et al 2001., Lang et al, 2002a., Chun et al 2003) to develop

cell cultures. For dissociation technique tissues are dissociated either by mechanical or enzymatic means.

Explant culture is most suitable for loose tissues such as hepatopancreas, ovary and testes (Mulford and Austin, 1998., Owens and Smith, 1999., Fraser and Hall, 1999) but loose tissues could also be dissociated mechanically (Toullec et al 1996). Though mechanical dissociation technique provides large number of cells it seems to reduce the ability of cells to attach and thus adhesion factor is sometimes needed to assist their attachment to the substrate (Toullec, 1999).

Mulford and Austin (1998) employed mechanical dissociation technique to isolate cells from the hepatopancreas and ovary of *Penaeus monodon*. For dissociating cells by Enzymatic method enzymes such as Trypsin, pronase, collagenase, and dispase are needed individually or in combination. This technique is suitable for compact tissues as digestion of connective tissue is required to separate cells. Trypsin and pronase seem to be too strong for crustacean tissues. Collagenase and dispase are weaker enzymes and cause less damage to the cells by being more specific to connective tissue (Toullec, 1999). Many researchers have shown the use of Trypsin and collagenase to dissociate crustacean tissues (Machii et a1, 1988., Owens and Smith, 1999., Toullec, 1999., Mulford et al, 2001., Maeda et al 2003., Chun et a1, 2003).

1.3.5 Media used in crustacean cell cultures

The choice of medium is an important factor for initiating cell culture, as each medium has different characteristics with regards to pH, salinity and osmolality. When analyzing the different studies, the choice of the culture medium appears to be more

controversial (Tolluec 1999). The nutritional requirements of crustacean cells are not understood exactly, therefore, media selection is based on presumptions (Mothersill and Austin, 2000). Most of the media used thus far are modifications of commercially available media such as M-199, Minimum essential medium (MEM), Eagle's minimal essential medium (EMEM), TC-100 insect medium, Dulbecco's Modified Eagle Medium (DMEM), CMRL, NCTC, RPMI 1640 and Leibovitz -15 (L-15). Many researchers have used Grace's insect medium for culturing crustacean cells as it was suitable for maintaining cells of other arthropods (Toullec et al 1996., Fraser and Hall, 1999). The media M199 and L-15 have consistently supported successful establishment of primary cultures from various crustacean tissues (Toullec, 1999., Wang et al 2000., Lang et al 2002a). Leibovitz's L-15 has been a popular choice for prawn and crab cultures (Roper et al 2001).

The adjustment of the media to the physio-chemical requirements of crustacean cells is also an essential factor that needs to be considered (Kerry, 2009). The main factor includes osmolality, pH and ionic strength. The osmolality for marine species are adjusted between 800 – 1000 mOsm/kg (Mothersill and Austin, 2000), while adjusting the osmolality the ionic strength should be taken in account as the use of sodium chloride alone may result in an excessive concentrations of sodium that hampers cell viability (Mothersill and Austin, 2000., Kerry, 2009). As far as pH of the media is concerned, it should be relatively alkaline at a range of pH 7.2 – 7.8. Kerry (2009) is of opinion that the culture media should be made up of a basal medium that includes essential amino acids, vitamins, species specific nutritional supplements and should have ionic strength and pH adjusted to the animals physiological needs.

1.3.6 Media supplements used

Addition of supplements to the basic culture medium is required to stimulate cultured cells to divide and proliferate. The uses of supplements are well documented but the nature and concentrations used varied among studies (Toullec, 1999). The use of FBS or FCS at concentrations of 5-20 % (v/v) as an ingredient in crustacean cell culture medium has been emphasized by some researchers (Tong and Miao, 1996., Mulford and Austin, 1998., Chen and Wang, 1999., Mulford et al 2001., Uma et al 2002). However, Leudeman (1990) and Frerichs (1996) suggested that serum could be toxic for crustacean cells. Toullec and Dauphin (1994) demonstrated that the secretory capacity of Y-organ cells of crab *Carcinus maenas* was improved by adding FBS.

Owens and Smith (1999) reported 48 hrs survival of hepatopancreatic explant culture in double strength L-15 medium with 20 % FBS. Though many researchers used FBS, some of them added hemolymph or tissue extracts. Chen et al (1986) used 30 % of muscle extract and 10 % hemolymph for *Penaeus monodon.* Rosenthal and Diamant (1990) used 5.0 % heat inactivated hemolymph in combination with more than 15 % FBS for enhancing proliferation of shrimp cells. Nadala et al (1993a, 1993b) tested different growth factors for improving culture efficiency. Thus, it is evident that the development of spontaneous cell lines from crustacean tissues would depend upon a better understanding of nature, chemistry and circulating forms of growth factors operating in marine invertebrates.

1.3.7 Antibiotics and Antimycotic treatments

One of the biggest difficulties encountered in cell cultures is microbial contamination in spite of a few successful attempts of animal cell culture without

antibiotics by Carrel and Rivers (1927). Significant advances in invertebrate cell culture were made in 1950 and 1960 largely due to availability of antibiotics (Mothersill and Austin, 2000., Kerry, 2009). Antibiotics like penicillin, streptomycin, neomycin, kanamycin, gentamicin, amphotericin B, fungizone and nystatin are used in cell culture. To deal with the mycoplasma, antibiotics like tylosin, tiamulin or ciprofloxacin are used (Schmitt et al 1988). Antibiotics are essential for long-term cell survival as bacteria, fungi and sometimes mycoplasma are found as common contaminants in cell cultures. However, increased concentrations of antibiotics pose the problem of negative impact of the antibiotics on the cultured cells (Toullec, 1999). Few researchers strongly discourage the use of antibiotics in culture (McGarrity, 1976., McGarrity et al 1985., Lincoln and Gabridge, 1998., Freshney, 2000). Contamination in cell culture can occur at any step which involves the handling or manipulation of the cultured cells or their media. The use of antibiotics can limit the contamination but may not eliminate it. Fungal and yeast contaminations are particularly difficult to control, thus aseptic primary culture techniques are imperative to reduce the chances of contamination.

1.3.8 Characterization of cell cultures

To date, characterization of crustacean cell culture is mainly based on determination of cell morphology by light microscopy (Tong and Miao, 1996., Mulford et al 2001). Mulford et al (2001) characterized the primary cell cultures of hematopoietic tissues of *Nephrops norvegicus* in terms of their morphology and proliferation. However, growth characteristics include passage level and doubling time.

Cell proliferation in culture has been detected by labelling the cells by tritiated-thymidine and BrdU as they get incorporated into the DNA during its synthesis (Gratzner, 1982). Maeda et al (2003) characterized the ovarian primary cells in terms of its

proliferation by incorporating 5-bromo-2'-deoxyuridine (BrdU) an analog of thymidine 5-bromo-2'-deoxyuridine. However, in comparison to the traditional radioactive assay and the time consuming BrdU assay, MTT (Methylthiazol tetrazolium) and XTT (2, 3-bis [2-methoxy-4-nitro-5-sulfopheny]-2H-tetrazolium-5-carboxyanilide inner salt) Tetrazolium hydroxide assays have been widely used for studying cell proliferation. Domart-Coulon et al (1994), Naganuma et al (1994) and Lebel et al (1996) employed MTT test for evaluating the viability of marine invertebrate cells.

Genetic stability of cultured cells is generally performed using molecular techniques such as Polymerase chain reaction (PCR) which includes RAPD, RFLP and Sequencing. Saiki et al (1985) and Mullis (1990) claimed that in nineteen eighty five Kary Mullis developed the Polymerase Chain Reaction technique. Within a short duration of its introduction PCR technique has been extensively used for analyzing genes. In cell culture (*in vitro*) the PCR techniques are widely employed to confirm if the cultured cells are derived form the parental tissue (that is species specific genes), to know the instability of culture and for identifying cross contamination. PCR related techniques have been extensively used to authenticate many invertebrate cell lines. PCR amplification of the mitochondrial 18S rRNA gene fragment has been used to characterize insect cell lines (Kshirsagar et al 1997)

1.3.9 Study of crustacean viral infectivity

Viruses are the most common microorganisms in the marine environment. Over the past two decades, marine virology has progressed from a curiosity to an intensely studied topic of critical importance to oceanography. Disease outbreaks due to viral infections are the major constraints to the crustacean aquaculture industries. Some crustacean viruses

such as the white spot syndrome (WSSV) (Inouye et al 1994., Inouye et al 1994., Nakano et al 1994), *Monodon baculovirus* (MBV) (Johnson and Lightner, 1988., Ramasamy et al 2000), *Baculovirus penaei* (BP) (Couch, 1974., Lightner et al 1983a), Baculoviral midgut gland necrosis virus (BMNV) (Sano et al 1981), Infection hypodermal and hemotopoietic necrosis (IHHNV) (Lightner et al 1983b), Hepatopancreatic parvovirus (HPV) (Flegel et al 1992., Lightner, 1993., Limsuwan, 1999), Yellow head disease (Boonyaratpalin et al 1993., Chantanachookin, 1993., Chen and Wang, 1999) are highly virulent and cause mass mortality to crustacean species. These viruses have a wide geographical distribution and host range.

White spot syndrome is one of the major infectious viral diseases, causing large scale mortality of commercially cultivated crustacean species; which include shrimp, prawn, crab and other related crustaceans (Nakano et al 1994., Takahashi et al 1995., Lo et al 1996., Lightner, 1996., Durand et al 1997., Flegel, 1997., Chang et al 1998., Wang et al 1998., Chen et al 2000., Shi et al 2000., Corbel et al 2001., Jiravanichpaisal et al 2001., Chakraborty et al 2002). Kanchanaphum et al (1998) have shown susceptibility of crab *Scylla serrata* to WSSV both in natural and experimental conditions. The virus has a wide host range and is highly lethal and contagious causing mass mortality of commercially important species. Outbreaks of this disease cause heavier losses to fisheries as there is no available treatments for WSSV. The appearance of disease in the farm usually requires destruction of infected stock before starting anew.

WSSV was first reported in Taiwan shrimp farm in 1991 (Chen et al 1995) and subsequently in China in 1993 (Huang et al 1995), where it led to a virtual collapse of the shrimp farming industry. This was followed by outbreaks in Japan, Thailand, India and

Malaysia in 1994 - 1995. In late 1995, the disease leapt over the Pacific Ocean and spread throughout the small-scale farms in North America and by 1996 it had severely affected East Asia and South Asia. In 1998 the disease began to ravage South America, first in Ecuador, then Peru, in 1999 Mexico and in 2000 Philippines. In less than ten years this disease appeared and spread globally, creating thus far the greatest economic damage. In India during 1994-95, white spot syndrome viral disease caused severe mortality in cultured shrimp *P. monodon* and *P. indicus* along the east coast of India. Karunasagar et al (1997) reported the disease outbreak on the west coast of India.

Previous studies indicate that *Scylla serrata* could be infected by parasites, *Vibrio* spp. and viruses (Parenrengi et al 1993., Lo et al 1996., Flegel, 1997., Kanchanaphum et al 1998., Quinitio et al 2001., Chakraborty et al 2002., Weng et al 2007., Li et al 2008). Recent research showed that WSSV was the main pathogen with high mortality to *Scylla serrata* in Zhejiang province in China (Liu et al 2011). The infection of WSSV has resulted in high mortality and economic losses in many regions of the world (Lo et al 1996., Magbanua et al 2000).

WSSV virons are ovoid to bacilliform shaped with a characteristic tail-like appendage at one end of the viron. Within these virons a rod shaped nucleocapsids with a clear striated appearance are located which are constructed out of 16 stacked rings of two parallel rows of globular subunits of about 10 nm (Wongteerasupaya et al 1995., Durand et al 1996, 1997). Each nucleocapsid contains one copy of the circular double stranded DNA genome of WSSV. Based on the morphology, genomic structure and composition and phylogenetic analysis, WSSV is a member of the genus Whispovirus within a few new

virus family called Nimaviridae, referring to the thread-like polar extension on the virus particle.

Various diagnostic methods have been developed in many different laboratories throughout the world to detect WSSV, because early monitoring of any disease is critical for disease management and control. Thus, much effort has been put into the detection of WSSV. Besides the traditional observation of gross and clinical signs and morphological pathology using light and electron microscopy, histopathology and histochemistry, a whole array of molecular technologies have been developed for the detection of WSSV.

Although, numerous studies on morphology, histopathology and gene sequence of WSSV are reported, the actual mechanism of WSSV infection is not understood (Jiang et al 2004). Though new information is available on WSSV, relatively less is known about host parasite interaction (Jiravanichpaisal et al 2006a). To understand and ultimately control the viral diseases in crustacea, a specific and sensitive diagnostic tool to investigate this pathogen need to be developed (Claydon and Owens, 2008).

Tissue culture is an important tool employed for the study of pathogenic infections, especially for those pathogens such as viruses that replicate intracellularly (Jiravanichpaisal et al 2006a). The use of cell cultures is vital not only to aid viral identification as sensitive diagnostic tool, but also for the analysis of interaction between viruses and their host cells in an effort to identify some of the mechanisms involved in the steps of viral infection. At present there is no crustacean cell line available for testing the effects of pathogens in spite of development of crustacean primary cell cultures since 1960s. Claydon and Owens (2008) reported that due to the presence of dominant

senescence genes, crustacean primary cell cultures have limited proliferation. A few primary cultures of crayfish and prawn tissues have previously been developed for the diagnosis of viruses infecting crustaceans (Uma et al 2002., Jiravanichpaisal et al 2006a., Li and Shields, 2007a) but long surviving and proliferating cell cultures were not available for evaluating viral infections.

1.3.10 Choice of test species (*Scylla serrata*) for cell culture

Among various crustacean species, *Scylla serrata* (Mud crab) was selected for the study purpose. The choice of crab *Scylla serrata* was mainly because of its growing commercial importance in many parts of the world. The wild catch of *Scylla* is estimated 10000 tons annually and contributes significantly to the costal fisheries of many developing Asian countries, like Bangladesh, India, Sri Lanka, Indonesia, Thailand, Vietnam and the Philippines. In India, mud crab, *Scylla serrata* has gained economic importance since early eighties with the commencement of live crab export to south East Asian countries. Aquaculture of the mud crab has been practiced for the past 100 years in China (Shen and Lai, 1994) and for the past 30 years throughout Asia.

The production of mud crab was 108,500 tons in China in 2004, which accounts for 88% of the total culture production of the world. Almost all crab aquaculture production relies on wild-caught stock, as larval rearing has not yet reached a commercially viable level for stocking into aquaculture farms. The farming of mud crabs *Scylla* spp. has received special interest in the past few years due to its importance as a source of high quality seafood. It is also very important to the economy of many Asian countries as it is an export commodity. In the Philippines the mud crab, *Scylla* spp has been identified as an export-winner in the country's agenda for national development. It is

believed that the improvement of the culture techniques for the mud crab will boost its production as mud crab production in many countries in Southeast Asia has not yet really been developed. Several attempts have been made in order to improve the culture techniques of the mud crab in several countries.

Though crab fishery is growing in India, it is blighted by disease outbreaks resulting from environmental and pathogenic factors. As a result of high culture densities and increasing extension of aquaculture farms, serious diseases causing large economic damages have occurred in recent years. Till now, there are no effective methods to prevent WSSV infection in crustacean culture. The study of molecular pathogenesis of WSSV would be a great benefit for the disease prevention and control. Mud crab reovirus (MCRV), associated with large economic losses was recently isolated from marine cultured mud crab, *Scylla serrata*, in southern China. The virus infects connective tissue cells of the hepatopancreas, gills and intestine in mud crab and develops in the cytoplasm.

This has resulted in growing need to develop cell culture techniques for understanding the pathogenicity. Earlier studies on crab were related to cytotoxicology and histology (Al-Mohanna and Nott, 1985, 1987). However, the review of literature clearly indicates that there is hardly anywork done on establishing a long lasting, sustainable primary culture as well as a crustacean cell line. Therefore, it was decided to develop a primary cell culture from few tissues of commercially important crab species such as *Scylla serrata.* Such cell culture system / cell lines are essential for isolation, cultivation of crustacean viruses, besides they increase reproducibility of biological investigation of pathogenesis.

1.4 Research Objectives :-

- To establish primary cell cultures from tissues such as Gills, Hepatopancreas, Hemocytes, Muscle, Heart, Brain, Ovaries and Testis.
- To identify suitable dissociation media.
- To develop suitable media composition and to identify the required growth factors.
- To identify suitable substratum.
- To characterize cultured cells.
- To assess pathogenicity of WSSV on cultured cells/ *cell* line of *Scylla serrata.*

CHAPTER - 2

Materials and Methods

CHAPTER 2

MATERIALS AND METHODS

Mud Crab (*Scylla serrata*)

Phylum	Arthropoda
Class	Crustacea
Sub-class	Malacostraca
Order	Decapoda
Family	Portunidae
Genus	*Scylla*
Species	*serrata*

Live mud crabs (*Scylla Serrata*) were obtained from local supplier. The crabs were housed in well aerated aquaria tanks (45 x 45 x 50 centimetres) having seawater (30 litres) maintained at salinity equivalent to 29 ‰ and temperature 27 to 28^{o} C. The choice of the crab size (carapace length and body weight) was based on the type of tissue selected for cell culture. For culturing cells from tissues such as hepatopancreas, heart and haemocytes, crabs of carapace length (6 - 7 cm) and weighing ~13 ± 0.60 gm were used. For testicular and ovarian cell culture crab carapace length of 8-10 cm and weight ~ 110 ± 0.60 gm was used. For Gill culture crab carapace length (4 - 5 cm), weighing (9 ± 0.60 gm) were used. Prior to the experiment the crabs were anesthetized and sterilized for 15 minutes in ice cold water containing 10 % sodium hypochlorite (to get rid of external contaminants). Further the crabs were surface sterilised with 70 % alcohol. The dissection of crabs was carried out inside the Laminar hood under sterile conditions. The dorso-branchial carapace of *Scylla serrata* was removed by splitting the dorsal seam towards the rostrum, then pulling each half of the carapace free of the thorax. The desired organs were located and aseptically removed (Figure 2.1, 2.2, 2.3, 2.4).

Figure 2.1 The dorso-branchial carapace of *Scylla serrata* removed so as to expose the organs such as hepatopancreas (H), gill (G) and heart (H)

Figure 2.2 The photograph showing the female reproductive system of *Scylla serrata*.

. O – ovary.

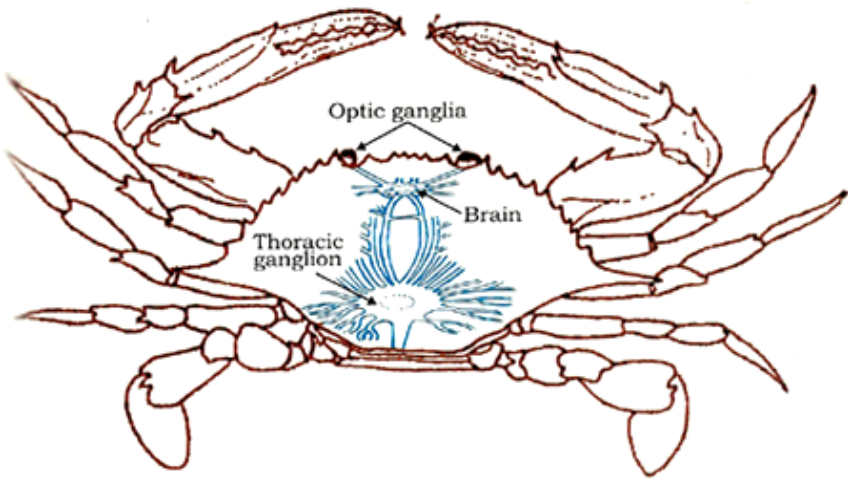


Figure 2.3 The diagram showing the neuroendocrine centre, optic ganglia, brain and thoracic ganglion of *Scylla serrata*.

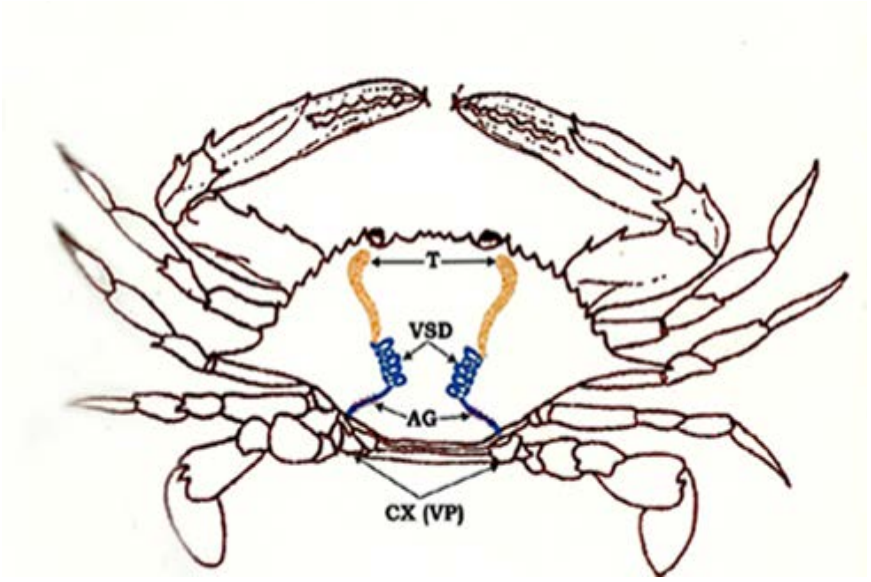


Figure 2.4 The relative position of male reproductive system of *Scylla serrata*.T : Testis, VSD-vas deference, AG-androgen gland, CX (VP)-coxopodite of Vth peripod.

2.1 PRIMARY CULTURE TECHNIQUES (Freshney, 2000, 2010., Mitsuhashi, 2002)

2.1.1 Primary culture of gill epithelial cells

Material :- Crab (*Scylla serrata*), Multi well plates (MWPs - 6 and 12 wells), Centrifuge tubes, Forceps, Scalpels, Scissors, Nylon mesh 100 µM, Syringe filter 5.0 µM, Syringe 10 ml, Cellulose acetate filter 0.45 µM, Cellulose membrane (0.22 µM pore size),

Chemicals :- Leibovitz 15 Medium (L15), Dulbecco's Modified Eagle's Medium (DMEM), Medium 199, Epidermal growth factor (EGF), Fetal bovine serum (FBS), Fetal calf serum (FCS), Potassium permanganate (0.05 %), Crab saline (NaCl - 440 mM, KCl - 11.3 mM, $CaCl_2$ - 13.3 mM, $MgCl_2$ - 26 mM, Na_2SO_4 - 23 mM, HEPES - 10 mM), Artificial sea water (ASW: NaCl - 480 mM, KCl - 10 mM, $MgSO_4.7H_2O$ - 30 mM, $MgCl_2.6H_2O$ - 20 mM, HEPES - 5.0 mM), Salt mixture (Na_2SO_4 - 8.3 mM, KCl - 10.3 mM, $MgCl_2$ - 12.9 mM, NaCl - 293 mM, $NaHCO_3$ - 4.1 mM, $CaCl_2$ - 33 mM), Trypsin 0.1 % in ASW, 1.0 N HCl, 1.0 N NaOH, Phosphate buffer saline (PBS), Antibiotics mixture (L- Glutamine – Penicillin-streptomycin solution (0.1 µl/ml)– PCN: A007, Antimycotic (0.1 µl/ml) – PCN: A002A + Gentamicin (0.1 µl/ml) – PCN: A005 + Amphotericin B (0.1 µl/ml) – PCN: A011, Himedia, Pune, India), Collagen, Laminin, Poly-L-Lysine, Trypan blue dye.

Protocol :-

2.1.1.a Media preparation :- Media such as Leibovitz 15 (Himedia, PCN: AT011), Dulbecco's Modified Eagle's Medium (Himedia, PCN: AT127), Minimum Essential

Medium (Himedia, PCN: AC003), Medium 199 (Himedia, PCN: AT042), were reconstituted using either Milli Q water or artificial sea water as shown in (Table 2.1). The media were stirred thoroughly until the powder dissolved. Once, the powder dissolved the required osmolality of the media was achieved either by adding sea water/ crab saline to increase the osmolality or Milli Q water to decrease the osmolality with the help of Osmometer (Model 3320). The pH was adjusted to 7.3 by adding required quantity of 1.0 N HCl or 1.0 N NaOH. All media were vacuum filtered through cellulose membrane having pore size 0.22 µM.

2.1.1.b Preparation of Media Supplement :-

Reconstitution of Epidermal growth factor (Sigma, PCN-E4127) :- EGF stock solution was prepared by mixing 1.0 mg of EGF with 5.0 ml of sterile crab saline. Further 0.01 ml of stock solution was diluted to 5.0 ml (v/v) with crab saline to achieve a sub stock solution having a strength equivalent to 400 ng. The sub stock solution (0.025 and 0.05 ml) of EGF was further diluted with crab saline to obtain concentrations equal to 10 ng/ml and 20 ng/ml.

2.1.1.c Coating of multi-well plates (MWP) with attachment factors

2.1.1.c.i Poly-L-lysine (Sigma, PCN-P7280) :-

- Poly-L-lysine powder (5.0 mg) was reconstituted in 50 ml of sterile Milli Q water.
- Using a 10 ml syringe, 1.0 ml of Poly-L-lysine was aseptically aspirated and added to each well of MWPs. The plates were gently shaken to ensure even coating of the well surface with Poly-L-lysine.

- After 5 minutes, the Poly-L-lysine solution was aspirated and the well surfaces were rinsed with sterile Milli Q water. The coated wells were allowed to dry for two hours before introducing the medium and cells.

2.1.1.c.ii Laminin (Sigma, PCN-L2020) :-

- Laminin stock solution was prepared by dissolving 1.0 mg in 1.0 ml of sterile Milli Q water and transferred to deep freezer.
- The stock laminin was slowly thawed at 2-8^{o} C prior to the use to avoid the formation of a gel.
- It was further diluted with sterile Milli Q water to a concentration of 2.0 µg/ml. Diluted laminin (1.0 ml) was added to the multi-well plates and then the plates were left undisturbed for two hours under the laminar flow hood. Subsequently, the plates were rinsed thrice with PBS and then air dried for at least 45 minutes in the Laminar flow hood before inoculating cells with the medium.

2.1.1.c.iii Collagen (Sigma, PCN-C7661) :-

- Collagen stock solution was prepared by dissolving 10.0 mg (w/v) in 10 ml of 0.1 M acetic acid. The mixture was stirred for 1 to 3 hours until dissolved at room temperature.
- The collagen solution was then filtered through a 5.0 µm syringe filter, followed by filtration through a 0. 45 µm cellulose acetate filter.
- Working solution was prepared by diluting collagen stock to obtain collagenase strength equal to 0.1µg /ml with MQ water.
- In each well of MWPs, 1.0 ml of diluted collagen was added and the plates were left in the Laminar flow hood for 12-18 hrs under UV light.

- The left over collagen solutions from the wells were aspirated and the wells were allowed to air-dry inside the hood. After drying the wells of MWPs, they were rinsed with sterile Milli Q water.

2.1.1.d Explant Culture :- The technique involves aseptic removal of the tissues and subsequent inoculation of tissue fragments in MWPs containing the select culture media.

a. The crab was surface sterilised as mentioned previously and aseptically dissected to extirpate the gills.
b. The gills were immersed in potassium permanganate solution (0.05 %) for a period of 2 minutes and then washed thrice in artificial sea water (osmolality 1050 mOsm/kg) containing 2.0 % antibiotic solution.
c. The gills were cut into tiny fragments of 1.0 mm^3 and the fragments were transferred to multi well plates coated earlier with previously mentioned three different attachment factors (protocol 2.1.1.c).
d. To each well of MWPs, 2.0 ml of plating media as shown in Table (2.1) were added followed by three to four gill fragments. The plates were sealed with parafilm, labelled and observed under microscope. Subsequently these MWPs were incubated at 20 – 24^o C until the migration of cells or confluent monolayer was formed. Then, the tissue fragments from the wells were removed and the initial plating medium was replenished.

2.1.1.e Culture of enzymatically dissociated cells :-

a. Aseptically excised gills were transferred to a sterile Petri dish containing artificial seawater (Osmolality 1050 mOsm/kg) having 2.0 % antibiotics.

b. The gills were then immersed in $KMnO_4$ solution (0.05 %) for 3 minutes and later rinsed with crab saline.

c. Gills were cut into ~ 2.0 mm fragments using a sterile surgical scalpel and again rinsed in artificial sea water having antibiotics.

d. Gill pieces of ~ 2.0 mm were transferred to a flask containing 0.1 % Trypsin prepared in artificial sea water. (Table 2.4)

e. The flask was manually stirred for 10 minutes at room temperature till the solution turned cloudy indicating formation of cell suspension.

f. The suspension thus obtained was filtered through a 100 µm nylon mesh and the filtrate was centrifuged at 1000 rpm for 3 minutes.

g. The supernatant was discarded and the cell pellet was rinsed with appropriate culture media (Table 2.2) containing 5.0 % fetal bovine serum.

h. The pellet was dispersed in culture media (Table 2.2) and the cells were assessed for cell viability by Trypan blue dye exclusion technique.

i. The viable cells at a density of 10^5 cells/ml were inoculated in the wells of MWPs and incubated at 20 – 24^o C. The media were replenished at two days interval.

j. The cultured gill cells were examined daily with an Olympus inverted microscope to observe growth and proliferation.

2.1.1.f Cell viability (Mc Limans et at 1957) and cell density count :-

a. Cell viability was determined by Trypan blue dye exclusion technique.

b. To one millilitre of cell suspension equal volume of 0.2 % Trypan blue dye was added and mixed properly.

c. Ten microliter of the above mixture was placed on the counting chamber of the hemocytometer, and a coverslip was placed on it without intervention of any air bubble.

d. The cells were allowed to settle for a period of 2 to 3 minutes and then examined under Olympus inverted microscope.

e. Trypan blue stained the dead cells, while the live cells excluded dye thereby allowing visual determination of living versus dead cells.

f. The procedure from cell loading to cell counting was repeated at least three times. The average number of cells/ mm^2 (M) was calculated as per the formula below.

Calculation (Mitsuhashi, 2002)

The cell density (number of cells/ ml) is calculated by the formula

M x P x 20,000

M = Average number of cells / mm^2

P = Dilution factor

2.1.2 Primary culture of hepatopancreas

Materials :- Crab (*Scylla serrata*), MWPs (6 and 12 wells), Nylon mesh (100 µM pore size), Scalpels, Forceps, Centrifuge tubes.

Chemicals :- Leibovitz 15 medium, Dulbecco's Modified Eagle's Medium, Medium 199, Epidermal growth factor, Fetal bovine serum, Fetal calf serum, Potassium permanganate (0.05 %), Artificial sea water, Crab saline, Citrate buffer (Citrate buffer - 27 mM, NaCl - 440 mM, KCl - 3.0 mM, NaH_2PO_4 - 5 mM, Na_2SO_4 -5.6 mM, EDTA - 10 mM, Poly vinyl

pyrrolidone - 0.25 mM), Antibiotics mixture (prepared as mentioned in protocol 2.1.1 under sub-heading chemical), Collagen, Laminin, Poly-L-Lysine.

Protocol :-

2.1.2.a Explant culture :-

a. The crab carapace was dissected open to expose the thoracic region having hepatopancreas arranged symmetrically on both the sides of the stomach (Figure 2.1).

b. The hepatopancreas was removed aseptically and transferred to a petri-dish containing artificial seawater and 2.0 % antibiotic solution (osmolality 1050 mOsm/kg). Subsequently the hepatopancreas was immersed in 0.05 % $KMnO_4$ for 3 minutes and then rinsed in ASW.

c. The hepatopancreas was cut into fragments of 1.0 mm^3. Three to four such tiny fragments per well were transferred to multiwell plates (MWPs) coated with three different attachment factors such as laminin, Poly-L-lysine and collagen (protocol 2.1.1.c).

d. To each well of MWPs, 2.0 ml of plating media as shown in Table (2.2) were added.. The MWPs were incubated at 20 – 24^o C for two days. After achieving the cell migration tissue fragments were removed aseptically and the media were replenished.

2.1.2.b Culture of disaggregated / dissociated cells :-

a. The hepatopancreas was separated with the use of sterile scalpel and forceps and transferred to a sterile centrifuge tube containing crab saline.

b. The tissue was rinsed thrice in sterile crab saline, and then was cut aseptically into 1.0 mm^3 fragments and transferred to 15 ml sterile centrifuge tube containing sterile citrate buffer, (pH 7.4).

c. The fragments of hepatopancreas were triturated by several passages through 10 ml sterile pipette to obtain cell suspension.

d. The suspension thus obtained was filtered through a 100 µm nylon mesh and spun for 3 minutes at 1000 rpm.

e. The resulting pellet had two strata one at the bottom was of white cell fraction and the other on top was yellow cell fraction. The two cell fractions were carefully aspirated separately into centrifuge tubes using Pasteur pipette and were washed twice with culture media.

f. Each cell fraction was assessed for cell viability by Trypan blue dye exclusion technique (2.1.1.f) and cultured separately in optimized media at a density of ~3 x 10^3 cells/ml.

g. All the cultures were incubated at 20 – 24^o C and the media was replenished at two days interval.

h. Cultured cells were examined daily with an Olympus inverted microscope to observe cell growth and proliferation.

2.1.3. Primary culture of hemocytes

Materials :- Crab (*Scylla serrata*), MWPs (6 and 12 wells), 27-gauge syringe, Nylon mesh (100 µM), Centrifuge tubes.

Chemicals :- Leibovitz 15 Medium, Dulbecco's Modified Eagle's Medium, Medium 199, Epidermal growth factor, Anticoagulant (Tris - HCl - 18 mM, EDTA - 50 mM, NaCl - 32.4

g/l, KCl - 1.0 g/l, and glucose - 0.3 g/l; pH 7.5), Potassium permanganate (0.05 %), Antibiotics mixture (prepared as mentioned in protocol 2.1.1. under sub-heading chemical), Collagen, Laminin, Poly-L-Lysine.

2.1.3.a Hemocyte culture :-

a. Hemolymph from sterile crabs' heart was obtained by introducing a 27-gauge needle of a syringe into it from the junction between the basis and ischium of the fifth walking leg.
b. In order to prevent the coagulation of hemolymph (0.5 ml) an equal volume of sterile anticoagulant was added.
c. The hemolymph was then centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the pellet was dispensed in appropriate culture media as referred in Table 2.2.
d. The cell viability and cell density counts were determined as mentioned in protocol (2.1.1.f). The cells were inoculated in MWPs at a density of 2×10^5 cells /ml/well.
e. The culture plates were incubated at $20 - 24^0$ C and observed daily under inverted microscope for cell proliferation.

2.1.4 Primary culture of heart cells (Cardiomyocytes)

Material :- Crab (*Scylla serrata*), MWPs (6 and 12 wells), Scalpels, Scissor, Forceps, Centrifuge tubes, Nylon mesh (100 µM pore size).

Chemicals :- Leibovitz 15 Medium, Dulbecco's Modified Eagle's Medium, Medium 199, FBS, Collagenase (Sigma, PCN: 27678), Potassium permanganate (0.05 %), Fibroblast growth factor (FBS), Antibiotics mixture (prepared as mentioned in protocol 2.1.1. under sub-heading chemical), Collagen, Laminin, Poly-L-Lysine.

Protocol :-

2.1.4.a Preparation of Media Supplement :-

Reconstitution of Fibroblast growth factor (FGF – Sigma, PCN: F3133) :- One milligram of fibroblast growth factor was dissolved in 4.0 ml of crab saline containing 1.0 % FBS and labelled as stock. This stock was reconstituted to FGF concentration equal to 200 ng by diluting 0.1 ml of it to 5.0 ml (v/v) with crab saline. The reconstituted stock was further serially diluted to give FGF solutions of a strength ranging from 5.0 - 20 ng/ml using desired culture media.

2.1.4.b Explant culture :-

a. The crabs (N=5) were dissected open aseptically so as to expose the heart, located in the centre of the crab between the two sets of gills (Figure 2.1). The heart of each crab was removed and transferred to ASW containing 2.0 % antibiotics.
b. Each dissected heart was immersed in potassium permanganate solution (0.05 %) for a period of 2 minutes and then washed thrice in artificial sea water (osmolality 1050 mOsm/kg) containing 2.0 % antibiotic solution
c. The heart was cut into fragments of 1.0 mm^3 and then the fragments were transferred to previously coated multi well plates as mentioned in protocol

2.1.1.c. Plating media (2.0 ml/ well) as shown in Table 2.2 were added in each well of the MWPs.

d. The plates were incubated at 20 – 24^{o} C until confluent monolayer was formed. The medium was replenished as and when required.

2.1.4.c Culture of enzymatically dissociated cells :-

a. The heart was aseptically removed and placed in a mixture of ASW and 2.0 % antibiotics. Subsequently, it was immersed in potassium permanganate solution (0.05 %) for 2 minutes, and then rinsed with ASW.

b. Further it was cut into tiny pieces (0.5 mm^{3}) which were transferred to a collagenase solution (0.3 mg/ml) prepared in ASW (Table 2.5) and incubated at 25-27^{0} C for 10 minutes with low agitation.

c. The cell suspension was carefully removed without disturbing the tissue pieces by a Pasteur pipette and transferred to a centrifuge tube. The remaining tissue pieces in the flask were further incubated for 5 minutes in collagenase solution and aforementioned steps were repeated till the tiny tissue pieces disappear.

d. The cell suspension was centrifuged at 1000 rpm for 3 minutes and the supernatant was discarded. The cell pellet was collected and bathed with ASW having 10 % FBS for 5 minutes.

e. The cell pellet was rinsed thrice and then re-suspended in media as presented in the Table 2.2.

f. Cell viability (Trypan blue method) and cell density counts (with hemocytometer) were made as described earlier in protocol (2.1.1.f) before cell inoculation. The cells were inoculated at a density of 10^{6} cells/ml and were incubated at 20 – 24^{o} C.

2.1.5 Primary culture of muscle fibroblasts

Materials :- Crab (*Scylla serrata*),), MWPs (6 and 12 wells), Scalpels, Forceps, Scissor, Centrifuge tubes, Nylon mesh (100 µM pore size).

Chemicals :- Leibovitz 15 Medium, Dulbecco's Modified Eagle's Medium, Medium 199, Fibroblast growth factor, Potassium permanganate (0.05 %), Antibiotics mixture (prepared as mentioned in protocol 2.1.1. under sub-heading chemical), Collagen, Laminin, Poly-L-Lysine.

Protocol :-

2.1.5.a Explant culture :-

a. The leg muscles from ischiopodites of *Scylla serrata* were aseptically removed and rinsed in ASW having 0.02 % antibiotics.
b. The muscles were chopped into 1.0 mm^3 pieces and were plated aseptically in MWPs coated with laminin / poly-L-lysine / collagen.
c. Two millilitre plating media per well were added to MWPs having muscle pieces referred above. These MWPs were incubated at 20 – 24^o C.
d. The MWPs were observed regularly under Olympus inverted microscope for detecting cell migration and proliferation..

2.1.5.b Culture of enzymatically dissociated cells :-

a. The muscles were removed as mentioned at 2.1.5.a.(a).
b. The muscles were cut into fine pieces (1.0 mm^3) and treated with collagenase (0.3 mg/ml) constituted with 3 x L15 at 25^0 C for 10 minutes with intermittent shaking.

c. The resulting cell suspension was filtered through nylon mesh (100 µM) and filtrate was centrifuged at 1000 rpm for 3 minutes. The supernatant was discarded and the pellet was dispensed in 3 x L15 having 5.0 % FBS for 5 minutes. Once again the dispensed cells were centrifuged at 1000 rpm for 3 minutes.
d. Cell viability test and cell density counts were done prior to cell inoculation.
e. The cells were inoculated at a density of 2 x 10^5 cell/ml and were observed daily under Olympus inverted microscope.

2.1.6 Primary culture of neurons

Material :- Crab (*Scylla serrata*), Forceps, Nylon mesh (100 µM pore size), MWPs (6 well and 12 wells), Scalpels, Centrifuge tubes.

Chemicals :- Leibovitz 15 Medium, Dulbecco's Modified Eagle's Medium, Medium 199, Nerve growth factor (NGF), Potassium permanganate (0.05 %), Antibiotics mixture (prepared as mentioned in protocol 2.1.1. under sub-heading chemical), Collagen, Laminin, Poly-L-Lysine.

Protocol :-

2.1.6.a Preparation of Media Supplement :-

Reconstitution of Nerve growth factor (NGF – Sigma PCN: N8133) :- One microgram of NGF was dissolved in 2.0 ml of crab saline containing 1.0 % serum and labelled as stock. The stock was further diluted before use to the final working concentration of 5.0 – 10 ng/ml.

2.1.6.b Explant culture :-

a. Cerebral ganglia of crabs (N=5) were aseptically removed and transferred for 5 minutes to ASW + antibiotics (2.0 %) mixture. Then they were bathed in 0.05 % $KMnO_4$ for 3 minutes followed by three rinses of ASW.
b. Desheathed ganglia were planted in MWPs previously coated with Poly-L-Lysine and loaded with appropriate culture media as referred in Table 2.2.
c. The MWPs were incubated at 20 – 24^{o} C for neuronal migration.

2.1.6.c Culture of enzymatically dissociated neurons :-

a. Cerebral ganglia of crabs (N=5) were aseptically removed and processed as mentioned at protocol 2.1.6 b.(a)
b. The desheathed ganglia were exposed to Trypsin (1.0 mg/ml) constituted with 3 x L15 solution set at 25 - 26^{0} C for 10 minutes on a magnetic stirrer set at 50 strokes per minute.
c. The cell suspension was collected in sterile centrifuge tubes and spun at 1000 rpm for 3 minutes. The pellet was rinsed with culture media containing 10 % FBS for 5 minutes to neutralise Trypsin action.
d. The neuronal viability and density counts were conducted as mentioned in protocol (2.1.1.f) prior to cell inoculation. The dissociated neuronal cells were inoculated at a density of 2 x 10^5 cells /ml.
e. The MWPs were incubated at 20 – 24^{o} C.
f. The media were replenished at two days interval and the cells were monitored daily under Olympus inverted microscope for detecting growth and proliferation and contamination if any.

2.1.7 Primary culture of Ovarian cells

Materials :- Crab (*Scylla serrata*),), MWPs (6 and 12 wells), Scalpels, Forceps, Nylon mesh (100 µM pore size), Centrifuge tubes.

Chemicals :- Leibovitz 15 Medium, Dulbecco's Modified Eagle's Medium, Medium 199, Epidermal growth factor, Potassium permanganate (0.05 %), Antibiotics mixture (prepared as mentioned in protocol 2.1.1. under sub-heading chemical), Collagen, Laminin, Poly-L-Lysine.

Protocol :-

2.1.7.a Explant culture :-

a. The ovary was removed aseptically and transferred to a mixture of ASW and 3.0 % antibiotics for 15 minutes (Figure 2.2). The osmolality of the mixture was maintained at 1050 mOsm/kg.
b. Subsequently the ovaries were cut into fragments of 1.0 mm^3. Three to four such tiny fragments per well were transferred to MWPs coated previously with attachment factors such as laminin, poly-L-lysine, and collagen as described earlier. Different media (Table 2.2) were added to MWPs containing tissue fragments.
c. The MWPs containing tissue fragments were incubated at 20 – 24^o C until cell migration leading to confluent monolayer was formed. Then, the tissue fragments from MWPs were removed followed by replenishment of the initial plating medium. The MWPs were examined daily under Olympus inverted microscope for cell growth and proliferation and contamination if any.

2.1.7.b Culture of disaggregated / dissociated cells :-

a. The ovaries of crabs were removed aseptically and sterilized as mentioned previously in protocol 2.1.7.a.(a).
b. These tissues were cut into tiny fragments and transferred to a 50 ml centrifuge tube containing artificial sea water (osmolality 1050 mOsm/kg). The tissues were triturated by allowing fifteen passages through a 10 ml pipette to obtain a cloudy cell suspension. This cell suspension was filtered through nylon mesh having a pore size of 100 µM. The filtrate was centrifuged at 1000 rpm to obtain cell pellet.
c. The cell pellet was suspended in desired culture media (Table 2.2)
d. Cell viability and density were assayed before plating the cells at a density of 10^5 cells/ ml in each well of MWPs containing appropriate media as shown in Table (2.2).
e. All the plated MWPs were incubated at 20 – 24^o C and the media were replenished at three days interval.

2.1.8 Primary culture of testicular cells

Material :- Crab (*Scylla serrata*), Forceps, Nylon mesh (100 µM pore size), MWPs (6 and 12 wells), Scalpels, Scissors, Centrifuge tubes.

Chemicals :- Leibovitz 15 Medium, Dulbecco's Modified Eagle's Medium, Medium 199, Epidermal growth factor, FBS, FCS, Accutase (Sigma A6964), Potassium permanganate (0.05 %), Antibiotics mixture (prepared as mentioned in protocol 2.1.1. under sub-heading chemical), Collagen, Laminin, Poly-L-Lysine.

Protocol :-

2.1.8.a Explant culture :-

a. The testes were aseptically removed and transferred to a Petri dish containing ASW + 2.0 % antibiotic solution (osmolality 1050 mOsm/kg).

b. Subsequently the testes were cut into fragments of 1.0 mm^3. Three to four such fragments per well were transferred to MWPs coated with three different attachment factors (laminin, poly-L-lysine and collagen). The plating media (2.0 ml/well) were added to the MWPs (Table 2.2).

c. The plates were then incubated at 20 – 24^o C for twenty four hours. Then, the tissue fragments from the wells were removed after the cell migration and the initial plating media were replenished.

2.1.8.b Culture of disaggregated / dissociated cells :-

a. Mechanical dissociation technique was employed to isolate testicular cells. Prior to dissociation the testicular tissues were immersed in 0.05 % potassium permanganate solution for 3 minutes and subsequently rinsed thrice with ASW.

b. The testes were cut into tiny pieces of about 1.0 mm^3. These tiny pieces were processed further as described in protocol 2.1.7.b (b -e).

c. The cells were sub cultured after five to six days of initiation of cell culture.

2.1.8.c Sub Culture technique :-

a. The primary cultured cells were viewed using inverted microscope to assess the proliferation and confluence formation.

b. The cells that formed confluence were separated by 30 minutes accutase (1.0 %) treatment.
c. The cells were then dispersed by slow pipetting and the resulting cell suspension was centrifuged at 1000 rpm for 3 minutes and then re-inoculated at a density of 2 x 10^5 cells /ml in appropriate culture media standardised for sub culture.

2.1.9 Development of Testicular cell line (Freshney, 2010)

Material :- Forceps, Nylon mesh (100 µM pore size), MWPs (6 and 12 wells), Scalpels, Scissors, Centrifuge tubes.

Chemicals :- The efficacy of various culture media for cell viability, growth and proliferation is tabulated in Table 2.2. The media supplements such as glucose (1g/l), epidermal growth factors (10 – 20 ng/ml), insulin (0.06 IU/ml), transferrin (5.0 µM), foetal bovine serum (Himedia 07-359), horse serum (HS - Himedia PCN: 07-381), heat inactivated sera (30 minutes at 56^o C) of different concentrations (v/v – 5.0 %, 10 %, 20 %) were used as supplements. The osmolality of the media was adjusted to 1050 mOsm/kg using artificial sea water and Osmometer.

Protocol :-

a. The primary cell culture of testes was established as described in protocol 2.1.8.b (a-c).
b. The first sub culture from the primary cell culture was attempted after four days of initiation of culture. The sub culture was performed as given in protocol 2.1.8.3.c.

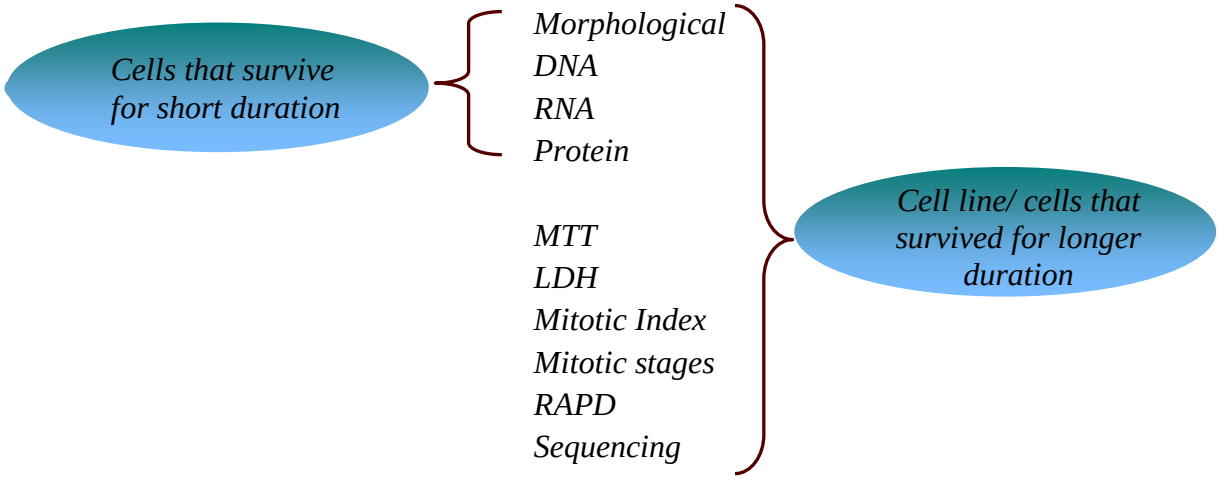
c. The sub culture was allowed to grow and proliferate in different media combination along with different supplements.

d. Subsequently, sub cultures were made after an interval of six days from each sub culture using a cell density equal to 10^5 cells/ ml.

e. The sub cultures were examined daily with an Olympus inverted microscope to observe growth, proliferation and contamination if any.

2.2 CHARACTERIZATION OF CULURED CELLS

Diagrammatic representation of cell characterization based on cell survivability/ longevity.



2.2.1 Morphological characterization :-

Cells were characterised morphologically in terms of their structures, shapes, sizes and colours. The following protocol was employed for slide preparation.

2.2.1.a Preparation of fixative, stains and mountant :-

a. Fixation: Fixation buffer as supplied by Sigma (PCN: F1797) containing formaldehyde - 29 % with glutaraldehyde - 2 %, Na_2HPO_4 -70.4 mM, KH_2PO_4 - 14.7 mM, NaCl - 1.37 M and KCL - 26.8 mM was used.
b. Acetocarmine stain: To a boiling acetic acid (45 %) 0.5 g of Carmine powder was added and further boiled for 15 - 20 minutes. The resulting solution was cooled, filtered and stored at 30 - 32^0 C.
c. Giemsa stain: Giemsa powder (3.8 gm) was dissolved in 250 ml of methanol kept stirring at ~ 60^0 C and to this 250 ml of glycerol was slowly added. The above mixture was filtered and stored at 30 - 32^0 C. The working solution was prepared by mixing 10 ml of above Giemsa mixture with 80 ml of distilled water.
d. Mountant: Gelatin powder (10 gm) was dissolved in 60 ml of boiling distilled water to which 70 ml of Glycerol and 0.25 g of phenol were added. This mixture was stored in a refrigerator after cooling.

2.2.1.b Preparation of microscopy slides :-

a. The coverslips were placed in a Teflon rack containing 65 % of nitric acid and incubated overnight on a shaker. Then the coverslips were washed several times with distilled water until pH was neutralised.
b. Further the coverslips were transferred to the Laminar flow hood for drying and sterilised under UV light.
c. Such coverslips were used for cell culture by placing them in Petri dishes after pre-coating with appropriate attachment matrices (substrate) mentioned earlier in protocol 2.1.1.c.

d. The cells were plated on the coverslips placed in sterile Petri dishes at a density of 10^6 cells/ml/well.

e. After the desired culture period (24 and 48 hr) the cells were exposed to 0.025 % colchicine for 4 - 5 hrs. These cells were then fixed using fixative buffer for 20 minutes. The fixed cells were carefully rinsed with ASW, and later transferred to another Petri dish for staining.

f. Acetocarmine staining – the cells anchored to the cover slips were exposed to acetocarmine stain for a period of 15 minutes, and then rinsed carefully with distilled water. The coverslips containing the stained cells were mounted on the mountant without any intervention of air bubbles. The slides thus prepared were observed under Olympus microscope and photographed.

g. Giemsa staining – The cells cultured on the coverslips and processed as mentioned above at 2.2.1.b.(e) were exposed to diluted Giemsa stain for 5 minutes and were subsequently rinsed with distilled water. The stained cells were mounted and observed as mentioned earlier.

2.2.2 Molecular Characterization :-

2.2.2.a DNA Extraction (Chomczynski et at 1997) :- The genomic DNA of parental tissue and cultured cells was extracted by using DNAzol® reagent kit (DN 127- Molecular Research Centre, Inc. Ohio) following the manufacturer's recommendation.

Cell lysis: DNAzol (1.0 ml) was added to a test tube containing 10^7 cells. This mixture was repeatedly pipetted for cell disruption. The disrupted cells were kept undisturbed for 10 minutes at 30-32^0 C.

Centrifugation: After 10 minutes, the disrupted cells - DNAzol mixture was centrifuged at 10,000 g for 10 minutes (at 4 - 5^0 C). Following centrifugation, the resulting viscous supernatant (lysate) was transferred to a fresh tube.

DNA precipitation: The DNA was precipitated from the above lysate by adding 0.5 ml of absolute ethanol per millilitre of the DNAzol used for the isolation. DNA was visible as cloudy precipitate. The precipitated DNA was sedimented by centrifugation at 5,000 g for 5 minutes at 4^0 C.

DNA Wash: The DNA precipitate thus obtained was collected in a centrifuge tube and washed twice with 1.0 ml of 75 % ethanol by inverting the tube 3 - 6 times during each wash. The DNA ethanol was centrifuged at 1000 rpm for 5 minutes and the supernatant was removed by pipetting leaving the DNA sediment.

DNA Solubilization: In the DNA sediment, 8.0 ml of NaOH was added and the mixture was lightly vortexed till DNA dissolved. The presence of DNA in the solution was ascertained by recording the absorbance at 260 nm wavelength using NanoDrop (ND-1000) spectrophotometer. The dissolved DNA was stored at - 20^o C.

2.2.2.b DNA estimation (Diphenylamine reaction – Plummer, 1979)

DNA reacts with diphenylamine under acidic condition to give a blue colour compound with sharp absorption maxima at 595 nm. In acidic solution, the straight chain of deoxypentose is converted to the highly reactive ß hydroxylevulinaldehyde which reacts with diphenylamine giving a blue complex. In DNA, only the deoxyribose of the purine

nucleotides reacts, so that the value obtained represents half of the total deoxyribose present.

Materials :- Buffer saline (Nacl 0.15 mol/l, sodium citrate 0.015 mol/l, pH 7.0), DNA (0.2 mg/ml buffer saline), Freshly prepared Diphenylamine reagent (10 g of pure diphenylamine dissolved in l.0 ml of glacial acetic acid + 25 ml of concentrated sulphuric acid).

Protocol :-

a. Diphenylamine reagent (4.0 ml) was added to DNA samples (2.0 ml) extracted from the cultured cell, and kept in a boiling water bath for 10 minutes, cooled and the optical density was read at 595 nm using NanoDrop (ND-1000) spectrophotometer.
b. Samples of varying concentrations of commercial DNA (0.02 to 0.14 mg/2.0 ml of buffer) were prepared from the stock (1.0 mg of DNA in 5.0 ml of buffered saline).
c. To each sample 4.0 ml of diphenylamine reagent was added and further processed as described in the protocol at 2.2.2.b.(a) The optical density (O.D) values thus obtained were used for plotting a standard graph in Microsoft excels. The following formula was used to find the unknown concentration.

$$X = Y/M.$$

X = concentration of unknown

Y = OD of the unknown

M = Y value in the standard graph

2.2.2.c RNA extraction (Chomczynski and Sacchi, 1987)

RNA was extracted from the parental tissue and cultured cells using EZ-RNA kit (PCN: 20-400-100- Life technologies (India) Pvt Ltd) following the manufacturer's recommendations.

Materials :- Denaturing solution (PCN: 20-400-100A – contains guanidine thiocyanate), Extraction and phase separation solution (PCN: 20-400-100B – contains phenol and chloroform), Isopropanol, Ethanol (75 %).

Protocol :-

Cells: Cells grown as monolayer were lysed directly in a culture dish using 0.5 ml denaturing solution per ten square centimetre area of a culture dish. The cell lysate was collected by a Pasteur pipette and transferred to a centrifuge tube and vortexed for three minutes.

Phase separation: To a vortexed cell lysate kept at 29^0 C for 5 minutes, 0.5 ml of RNA extraction reagent was added and further vortexed for 15 minutes. The vortexed mixture was kept undisturbed for 10 minutes and then centrifuged at 4^0 C for 15 minutes at 12,000 g so as to obtain the lower viscous and upper aqueous transparent phase (UATP).

RNA precipitation: The UATP was collected in a centrifuge tube and 0.5 ml of isopropanol was added. This mixture was kept overnight at 4^0 C for precipitating RNA. The precipitate was cold (4^0 C) centrifuged for 8 minutes at 12,000 g to obtain RNA sediment.

RNA Wash: To the RNA sediment 1.0 ml ethanol (75 %) was added and the RNA ethanol mixture was vortexed followed by cold centrifugation at 7500 g for 5 minutes. The RNA precipitate was stored at 4^{o} C in ethanol (75 %) until used.

2.2.2.d RNA estimation (Orcinol reaction – Plummer, 1979)

Pentose reacts with concentrated hydrochloric acid to form furfural. Orcinol reacts with the furfural in the presence of ferric chloride as a catalyst to give a green colour compound with absorption at 665 nm. Only the purine nucleotides give significant reaction.

Material :- Buffer saline (Nacl 0.15 mol/l; sodium citrate 0.015 mol/l, pH 7.0), RNA solution (0.2 mg/ml buffer), Orcinol reagent [0.1 g of ferric chloride dissolved in 100 ml of concentrated HCl + 3.5 ml of ethanolic Orcinol (0.6 g Orcinol dissolved in 10 ml ethanol) added while heating in boiling water bath].

Protocol :-

a. Orcinol reagent (3.0 ml) was added to RNA sample (2.0 ml) extracted from the cultured cells. This mixture was heated in a boiling water bath for 20 minutes, cooled and optical density was read at 665 nm using NanoDrop (ND-1000) spectrophotometer.
b. Samples of varying concentrations of commercial RNA (0.02 to 0.14 mg/ 2.0 ml of buffer) were prepared from the stock (1.0 mg of RNA in 5.0 ml of buffered saline). Orcinol reagent (3.0 ml) was added to the samples (2.0 ml) and the mixture was processed further as described at 2.2.2.d.(a). The optical density (O.D) values thus

obtained were used for calculating RNA concentrations of unknown samples following the protocol given at 2.2.2.b.(b).

2.2.2.e Protein Assay (Lowry, 1951)

Protein reacts with the Folin-Ciocalteu phenol reagent to give a coloured complex. The colour formed is due to the reaction of the alkaline copper with the protein and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein. The intensity of colour depends on the amount of aromatic amino acids present in the protein.

Materials :-

d. Alkaline sodium carbonate solution (20 g/l Na2CO3 in 0.1 mol/1 Na, K tartrate).
e. Copper sulphate (5.0 g/l CuSO4.5H2O in 10 g/l Na, K tartrate).
f. Alkaline solution was prepared on the day of use by mixing 50 ml of alkaline sodium carbonate and 1.0 ml of copper sulphate.
g. Folin–Ciocalteu phenol reagent (Folin reagent was diluted with equal volume of Milli Q water on the day of use.)
h. Standard protein (0.2 mg Bovine serum albumin per millilitre of Milli Q water).

Protocol :-

a. Alkaline solution (5.0 ml) was added to sample solution (0.1 ml + 0.9 ml Milli Q water), mixed and allowed to stand at room temperature for 10 minutes. Subsequently diluted Folin-Ciocalteu reagent (0.5 ml) was added while shaking.
b. The above mixture was incubated for 30 minutes at 27^0 C and extinction was read against the appropriate blank at 750 nm.

c. The protein concentrations of samples were measured using a protein standard curve. For standard curve bovine serum albumin concentrations in the range of 0.02 - 0.14 mg/ml were used. The calculations were done using the method described at protocol 2.2.2.b.(b).

2.3 TESTICULAR CELL LINE

The primary cell cultures obtained from different tissues of *Scylla serrata* were characterised with reference to morphology, DNA, RNA and Protein contents while the testicular cell lines were characterised further on the basis of MTT and LDH assays, Mitotic Index, Mitosis, RAPD analysis as well as 18 S rRNA Sequencing.

2.3.1 Biochemical characterization

2.3.1.a Methylthiazol Tetrazolium Assay (Mosmann, 1983)

The Cell proliferation and viability was assessed by using MTT kit (Sigma, PCN: TOX1). The assay is based on the intrinsic ability of mitochondrial dehydrogenases of viable cells to cleave MTT (*3-[4,5-dimethylthiazol-2-yl]-2 diphenyltetrazolium bromide*) to a purple-coloured formazan crystal. The crystals thus obtained were dissolved in acidified isopropanol. The optical density (O.D) of resulting solution was measured at 570 nm wavelength

Materials :- MTT (PCN: M5655), MTT Solubilization Solution (PCN: M8910) – (10 % Triton x-100 plus + 0.1 N HCl in anhydrous isopropanol - 125 ml).

Protocol :-

a. The culture medium was aspirated from the wells with a 5.0 ml pipette, and the cells were rinsed thrice with crab saline.
b. MTT assay mixture (M-5655) was reconstituted by adding 3.0 ml of crab saline to each vial content.
c. Further the reconstituted MTT mixture in an amount equal to 10 % of culture medium volume was added to the wells and incubated for 3 hours to form the formazan crystals. After the incubation period, the formazan crystals were dissolved by adding MTT solubilization solution (acidified isopropanol) equal to the volume of original culture medium.
d. Pipetting up and down of MTT solubilization solution was required for dissolving formazan crystals. The optical density of resulting solution was measured at 570 nm wavelength using Nanodrop (ND-1000) spectrophotometer.

2.3.1.b Lactate Dehydrogenase Assay (LDH - PCN: Sigma TOX 7) (Decker and Lohmann, 1988)

Materials :- LDH Assay substrate Solution (PCN: L2402), LDH Assay Cofactor (PCN: L2527), LDH Assay Dye Solution (PCN: L2277), LDH Assay Lysis Solution (PCN: L2152)

Preparation of reagents :-

- **LDH Assay Cofactor** – 25 ml of Milli Q water was added to the bottle of lyophilized cofactors. The cofactor was stored at -0^{o} C as working aliquots so as to avoid repeated freezing and thawing procedure.

- **Lactate Dehydrogenase Assay Mixture** – This was prepared by mixing equal volume of LDH assay substrate cofactor and dye Solution.

Protocol :-

a. The culture medium from MWPs was aspirated and transferred to centrifuge tubes labelled appropriately.
b. Culture medium thus obtained was centrifuged at 5000 rpm for 10 minutes at 4° C in order to remove cells if any in the aspirated medium and the supernatant was collected in separately labelled centrifuge tubes.
c. The cultured cells from the MWPs and from the sediments collected after centrifugation as referred at 2.3.1.b.(b) were lysed using lyses solution and the lysates were mixed. This mixed cell lysate was centrifuged at 5000 rpm for 10 minutes and the supernatant was used as the non-damaged control.
d. The obtained supernatant at 2.3.1.b.(a) and cell lysate at 2.3.1.b.(b), were mixed with working solution from the kit. The mixture was stirred and incubated at room temperature for 30 minutes.
e. The reaction was stopped by adding 1N HCl solution. Absorption of the reaction mixture was measured at 490 nm using Nanodrop (ND-1000) spectrophotometer.

LDH leakage was calculated using the following formula:

$$\text{LDH leakage (\%)} = 100 \times (CS - BC) / (NDC - BC + CS - BC).$$

CS - Culture supernatant

BC - Background control

NDC - Non-damaged control

2.3.1.c Cell Senescence Test (PCN: CS0030 – Dimri et at 1995)

The test is based on histochemcial staining technique for β- galactosidase activity at pH 6.0. β- galactosidase activity is easily detectable in senescent cells, but undetectable in quiescent, immortal and normal healthy cells. For detection of β-galactosidase activity senescence cell staining kit was used following manufacturers recommendation.

Materials :- Fixation buffer 10 x (PCN: F1797), formaldehyde - 29 %, glutaraldehyde - 2.0 %, Na_2HPO_4 - 70.4 mM, KH_2PO_4 - 14.7 mM, NaCl -1.37 M and KCL - 26.8 mM), Reagent B (PCN: R5272 - 400 mM Potassium ferricyanide), Reagent C (PCN: R5147 - 400 mM Ferrocyanide), X-gal solution (PCN: X3753 - 40 mg/ml), Staining solution 10 x (PCN: S5818), Phosphate buffer saline 10 x (PCN: P3621).

Preparation of reagents :-

- **Fixation Buffer** – Fixation Buffer 10 x was diluted 10-fold using Milli Q water and then filtered through 0.2 µm sterile filter fitted to a syringe and the filtrate was stored at – 20° C until used.
- **Phosphate buffer saline** –The PBS 10 x was diluted 10-fold with 0.2 µm filtered Milli Q water. After preparation, the 1 x PBS was stored at 2–8° C.
- **Staining Mixture** – was prepared just prior to use. The following chemicals were mixed for preparing 10 ml of the Staining mixture.
    - 1.0 ml of Staining Solution 10 x
    - 125 µl of Reagent B
    - 125 µl of Reagent C
    - 0.25 ml of X-gal Solution and 8.50 ml of Milli Q water.

Staining Mixture was then filtered through 0.2 µm filter to ensure that there was no aggregates in the solution.

Protocol :-

a. The culture medium was aspirated from the culture plates carefully, so that cells do not detach.
b. The cells were rinsed twice with 1.0 ml of PBS per well/plate and then fixed in 1.5 ml fixation buffer for 6–7 minutes at room temperature.
c. After the incubation period the cells were rinsed with 1.0 ml of PBS and to that rinsed cells 1.0 ml of the staining mixture was added and incubated for 24 hrs at 37° C until the cells were stained blue, due to senescence.
d. The cells were observed under microscope. Cells expressing β- galactosidase were detected by the presence of blue coloration located in the nucleus of the cells.
e. For long-term storage of the stained plate, the staining mixture was aspirated, and then overlaid with a glycerol solution (70 %), and stored at 2–8° C.

2.3.2 Mitosis

2.3.2.a Mitotic Index :-

The percentage of cells in mitosis was determined by counting mitotic cells in a culture as a proportion of the whole population. The cell cultures were stained with acetocarmine.

Formula : (Number of cell in mitosis / Total number of cells counted) x 100.

2.3.2.b Mitotic stages :-

Material :- Colchicine 0.025 %, Chemically defined medium [CDM = L15 - crab saline (1:1 v/v) + EGF 20 ng/ml + glucose, 1.0 mg/ml], acetocarmine stain (0.5 %), Artificial sea water, Mountant for histology (prepared as mentioned in protocol 2.2.1.a-d), slides, coverslips, fixation buffer (protocol 2.2.1.a-a).

Protocol :-

a. The cells isolated from testes by mechanical dissociation as described in protocol 2.1.8.b(a-b) were inoculated at a density of 10^5 cells/ml on the coverslips placed in a sterile Petri dishes having CDM (L15 + crab saline (1:1 v/v) supplemented with epidermal growth factor (20 ng/ml) and glucose (1.0 mg/ml)
b. After 48 hours of initiation of culture the proliferating testicular cells were exposed to 0.025 % colchicine for 4-5 hours. These cells were then fixed using fixation buffer for 20 minutes.
c. The fixed cells were carefully rinsed with ASW, and then exposed to acetocarmine stain for a period of 15 minutes and then rinsed carefully with distilled water. The coverslips containing the stained cells were mounted on the histology mountant without any intervention of air bubbles. The slides thus prepared were viewed under Olympus microscope for mitotic stages and photographed.

2.3.3 Genomic analysis

2.3.3.a RAPD Analysis using primer YNZ22 (Klinbunga et at 2000) **:-** In order to confirm that the cultured cells were derived form parental tissue of *Scylla serrata*, RAPD

analysis was done using primer (YNZ22) specific for *Scylla serrata* (Klinbunga 2000) under PCR conditions. The sequence of this primer (YNZ22) is 58-CTCTGGGTGTCGTGC-38.

Amplification reaction was carried out in 25 µl of reaction mixture stated below.

- Taq Buffer A (10X) : 2.5 µl
- 25 mM$MgCl_2$: 0.5 µl
- dNTP mix (10 mM each) : 1.5 µl
- Primer (100 ng) : 1.0 µl
- Taq polymerase (3U) : 1.0 µl
- Template (100 ng/ul) : 1.0 µl
- MQ distilled water : to make up the volume 25ul

The PCR reaction was initiated as below.

Initial denaturation	Denaturation	Annealing	Extension	Extension	Final Extension
	40 Cycle				
94° C	94° C	35° C	72° C	72° C	72° C
4 min	60 sec	60 sec	2 min	10 min	End point

To analyse the PCR products, 12 µl of PCR reaction mixture was used for electrophoresis on 1.5 % agarose gels containing 1.0 mg/ml ethidium bromide and visualized under ultraviolet transilluminator.

2.3.3.b RAPD Analysis using OPD20, OPD08 primers (Yoon and Park, 2006) :-

The parental tissue and cultured cells' genomic DNA was isolated using DNAzol® reagent (DN 127- Molecular Research Centre, Inc. Ohio) following the manufacturer's

recommendation (Protocol 2.2.2.a). The sequence of the primer was 5'ACCCGGTCAC' (OPD20) and 5' GTGTGCCCCA3' (OPD08). PCR amplification was carried out following the method of Yoon et al (2006). Amplification reaction was carried out in 20 µl reaction mixture using biometra thermal cycler that consisted the following.

- Taq Buffer A (10X) : 2.5µl
- Each primer : 2.5µl
- 2.5 mM of each dNTP : 2.5µl
- Taq DNA polymerase : 2.0µl
- Template DNA : 1.0 µl
- Nuclease free water : 9.5 µl

The PCR reaction was initiated as mentioned below:

Initial denaturation	Denaturation	Annealing	Extension	Final Extension
	35 Cycle			
94° C	94° C	34° C	72° C	72° C
5 min	1 min	1 min	2 min	7 min

The resulting amplified products (12 µl) were loaded onto 1.4 % agarose gels, subsequently stained with ethidium bromide and visualized under ultraviolet transillumination.

2.3.3.c Protocol for Electrophoresis

Materials :- Tris-Borate EDTA buffer (TEB) [(Boric acid 89 mM, Tris- HCL 89 m M, EDTA 2.0 mM (pH 8.0)], Agarose (1.0 to 1.5 % (w/v) in TEB**),** Loading dye (40 % (w/v)

sucrose, 0.25 % (w/v) bromophenol blue in 1.0 ml of TEB (10 x) pH 8.0), Staining solution (1.0 mg ethidium bromide per millilitre of distilled water), DNA marker (100 bp).

Protocol :-

a. Agarose gels that ranged from 1.0 to 1.5 % were used for separation of DNA fragments. Gels were prepared with 1 x TEB buffer.
b. The PCR amplified product mixed in one by three volume of loading dye was loaded along with the DNA marker.
c. Electrophoresis was performed at 10 V/cm until the dye reached the end of the gel. The respective gel was then gently removed and stained in ethidium bromide staining solution for 15 minutes.
d. Destaining was done in TEB solution with gentle shaking the gel for 15 to 30 minutes. The electrophoresed DNA gel was photographed under UV light source for a permanent record of the results.

2.3.3.d 18S rRNA Sequencing :- The cultured cells' and parental tissue genomic DNA was isolated using DNAzol® reagent kit (PCN: DN 127- Molecular Research Centre, Inc. Ohio) as described in protocol 2.2.2.a. The DNA extracts were stored at -20^{o} C until used. PCR amplification was carried out following the method of Williams and Ozawa (2006). The 18S rRNA gene was amplified using primers 18S-5_(F) and 18S1100R (R), the sequences of forward and reverse primers were 5'-CTG GTT GAT YCT GCC AGT-3', 5'-CTT CGA ACC TCT GAC TTT CG-3'. Amplification reaction was carried out in 50 µl of reaction mixture stated below.

- 2 x PCR master mix (Sigma-P 4600) : 26 µl
- Forward primer 100 pM/µl : 2.0 µl

- Reverse primer 100 pm/µl, : 2.0 µl
- Template DNA 80 ng/µl : 1.0 µl
- PCR water : 19 µl

The PCR amplification reaction was initiated as mentioned below

Initial denaturation	Denaturation	Annealing	Extension	Extension	Final Extension
	30 Cycle				
94° C	94° C	54° C	72° C	72° C	4° C
5 min	1 min	1 min	2 min	7 min	End point

The resulting amplified products (10 µl) were mixed with 2 µl loading dye (6X DNA loading Dye of Fermentas # PCN: R0611) and loaded onto 1.2 % agarose gel, subsequently stained with ethidium bromide and viewed under UV-light.

All the PCR products were sequenced at MWG Biotech private limited, Bangalore, India and chromatograms were obtained. The sequences of PCR products were analyzed by using Basic Local Alignment Search Tool (BLAST). The sequence was deposited in Gene bank, [National Centre for Biotechnology Information, (NCBI)] Maryland, USA.

2.4 CHARACTERIZATION OF HEPATOPANCREATIC GRANULAR CELLS

2.4.1 Preparation of Granular cell suspension :- Hepatopancreatic granular cell suspension was prepared according to the protocol described in 2.1.2.b.(a-e). Briefly, the hepatopancreas was removed and transferred to a beaker containing sterile crab saline + 0.2 % antibiotic (Himedia PCN A007-5 x 50 ml) mixture having osmolality equal to 1050

mOsm/kg and pH 7.3. After the final wash the tissue fragments were transferred to a citrate buffer having osmolality equivalent to 1122 mOsm/kg and pH 7.3. The tissue fragments were dissociated by mechanical dissociation involving fifteen passages through a 10 ml pipette. The suspension was filtered through a nylon mesh (pore size - 10 µm) and spun for 3 minutes at 1000 rpm. The resulting two layer pellet had granular cells at the bottom. The granular cells were aspirated after removing the upper layer using Pasteur pipette and rinsed twice with crab saline.

2.4.2 Morphological characterization

2.4.2.a Estimation of cell volume :- The Granular cells' shape, size and diameter were determined using Image Analyzer Programme (Image Pro Express US, Nikon microscope 102 Eclipse TS100, TI-SM Japan).

2.4.2.b Light and Confocal Microscopy :- The granular cells obtained by mechanical dissociation were resuspended in ASW containing 2.0 % glutaraldehyde. These cells were centrifuged at 150 g for 1 minute and then dehydrated in a series of diluted ethanol (15-100 %). The dehydrated cells were mounted on a glass slide with DPX mountant. Digital images were acquired using Olympus microscope (Bx41) fitted with Olympus digital camera (DP12). Some of the fixed granular cells were used for confocal imaging by spreading them on slides. The confocal images were obtained by using Olympus confocal microscope (FV1000 Confocal).

2.4.2.c Scanning Electron Microscopy :- The granular cell suspension (2.0 µl) was spread on coverslips and was allowed to dry for two hours. Each of these coverslips was

mounted on a bullet and then introduced into a sputter chamber/ coater (SPI module) for sputtering of gold particles to neutralize the charge on the cells. The prepared samples on the bullet were placed into the chamber of SEM (JSM – 5800 LV, JEOL Company) meant for loading samples. The machine was run and photographs of the scanned granular cells were taken to estimate average size (diameter of the cells).

2.4.2.d Fluorescent Microscopy :- Fura 2-AM (Sigma PCN: F0888) was diluted to a final concentration of 5.0 µl using 0.25 % pluronic acid after initial dilution with DMSO to 1.0 mM. The granular cell suspension was incubated for 45 minutes at 21^0 C with diluted Fura-2 AM. After incubation the cells were rinsed thrice in a Calcium free saline buffer (NaCl - 467 mM, KCl 10 - mM, NaH_2PO_4 - 1.0 mM, $NaHCO_3$ - 4.0 mM, Na_2SO_4 - 8.4 mM, HEPES - 30 mM, EDTA - 10 mM) pH 7.4. The cell suspension was then spread on a slide and observed under Nikon fluorescent microscope (Elise E 800) and the images were taken using Nikon Coolpix 4500 digital camera at 340 nm UV excitation.

2.4.3 Molecular Characterization

2.4.3.a DNA, RNA and Protein assay :- DNA was isolated using DNAzol® reagent (DN 127- Molecular Research Centre, Inc. Ohio) according to the manufacturer's recommendation. DNA concentration was determined by following the protocol described at 2.2.2.b RNA was extracted using EZ- RNA reagent (Cat No. 30-400-100 – Bimolecular Industries Israel) and RNA concentration was assayed as described earlier (Protocol 2.2.2.d). Similarly protein assays were performed as described earlier (Protocol 2.2.2.e)

2.4.3.b Assay of cytoplasmic calcium :-

Materials :- Fura -2 AM, Calcium, Calcium free saline buffer.

Protocol :-

The intracellular calcium concentration was measured spectrofluoro-photometrically (Shimadzu RF-1501) by following the protocol of Zilli et al (2007). Calcium buffer was used to calibrate binding of calcium to Fura – 2 AM. Buffer mixtures (N=6) were prepared by adding 0.2 ml of Fura 2-AM (5.0 µmol) to 4.0 ml of predetermined concentration of calcium. Each buffer mixture had a different concentration of calcium (0.2 – 0.8 mg/ml).

The harvested cells (4 x 10^6 cells) were centrifuged at 1000 rpm for 5 minutes and then incubated with Fura 2-AM (5.0 µmol) for 30 minutes at 37^0 C. After incubation, the cells were rinsed thrice with calcium free saline buffer before resuspending them in 4.0 ml of the same saline buffer. The excitation wavelength (340 nm) and emission wavelength (510 nm) were set for the assay. The maximum fluorescence was recorded by using 10 µl of 2.0 mol/l $CaCl_2$ + 20 µl of 10 % SDS; the minimal fluorescence was recorded by using 20 µl of 0.5 mol/l EDTA solution, pH 9.0. (Zilli et at 2007).

2.4.3.c MTT (Methylthiazol Tetrazolium) assay for cell proliferation

Material :- MTT Kit (Sigma, PCN: M5655), L15 + crab saline (1:1 v/v), L15 + crab saline (1:1 v/v) + EGF (20 ng/ml) + glucose (1.0 g/l).

Protocol :-

The granular cells were cultured in MWPs as described earlier in protocol 2.1.2.b. Control cultures were maintained in L15 + crab saline (1:1 v/v) + glucose (1g/l), while experimental cultures were maintained in L15 + crab saline (1:1 v/v) supplemented with epidermal growth factor (EGF - 20 ng/ml) + glucose (1.0 g/l). All the cultures were incubated at 20 – 24^{o} C and the media were replenished at two days interval. For detecting growth and cell proliferation, MTT assay was employed after 24 hr and 5 days of initiation of culture following the protocol given in 2.3.1.a.

2.4.3.d Ecdysterone mediated calcium release :-

The effect of ecdysterone (Sigma, PCN: 44658) on granular cells with respect to release of calcium was tested by exposing the cultured granular cells to different concentrations of ecdysterone (5.0, 10, 20 µg/ml) in order to determine the optimal dose of ecdysterone for calcium release.

Further, the optimal dose was used to evaluate the calcium release by the cultured granular cells. Briefly the granular cells were isolated as mentioned previously and were inoculated at a density of 2 x 10^{8} cells per well. The controls were maintained in a calcium free crab saline while the experimental received calcium free crab saline loaded with the optimal dose of ecdysterone. After exposure to ecdysterone for one hour the cells were rinsed and maintained in calcium free crab saline. From this crab saline 0.5 ml of a sample was aspirated after one hour and then subsequently 0.5 ml of the sample was aspirated at an interval of two hours for a total period of 10 hrs. Each sample thus obtained was analysed for calcium contents using Fura 2-AM as described previously (protocol 2.4.3.b).

2.4.3.e RAPD analysis (Klinbunga, 2000) :-

The genomic DNA was isolated from Granular cells as well as from parent tissue using DNAzol® reagent (DN 127- Molecular Research Centre, Inc. Ohio) following manufacture's recommendation described earlier at 2.2.2.a. The isolated DNA was stored at 4^{o}C until used. Two specific 10-mer and 15-mer RAPD primers (UBC457, YNZ22) specific for *Scylla serrata* were designed based on Klinbunga (2000) recommendation. The sequences of these primers were (UBC457) 58-CGACGCCCTG-38 and (YNZ22) 58-CTCTGGGTGTCGTGC-38.). Amplification reaction was carried out in 25 µl of reaction mixture having below mentioned composition:

- Taq Buffer A (10X) : 2.5 µl
- $MgCl_2$ (25 mM) : 0.5 µl
- dNTP (10 mM) : 1.5 µl
- primer (100 ng) : 1.0 µl
- Taq polymerase (3U) : 1.0 µl
- Template (100 ng/ul) : 1.0 µl
- Milli Q water : 12.5 µl

RAPD – PCR reaction was initiated as given in the Table below

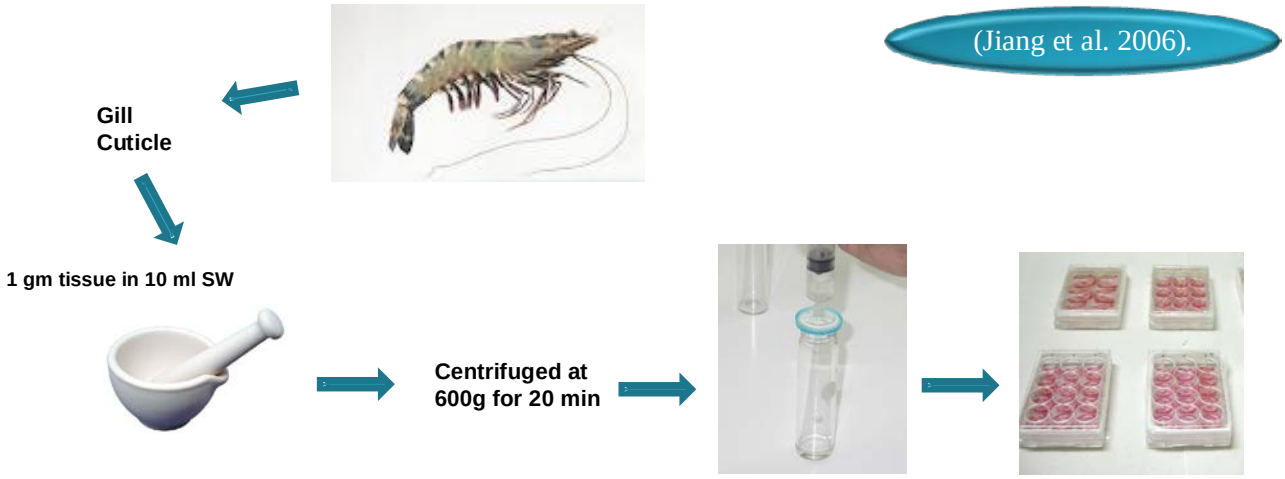
Initial denaturation	Denaturation	Annealing	Extension	Extension	Final Extension
	40 Cycle				
94° C	94° C	60° C	72° C	72° C	4° C
4 min	60 sec	35 sec	2 min	10 min	End Point

The resulting amplified products (12 µl) were loaded onto 1.5 % agarose gels, stained with ethidium bromide, and visualized using a UV transilluminator.

2.5 UTILITY OF TESTICULAR CELL LINE FOR TESTING WSSV INFECTIVITY

2.5.1 Preparation of white spot syndrome virus inoculum

WSSV suspension was prepared from gills and cuticles of heavily infected *Penaeus monodon* stored at -80^{o} C following the protocol described by Jiang et al (2006). Briefly the tissues (1.0 gm) were homogenized in artificial sea water (pH-7.4, osmolality - 1050 mOsm/kg) and then centrifuged at 600 g for 20 minutes at 4^{o} C. The supernatant was passed through 0.2 µm sterile filter fitted to a syringe and the filtrate was stored at -80^{o} C until used.



2.5.2 Dilution of virus suspension and determination of ID50

The above viral suspension was serially diluted to 10^{-1}, 10^{-3} and 10^{-6} dilutions with L15 - Crab saline (1:1, v/v) medium. To evaluate the virus concentration that infects 50 % (ID50) of the cell cultures, the viral titers were prepared from the gill and cuticle

suspensions by the end point dilution assay following the method of Reed and Muench (1938). In brief, the testicular cell cultures (ten monolayers) were infected with each serial dilutions of viral stock as mentioned above. The number of cell cultures that were infected was then determined for each virus dilution, by looking for cytopathic effect.

2.5.3 Exposure of testicular cell line to WSSV (Jiang et at 2006) **:-**

The testicular cell line was prepared as described in protocol 2.1.9. The cultured testicular cells were inoculated with different dilutions (10^{-1}, 10^{-3} and 10^{-6}) of WSSV suspension (Gill, Cuticle). Control culture plates were inoculated with heat inactivated (at 95^{o} C for 5 minutes) viral suspension of 10^{-1}, 10^{-3} and 10^{-6} dilution. The negative control culture plates received only plain medium used for dilution of virus in the identical volume. The extract of non infected *Penaeus monodon* was used in the similar dilution as another control to check whether a factor other than the virus in a prawn is responsible for cytopathy. The virus inoculated culture plates were incubated at 28^{o} C for 24 hrs and then rinsed repeatedly (N=5) with culture medium to divest the cultures of virus. Subsequently such cells were grown in chemically defined cultured medium (L15 – crab saline (1:1, v/v) medium, osmolality - 1050 mOsm/kg supplemented with EGF (20 ng/ml) + antibiotics mixture (0.3 %) devoid of any viral contamination. The control and experimental cells were observed daily for cytopathic effects for a period of one month.

2.5.4 MTT (Methylthiazol tetrazolium) Assay for evaluating viral toxicity :-

The toxic effects of viral infection to the cultured cells were analyzed using MTT - *In vitro* toxicology assay kit (TOX–1, Sigma-Aldrich). Before the assay the cells were rinsed five times with crab saline to remove WSSV from the medium. The assay mixture contained tetrazolium salt. Mitochondrial dehydrogenase of viable cells cleave tetrazolium

(3-[4,5-dimethylthiazol-2-yl]-2 diphenyltetrazolium bromide) to yield formazan crystals. The formazan crystals thus obtained were dissolved in acidified isopropanol. The optical density (O.D) of resulting solution was measured at 570 nm wavelength using NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies, USA).

2.5.5 Nested PCR with WSSV specific primers :-

For Nested PCR assay, WSSV detection Kit was used (Genei- 106070). The first step - PCR reactions were carried out in 25 µ1 of reaction mixture containing the following reagents.

- DNA : 1.0µl
- PCR pre mix : 23 µl
- Taq Polymerase enzyme : 1.0 µl

The PCR reaction was performed in a thermocycler with the following PCR conditions.

Initial denaturation	Denaturation	Annealing	Extension	Final Extension
	28 cycles			
95^0 C	95^0 C	58^0C	72^0C	72^0C
3 min	30 Sec	30 Sec	30 Sec	5 min

Second step - PCR reaction was initiated by using 1.0 µ1 of first PCR product, 23 µl Nested PCR pre mix, 1.0 µ1 Taq Polymerase enzyme. Thermocycle condition was same as first step - PCR reaction. PCR reaction products were separated on 1.0 % agarose gel and stained with ethidium bromide for band visualization.

2.5.6 RAPD analysis for detecting WSSV:-

Randomly amplified polymorphic DNA profiles were generated using 10-mer RAPD primers such as RPL 304, B2, B9, and B19. Under PCR conditions, the sequences of these primers were (RPL 304) 5'- AAAGCTGCGG - 3', (B2) 5'-ACCCAAGTGG - 3', (B9) 5'-ACCCTTGTGG - 3', (B19) 5'- AAAGCTGCGC - 3'. Amplification reaction was carried out in 25 µl of reaction mixture that consisted of following mix composition.

- DNA : 1.0µl
- dNTP mix (2.5mM each) : 1.0µl
- Primer : 1.0µl
- 25mM$MgCl_2$: 0.5µl
- XT5 DNA polymerase assay buffer(10X) : 2.5µl
- XT5 DNA polymerase enzyme : 3U
- Glass distilled water : to make up the volume 25ul

The PCR reaction was initiated as mentioned below.

Initial denaturation	Denaturation	Annealing	Extension	Denaturation	Annealing	Extension	Final Extension
	8 cycle			35 cycle			
94^0C	94^0C	35^0C	72^0C	94^0C	38^0C	72^0C	72^0C
4 min	45 sec	60 sec	90 sec	45 sec	60 sec	60 sec	7 min

After amplification, 12 µl of the amplified products were loaded on to 1.5 % agarose gel and resolved by electrophoresis. The gel was subsequently stained with ethidium bromide and viewed under UV-light.

Table 2.1 Reconstitution of culture media

Media	Reconstitution
Leibovitz (Himedia, PCN: AT011)	Leibovitz media was reconstituted at single (14.0 g/ l), double (28.0 g/l) and triple (42 g/l) concentration using sterile Milli Q water or Artificial sea water.
DMEM (Himedia, PCN: AT-007)	13.4 gm of DMEM was reconstituted in one litre of Artificial sea water having an osmolality of 1050 mOsm/kg.
Mccoy medium modified (Himedia, PCN: AT-071)	23.37 gm was reconstituted in one litre of Artificial sea water having an osmolality of 1050 mOsm/kg.
F-12 Nutrient mixture (Himedia, PCN: N-4388)	10.64 gm reconstituted in one litre of Artificial sea water having an osmolality of 1050 mOsm/kg
M-199 (Himedia, PCN: AT-014)	12.45 gm was reconstituted in one litre of Artificial sea water having an osmolality of 1050 mOsm/kg
HBSS (Himedia, PCN: TS-1003)	9.76 gm was reconstituted in one litre of Artificial sea water having an osmolality of 1050 mOsm/kg

Table 2.2 Media tested for initiating cell culture from different tissue of *Scylla serrata*

Sr. No:	Media	Diluents	Osmolality
1.	1x L15	ASW*	**Osmolality of the media ranged between 902 to 1088 mOsm/kg.**
2.	2xL15	ASW*	
3.	3xL15	MQW	
4.	3xL15	ASW*	
	3xL15 + Nutrient mixture F12 (HAM) + ASW*	MQW	
5.	L15 + Crab saline	MQW	
6.	F-12 - Nutrient mixture + ASW*	MQW	
7.	McCoys medium modified + ASW*	MQW	
8.	DMEM	ASW*	
9.	M-199	ASW*	
10.	M-199 + salt mixture	ASW*	

ASW* - Artificial sea water with an osmolality equal to 1050 mOsm/kg

Table 2.3 Media tested for dissociating tissues of *Scylla serrata* by mechanical dissociation technique

Media tested for mechanical dissociation technique.			
Sr. No:	**Media**	**Diluents**	**Osmolality**
	ASW*	MQW	
	Citrate buffer	MQW	**Osmolality of**
	Crab saline	MQW	**the media**
	L15 (1x, 2x, 3x)	ASW*	**ranged between**
	L15 + crab saline	MQW	**902 to 1088**
	M-199	ASW*	**mOsm/kg**
	3xL15 + Nutrient mixture F12 (HAM) + ASW	MQW	

ASW* - Artificial sea water with an osmolality equal to 1050 mOsm/kg

Table 2.4 Trypsin in combination with different media tested for dissociating tissues of *Scylla serrata*

Dissociating media	Diluents	Heart	Gill	Muscle	Neurons.
A. Trypsin.					
Trypsin (0.05 %)	ASW*	-	**	-	*
Trypsin (0.1 %)	ASW*	-	***	-	**
Trypsin (0.2 %)	ASW*	-	-	-	-
Trypsin (0.25 %)	ASW*	-	-	-	-
Trypsin (0.1 %) + EDTA (0.1 gm)	ASW*	-	**	-	-
Trypsin (0.25 %) + EDTA (0.1 gm)	ASW*	-	-	-	-
Trypsin (0.05%) + EDTA (0.1 gm)	ASW*	-	-	-	-
Trypsin (0.1 %) + PVP (0.1 gm)	ASW*	-	*	-	-
Trypsin (0.1 %) + EDTA (0.02 gm + glucose (0.1 gm) + PVP (0.1 gm)	ASW*	-	-	-	-
Trypsin 0.1 % + 3 x L15	MQW	-	**	-	***
Trypsin (0.5 %) + EDTA 0.02 gm	ASW*	-	-	-	-
Trypsin 0.1 gm + PVP 1.5 gm.	ASW*	-	-		
Trypsin (0.1%) + PVP (1.0 gm) + EDTA (0.02 gm) + glucose (0.1 gm).	ASW*	-	-	-	-
Trypsin (0.1 gm) + EDTA (0.02 gm)+ PVP (0.1 gm) + HBS (100 ml).	ASW*	-	**	-	-
Trypsin (0.1 %) + L15 + Crab saline (1:1 v/v)	MQW	-	*	-	**

******* : 90% cell survivability., ****** : 60% cell survivability., ***** : 30 % cell survivability,

- : enzyme media combination was not effective for dissociating cells. **ASW*** : Artificial sea water with an osmolality equal to 1050 mOsm/kg.

Table 2.5 Collagenase and Hyaluronidase in combination with different media tested for dissociating tissues of *Scylla serrata*

Dissociating media	Diluents	Heart	Gill	Muscle	Neurons.
B. Collagenase.					
Collagenase 0.1 mg/ml of 3x L15.	MQW	-	-	-	**
Collagenase 0.3 mg/ml of 3x L15.	MQW	***	-	***	*
Collagenase 0.1 mg/ml of 2xL15.	ASW*	-	*	-	**
Collagenase 0.3 mg/ml of 2xL15.	ASW*	**	-	*	**
Collagenase 0.3 mg/ml + + L15 + Crab saline (1:1 v/v)	MQW	-	-	-	*
Collagenase 0.2 mg/ml + EDTA (0.001 gm/ml)	ASW*	**	-	**	**
Collagenase 0.2 mg/ml + EDTA (0.001 gm/ml) + PVP (0.001 gm/ml)	ASW*	-	-	-	*
C. Hyalorinidase.					
Hyalorinidase 0.05 mg/ml of 3x L15	MQW	-	-	-	-
Hyalorinidase 0.1 mg/ml of 3x L15.	MQW	-	*	-	**
Hyalorinidase 0.3 mg/ml of 3x L15.	MQW	*	-	-	-
Hyalorinidase 0.05 mg/ml of 2xL15	ASW*	-	-	-	-
Hyalorinidase 0.1 mg/ml of 2xL15.	ASW*	-	-	-	-
Hyalorinidase 0.3 mg/ml of 2xL15.	ASW*	-	-	-	-
Hyalorinidase 0.2 mg/ml + EDTA (0.001 gm/ml)	ASW*	*	-	-	**
Hyalorinidase 0.2 mg/ml + EDTA (0.001 gm/ml) + PVP (0.001 gm/ml)	ASW*	-	*	-	-
Hyalorinidase 0.3 mg/ml of + L15 + Crab saline (1:1 v/v)	MQW	*	-	-	**

***** :** 90% cell survivability.

**** :** 60% cell survivability.

*** :** 30 % cell survivability.

- : enzyme media combination was not effective for dissociating cells.

ASW* : Artificial sea water with an osmolality of 1050 mOsm/kg.

CHAPTER - 3

Results

RESULTS

3.1 Primary cell culture

In the present study an attempt is made to develop an effective technique for cell culture from different tissues of a mud crab *Scylla serrata*. Since the mud crabs are picked up from the natural environment and brought to the laboratory, their acclimation to the laboratory conditions was performed as described in the methodology. The washing of the crabs with sea water removed the mud on the body and thereafter rinsing with ASW cleaned it further. Keeping animals in well aerated tanks containing filtered natural sea water with ad-libitum food supply in the form of bivalve tissues, helped crabs get acclimated to the laboratory conditions. The feeding behaviours such as searching and grabbing the food, cracking the bivalves, moving in water, climbing on the stones kept in the tank, climbing on each other indicated their acclimation to the laboratory conditions. Such acclimated crabs were surface sterilised as described in the methodology section and were subjected to paper swabbing followed by transferring the tiny swabs into the test tubes having culture medium. The culture medium's optical density (OD) was taken at 600 nm at initial stage (O.D - 0.004 ± 0.00057) and then they were transferred to the incubator set at 37^0 C for six hours. Subsequently the incubated medium's OD was taken (0.0046 ± 0.0003). As there was hardly any change in the OD it was assumed that crab surfaces were sterilised properly.

The present work has established a technique to isolate cells from different tissues of crab with higher viability. Among the two dissociation techniques employed, mechanical dissociation technique was more effective for tissues such as hepatopancreas, testis and ovary while enzymatic dissociation technique was effective for gills, heart,

neurons and muscles. The cultured cells from different tissues exhibited variation in survival pattern. Among the various tissues tested (Gills, Muscles, Heart, Ovaries, Testes, Neurons, Hepatopancreas, and Hemocytes) the most suitable tissue for longer survival of dissociated cells was testis followed by hepatopancreas (with reference to granular cells) and ovaries. Explant culture was found to be effective for tissues such as hepatopancreas, ovaries and testes while for other tissue types (Gills, Muscles, Heart, Neurons) dissociation technique was suitable. The cells obtained from explant and through dissociation of tissues were exposed to different media and growth factors in different permutation combination to find our their pluripotency. All the cell types obtained from different tissues did not exhibit any pluripotency instead they always developed their own cell types.

The cell culture was maintained at a pH range of 7.2 – 7.5 which is a normal pH range of hemolymph of a crab. The incubation temperature for primary cell cultures was kept to 25-28^0 C as this is the optimum temperature range of a habitat of crabs. Microbial contamination is always a problem with cell culture however; the bacterial contamination was overcome with the use of surface sterilization procedure and use of antibiotics such as Penicillin-streptomycin solution, Antimycotic + Gentamicin + Amphotericin B. Several cultures with no antibiotics exhibited very low survivability (24 hours) as compared with those set up with antibiotics.

3.1.1 Primary culture of gill epithelial cells

3.1.1.a Explant culture :- The tissue fragments placed in the culture medium showed tiny projections (blebs) emerging from them after 24 hours (Figure 3.1.A). These projections / blebs contain cells which come out of the tissue in the process of migration.

These projections grow in size as a result of cell migrations. Subsequently the cells from these blebs moved out and anchored to the substratum. These cells were round with a size ranging from ~ 38 to 52 μm (Figure 3.1.B). However, these cells survived for two days and later disappeared from the culture medium. Different media preparations with diluents like Milli Q water (MQW) and artificial sea water (ASW) with a lesser variation in osmolality had no influence on cell migration as well as cell proliferation and survivability. Even the use of crab saline with L15 had negligible influence on cell survival as well as on cell migration. The use of two media with two extreme osmolalities had no adverse influence on cell survivability as compared to those media prepared in the range of 1050 mOsm /kg. Besides the use of different growth factors and fortification of media with insulin, transferrin, or even with glucose had no apparent influence on bleb formation, cell migration, cell attachment to the substratum and cell survivability. The cultures grown with ASW and Glucose as a medium had a survivability comparable to that observed with other different media or their combinations. The cells showed best survivability in 2 x L15 medium for a period of four to five days (Table 3.1-A)

3.1.1.b Cell culture by disaggregation :- In order to disaggregate the tissue viz-a-viz to dissociate the cells from the tissue, mechanical and enzymatic procedures were adopted. The mechanical dissociation technique which involved trituration through several passages in and out of pipette resulted in dissociating cells in a less viable state as evidenced by Trypan blue dye staining that showed less than 30 % cells in a viable state. These cells could not survive for more than 2-3 days. Any permutation combination of culture media and growth factors or supplements had no influence on survivability of cells. For gills enzymatic dissociation techniques were employed using enzymes such as trypsin, collagenase and hyaluronidase in different concentrations. Subsequently cell viability was

examined using Trypan blue dye exclusion technique. Enzyme solutions were prepared in ASW, crab saline, L15 and other media (Table 2.3, 2.4) to test their suitability while keeping the osmolality nearly constant. However except 0.1 % Trypsin prepared in ASW (osmolality 1050 mOsm/kg) none of these enzymes provided viable cells, even at low concentration, at low reaction temperature or when short reaction times were used, as evidenced by cell viability tests. The enzymatically dissociated cells of gills did not anchor to the substratum though they exhibited a tendency to settle. Non anchored cells die within 1-2 days. The best result in terms of cell survivability was obtained when cells were cultured in 2 x L15 medium prepared in ASW with an osmolality of 1050 mOsm/kg. Though supplements were added to the media, cell division / cell proliferation was not observed. These cells remain viable for a period of five days and thereafter as time progressed the cells viability deteriorates. However, during this short period of survival four different types of cells can be observed (Figure 3.2).

3.1.1.c Characterization :- The dissociated cells of the gills exhibit four different types of cells such as nephrocytes, glycocytes, epithelial cells and hemocytes (Figure 3.2). The nephrocytes are large in size ~ 38 to 52 µm in diameter with the cytoplasm filled with many tiny excretory vesicles. The glycocytes are smaller in size ~ 34 to 37 µm in diameter and contain tiny globules in the cytoplasm however; the presence of nucleus in the cytoplasm could be seen. The globules appeared to be around the nucleus. Epithelial cells vary in size (~ 19 to 22 µm in diameter) but they have very distinct centrally located nucleus. These cells have clear endoplasm and darker ectoplasm surrounding it. Besides these cells, hemocytes, a relatively larger cell of ~ 30 to 38 µm are also seen. This cell's cytoplasm appears darker and nucleus gets obliterated due to the darker cytoplasm.

The concentrations of DNA, RNA and proteins were equivalent to ~ 2.1 pg/cell, 0.6 pg/cell and 1.8 ng/ cell respectively (Table 3.8).

3.1.2 Primary culture of hepatopancreas

3.1.2.a Explant culture :- The explants of hepatopancreas appeared as finger like projections under light microscope (Figure 3.3.A). The enlarged view of finger like projections showed the presence of Restzellen and Blasenzellen cells on the surfaces (Figure 3.3.B). In general, the surface looked leathery in appearance (Figure 3.3.B). Cell migration from the explants occurred within 4 hours of initiation of culture and was dependent on the medium used for explant culture. Media such as 3 x L15, L15 + crab saline + glucose with an osmolality of 1050 mOsm/kg were found to be effective for cell migration and survivability of hepatopancreatic cells. The explants gave rise to blebs (Figure 3.3.D) in the initial phase of cell culture and cells from the blebs progressively migrated (Figure 3.3.C,D) and formed a confluence. The cells forming the confluence were identified under phase contrast microscope on the basis of morphology and histology. The morphological and histological analyses of these cells revealed that there existed five different types of cells such as restzellen (R), fibrenzellen (F), blasenzellen (B), embryonalzellen (E) and granular (G) cells (Figure 3.5). The study of cell migration pattern showed that the granular cells migrated first from the explants, followed by blasenzellen and subsequently the remaining cell types (E, R and F cells) moved into the culture medium. Granular and embryonalzellen cells of hepatopancreas survived for longer period in most of the media tested, however B, R, F cells showed best survivability in 3 x Leibovitz - 15 medium supplemented with epidermal growth factor (EGF). The granular

cells formed a monolayer after five days of initiation of explant culture. However, rest of the cells did not survive for long.

3.1.2.b Cell Culture by disaggregation :- The cell suspension was obtained in centrifuge tubes containing citrate buffer after trituration of the hepatopancreas of *Scylla serrata.* Upon centrifugation of cell suspension at 150 g, two-layered pellet was obtained. The upper yellow layer was formed of mixture of E, F, B, R cells (Figure 3.4 - A,B,C), while the bottom white layer contained granular cells (Figure 3.26). The precisely separated layers of the pellet when inoculated in the culture media revealed the presence of different types of cells which had varying survivability (Table 3.4). After 8-10 days of initiation of cell culture B and F cells disintegrated from the culture while, E and R cells survived for 15 and 37 days respectively (Table 3.4.B). The granular cells survived for 192 days with intermittent replenishment of culture medium (Table 3.4.B). Among the various media tested, 3 x L15 medium supplemented with EGF and glucose, with an osmolality of 950 mOsm/kg was the most effective medium for the survival of granular cells (Table 3.4.B). Though mixed populations of F, B, R, E cells, have low survivability in comparison with G cells, their survivability enhanced relatively in 3 x Leibovitz 15 medium supplemented with EGF and glucose (Total osmolality 950 mOsm/kg).

Use of sera like Fetal bovine serum (FBS) and Fetal calf serum (FCS) as standard supplements to the culture media resulted in arrest of cell growth and cell viability. Hepatopancreatic cells cultured in the serum free media had higher cell viability than those grown in media containing sera. Fibrenzellen, blasenzellen, and restzellen cells were loosely attached to the substratum but embryonalzellen and granular cells showed firm attachment to the substratum with occasional cell proliferation.

3.1.2.c Characterization :-

E cells: These were round cells having a diameter of 23-28 µm. These cells had larger circular nucleus which appeared acentric (Figure 3.5.D).

R cells: These cells appear round or oblong and large with a size range of 21-42 µm. The cytoplasm is largely multi-vacuolated and each vacuole appears filled with lipid droplets as a result the cytoplasm is pushed to the periphery. However, in general one third of the cytoplasm looks denser with the presence of tiny granules. Many times the nucleus gets obliterated due to the presence of vacuoles (Figure 3.5.A).

F cells: These are generally oblong in appearance. However, sometimes they appear round depending upon the presence of cytoplasmic lipid droplets. These cells are somewhat look a-likes of R cells but they have a fewer lipid droplets in the cytoplasm. The cells range in size from 30-50 µm. The cytoplasmic vacuoles obliterate the nucleus (Figure 3.5.B).

B cells: These cells are 50-71 µm in diameter having many tiny vacuoles occupying about 80-90% of cytoplasm, and appear golden brown in colour (Figure 3.5.C). These cells are spheroid and the accumulation of large number of tiny vacuoles, obliterate cytoplasm and nucleus.

Granular cells: These are tiny cells having a diameter of ~1-5 µm and appear whitish in colour (Figure 3.26.B).

The hepatopancreatic cells' biochemical analyses (except of granular cells) revealed that the concentrations of DNA, RNA and proteins were equivalent to 1.77

pg/cell, 0.47 pg/cell and 1.17 ng/cell respectively in general (Table 3.8). The biochemical assays of granular cells are described separately under the characterization of granular cells. Instead of characterizing each cell with reference to RAPD analysis it was preferred to perform RAPD analysis of the mixed cell population of cultured hepatopancreatic cells and then compare with RAPD of hepatopancreas. The RAPD profile of hepatopancreas was generated using primer UBC457 and YNZ22 (Figure 3.6). Lane M depicts expression of 100 bp ladder in the range of 100-5000 bps. Lane 1 presents expression of RAPD bands of parental tissue amplified using primer UBC457. Lane 2 shows RAPD bands of cultured hepatopancreatic cells using the above mentioned primer. Lane 3 and 4 represent expression of RAPD bands of parental tissue and cultured cells of hepatopancreas which were amplified using YNZ22 primer. Both Lane 3 and 4 share identical bands with reference to band size (Figure 3.6). After the closer and careful observation of RAPD profiles, it is realised that the bands appear quite lighter for hepatopancreas and cultured cell mixture (lane 1 and 2) when amplified with primer UBC457. However, there was expression of bands beyond doubts. These bands appear at 500, 700 and 1000 bps levels. Further, the RAPD profiles obtained with Primer YNZ22 exhibit the presence of two amplified bands of about 1400 and 2000 bps. These results indicate that there is better expression of bands with YNZ22 in comparison to what is expressed with UBC457.

The sequences of primers (UBC457 and YNZ22) as well as the size range of parental and cultured cell DNA generated using each primer is presented in Table 3.9. The parental size range matched with the cultured cells DNA range indicating that the cultured cells were derived from parental tissue of *Scylla serrata.* Since the RAPD profile of cells of gills, cardiomyocytes, muscles, hemocytes, neurons and ovaries were exactly identical

to that obtained with hepatopancreatic cells they are neither described separately nor presented separately in the form of figures and tables.

3.1.3 Primary culture of hemocytes

The hemocytes in general exhibit anchorage to the substratum but have a tendency to settle like the formed elements of mammalian blood. These cells showed maximum survivability in 2 x Leibovitz 15 medium (osmolality, 1050 mOsm/kg) for a period of 14 days. The addition of EGF (10 ng/ml) to culture media promoted cell longevity to some extent (16 days), however, increased concentration of EGF (20 ng/ml) did not enhance the cell longevity. The hemocytes in general vary in size range from ~ 8 to 50 μm (Figure 3.7.A,B).

3.1.3.a Characterization :- The culture of hemocytes from the hemolymph of the crab revealed the presence of three types of hemocytes namely hyalinocytes, semi-granulocytes and granulocytes. Hyalinocytes were spheroid and oval in shape. The hyalinocytes have a distinct centric nucleus which is surrounded by either clear or slightly granular cytoplasm. The cell size ranged from ~ 8 to 18 μm (Figure 3.7-B, h). Semi-granulocytes: are oblong and spheroid in shape. A faint oblong nucleus is seen in the semi-granulocytes which are oblong while in spheroid types it is distinct but acentric. The cell size ranged from ~ 12 to 20 μm in diameter (Figure 3.7-B, s). The granulocytes of hemocytes are relatively large spheroidal cells with evenly distributed granules in the cytoplasm. Many a time the nucleus is not seen clearly owing to the granular cytoplasm. Granulocytes vary in range from ~ 30 to 50 μm in diameter (Figure 3.7-B, g).

In general the hemocytes have DNA, RNA and proteins in concentrations equivalent to ~0.4 pg/cell, 0.1 pg/cell and 1.3 ng/ cell respectively (Table 3.8).

3.1.4 Primary culture of heart cells (Cardiomyocytes)

3.1.4.a Explant culture :- Within one hour of planting heart tissue fragments (Figure 3.8.A) in culture medium, hemocytes of varying sizes began to migrate out instead of cardiomyocytes (Figure 3.8.B). These cells showed tendency to anchorage to any type of substratum (Collagen, Laminin, and Poly-L-Lysine) offered to them. Small blebs (outgrowth) started projecting from these explant fragments after four days of initiation of culture (Figure 3.8.C). No distinct migration of cardiomyocytes was observed from heat explants, however, a few fibroblast like cells migrated into the culture media, these cells showed anchorage to all the afore mentioned substrata. These cells survived for 6 to 8 days in 3 x Leibovitz 15 + FGF (10 ng/ml) + glucose (1.0 gm^{-1}) while use of other media and different combinations of supplements did not promote much migration, anchorage, proliferation and survival of cells. This led to use 3 x Leibovitz 15 + FGF (10 ng/ml) + 1.0 mg/ml glucose for testing the proliferation and survivability beyond 6 to 8 days (Table 3.7). However, this attempt to use different combinations of media and supplements yielded no significantly different results.

3.1.4.b Cell culture by disaggregation :- Mechanical and enzymatic dissociation techniques were employed to isolate cells from heart tissues. Among the two techniques, enzymatic dissociation was more suitable for cell isolation. Collagenase at a concentration of 0.3 mg/ml was effective for isolating cardiomyocytes in comparison to other enzymes tested (Table 2.5). Such isolated viable cells obtained by collagenase treatment survived in 3 x Leibovitz 15 medium supplemented with FGF (10 ng/ ml) + glucose (1.0 gm^{-1}) for a period of eleven days. The increase of FGF concentration beyond 10 ng/ ml did not enhance cell longevity (Table 3.3). Further, any medium with different combinations of

supplements did not produce any change in survivability rate or induce proliferation coupled with healthy longevity.

3.1.4.c Characterization :- The cardiomyocytes of crab appeared spindle shaped with a size ranging from ~11 to 13 μm in diameter particularly with reference to the main cell body. The cells exhibited fibre like extensions from the spindle and the length of the extension fibres varied from cell to cell giving a different look to the cells (Figure 3.9). These spindle shaped cells in a true sense had a centrally located distinct nucleus surrounded by compactly packed cytoplasm. Some of these cells had thick and long extensions while others had short. The nucleoplasm appeared granular with distinct chromatin. Those cells who lost cytoplasmic extensions during the slide preparation appeared round while others exhibited only one side extension.

The DNA, RNA and protein concentrations of cardiomyocytes were equivalent to ~ 1.3 pg/ cell, 0.3 pg/ cell and 1.23 ng/ cell respectively (Table 3.8).

3.1.5. Primary culture of muscle fibroblasts

3.1.5.a Explant culture :- The muscle explants kept in different media with different combination of supplements along with provision of different types of substrata did not exhibit any bleb formation or any kind of outgrowth for a period extended to one month from the initiation of primary culture as explant culture. This indicates the rigidity of the muscle explants towards bleb formation as well as migration of cells. Further use of 3 x Leibovitz 15 medium supplemented with FGF and glucose; a medium which was effective for cell longevity of fibroblast cells obtained by disaggregation technique had no influence on muscle explants (Figure 3.10.A).

3.1.5.b Cell culture by disaggregation :- Mechanical disaggregation technique was not effective for isolating muscle fibroblasts, but collagenase (0.3 mg/ml) solution prepared in 3 x Leibovitz - 15 resulted in obtaining viable fibroblast cells. These cells when cultured in 3 x Leibovitz - 15 medium supplemented with FGF (10 ng/ml) and glucose (1.0 mg/ml) with an osmolality of 902 mOsm/kg showed maximum survivability of 6 to 7 days. However, increased concentration of FGF to 15 ng/ml didn't enhance cell longevity. The other media in different combinations with supplements did not promote change in survivability, longevity as well as any proliferation of these fibroblasts. Therefore, these cells appear to be short living without any proliferation (Figure 3.10.B).

3.1.5.c Characterization :- The collagenase mediated separation of muscle fibroblasts yielded large number of cells. These muscle fibroblasts appeared thread like and criss-crossed in a culture medium. Some muscle fibroblasts were short while others were long but overall all of them appeared identical in shape (Figure 3.10.B). The cell size ranged from 10 to 15 μm in length. The light and phase contrast microscopy could not reveal the nuclear and cytoplasmic characteristics of these muscle fibroblasts.

DNA, RNA and protein concentrations of these muscle fibroblasts were equivalent to ~ 0.2 pg/cell, 0.09 pg/cell and 0.92 ng/ cell respectively (Table 3.8).

3.1.6 Primary culture of neurons

3.1.6.a Explant culture :- The success of neuronal explant culture of *Scylla serrata* depended upon the extent of removal of the ganglionic sheath surrounding the neurons present in the ganglion. When the ganglionic sheath was removed without disturbing the neurons in the ganglion followed by planting of such neuronal tissues in the culture plates

coated with either collagen, laminin or poly-L-lysine in different media with different combinations of supplements resulted in limited neuronal migration. The migrated neurons ranged in size from ~ 26 to 38 μm (Figure 3.11.A, B). The migrated neurons exhibited better survivability in 2xL15 medium supplemented with NGF (5 ng/ml) and glucose (1.0 mg/ml) with an osmolality equivalent to 1050 mOsm/kg

3.1.6.b Cell Culture by disaggregation :- The neuronal cell viability was less when mechanical dissociation technique was employed. However the use of enzymatic (Trypsin 0.1 % dissolved in 3 x L15) dissociation technique yielded better results. The enzymatically dissociated cells exhibited cell survival for 12 days when confronted with 2 x L15 medium supplemented with NGF (5 ng/ml) and glucose (1.0 gm^{-l}) (Table 3.3). No other media and combination of supplements promoted survivability for more than 12 days with some proliferation. The neurons tended to attach to Poly-L-Lysine substratum after forming aggregations (Figure 3.12). The neurital outgrowth even as stems was not observed during the survival period of 12 days.

3.1.6.c Characterization :- Cerebral ganglionic neurons after plating appeared round with a distinct and large nucleus. The nucleoplasm appeared darker than the surrounding cytoplasm. The ratio of cytoplasm to nucleoplasm was less than one. The early telophase stage of mitosis was observed in the cultured neurons that were undergoing mitosis. Such cells showed a prominent groove on the sides indicating the beginning of formation of cytokinetic plate. The neurons undergoing mitosis exhibited equal and unequal type of cell divisions. However, most of the neurons exhibited equal cell division. The nuclei of the dividing neurons appeared darker than those of non dividing neurons. The cytoplasm of the

neurons appeared granular. The cell size ranged from 26 to 38 μm and by virtue of the cell size one may broadly classify the larger cells as glia and smaller cells as neurons.

The DNA, RNA and protein concentrations of the neurons were equivalent to ~0.8 pg/cell, 0.14 pg/cell and 1.91 ng/ cell respectively (Table 3.8).

3.1.7 Primary culture of ovarian cells

3.1.7.a Explant culture :- The explants of ovaries survived for different length of time in different types of culture media provided to them. The explants showed bleb like projections from which subsequently the cells migrated. The cell migration from the blebs of the explant began within a few hours of initiation of explant primary culture (Figure 3.13.A, B, C). The migrated cells had a tendency to anchor to the substratum and proliferate very slowly. However, these cells did not show any substratum specificity for anchorage and they formed a monolayer within 48 hours of planting the tissue fragments in the medium. Since, mitosis of cells was not observed in the migrating cells, it appears that all the confluent monolayer forming cells had come into the medium by the process of cell migration from the explant. The most suitable medium for the cell growth and longevity of ovarian culture was L15 + Crab saline (1:1 v/v) as the cells survived healthily for 27 days. The addition of epidermal growth factor enhanced cell longevity to a period of 40 days.

3.1.7.b Cell culture by disaggregation :- Ovarian cells were isolated from the tissue by mechanical dissociation technique using artificial seawater having osmolality equal to 1050 mOsm/kg. Among the various media tested L15 + Crab saline (1:1 v/v) gave a better cell survivability in comparison to other media as shown in Table 3.2.F. In order to determine the optimum conditions viz-a-viz combination of media and supplements for

promotion of cell growth and proliferation, the various concentrations and / or proportions of epidermal growth factors, FBS, FCS, Insulin, transferrin were tested. These permutation combinations of media and the supplements revealed that Leibovitz 15 + Crab saline (1:1 v/v) medium supplemented with EGF (20 ng/ ml) and 1mg/ml glucose was the most suitable medium combination at osmolality equivalent to 1050 mOsm/kg. In this combination of medium and supplements, the cells remained viable with proliferation for a period of 40 days.

These cells showed firm anchorage to the substrata like Laminin, collagen and poly-L-lysine. The comparison of cell survivability and proliferation of cells obtained through explant culture and enzymatic dissociation culture techniques indicated that the enzymatic cell dissociation technique was most suitable.

3.1.7.c Characterization :- Ovarian cells were of different shapes, most of these cells were round, nearly round and some were slightly elongated (Figure 3.14.A). The cytoplasm was evenly distributed in the cells. The cell size ranged from ~ 7 to 14 μm in diameter. Though, the nucleus is prominent and nearly centrally located it is not always so (Figure 3.14.B). Many times the larger cells had smaller centric nuclei. The medium sized cells had larger nuclei than those found in smaller cells. However, the smaller cells had tiny nuclei at the centre or off the centre. In all the cells the plasma membrane appeared distinct, and darker under phase contrast. The ratio of cytoplasm to nucleoplasm appeared to be more than one.

The DNA, RNA and protein concentrations of ovarian cells were equivalent to ~1.8 pg/cell, 0.6 pg/cell and 1.32 ng/ cell respectively (Table 3.8).

3.1.8 Primary culture of testicular cells

3.1.8.a Explant culture :- When testicular tissue explants were planted in MWPs, within half an hour the explant tissues liberated cells in all directions in the culture medium (Figure 3.15.A). These liberated cells migrated further to form a confluence monolayer and during this process they attached to the substratum (Figure 3.15.B). The migrated cells did not exhibit any substratum specificity, particularly with reference to chemical nature as well as the thickness of the material used to coat the MWPs. When confronted with collagen, laminin and Poly-L-Lysine the migratory cells responded to them equally well as evidenced by their attachment. A rapid migration of spermatogonial cells occurred from the explant tissues and when explant had epididymal region (Figure 3.16.A) associated with it, vesicle like structures loaded with cells emerged from the main tissue fragments and liberated more cells (Figure 3.16.B). Within 24 hours, 70 % of cell confluence was achieved by cells ranging from ~ 10 to 38 µm in size. These cells had nuclei of varying sizes and shapes (Figure 3.17.A, B). The explants of testes implanted in different media with permutation combinations of supplements as given in Table 3.1 exhibited variations in cell migration patterns, cell survivability and proliferation. The explants which were inoculated in media such as 1 x, 2 x and 3 x Leibovitz 15 exhibited cell migration after half an hour of inoculation but as the time progressed less cell migration was observed, however, in Leibovitz 15 + crab saline medium the cell migration continued till a confluent monolayer was formed. The addition of supplement such as EGF enhanced cell migration, formation of confluent monolayer and cell proliferation. The concentration of EGF supplement also had influenced cell proliferation especially any concentration less than 20 ng/ml had no significant influence on cell proliferation. The use of insulin, transferrin did not enhance cell migration, cell survivability as well as cell proliferation.

3.1.8.b Cell culture by disaggregation :-

Mechanical dissociation of tissue using artificial sea water was found to be effective technique to isolate cells with 95 % viability. These viable cell showed variation in survival periods when different media combinations were used for culture purpose. Different media combinations and supplements (Epidermal growth factor - EGF, transferrin and Insulin) were tested to find a suitable media formulation that can promote proliferation and longevity of primary cultures. Leibovitz 15 medium prepared in crab saline with osmolality of 1050 mOsm/kg promoted survivability of <150 days in comparison to other culture media (Table 3.5), and therefore it was used for subsequent studies for testing cell proliferation using supplements. The variation in the concentrations of EGF, transferrin and insulin in the media individually as well as in different permutation combinations indicated that individually supplements like transferrin and insulin had no apparent influence on cell survivability and cell proliferation. Further, MTT assays on cells grown with suitable media and supplements in combination indicated L15 - crab saline supplemented with EGF (20 ng/ml) and glucose (1.0 mg/ml) facilitated maximum proliferation of cultures (Table 3.6.A). With insulin, transferrin supplements the proliferation of cells was poor. The chemically defined medium (CDM = Leibovitz 15 + crab saline supplemented with EGF 20 ng/ml, glucose 1.0 mg/ml) supplemented with different concentrations and types of sera when tested indicated that all sera diluted to 5 % (v/v) support cell proliferation. Therefore, CDM and 5 % serum combination when used yielded higher rate of cell proliferation and longevity of the cultures in comparison to those tested with higher concentrations of sera. Among the sera supplements, 5% horse serum promoted better results (Table 3.6.B). However, MTT assay revealed that CDM was most suitable for cell proliferation and longevity in comparison with sera supplements (Table 3.6.B).

3.1.8.c Sub culture of testicular cells :-

The sub cultures of testicular cells were attempted on fourth, fifth and sixth day after initiation of primary culture at a requisite density of 2×10^5 cells / ml in a chemically defined medium for developing a cell line. It was observed that the sub cultures executed on the sixth day of primary culture gave better results with reference to health, survivability and proliferation of cells. The health of the sub cultured cells was assessed by microscopic examination for morphological changes such as cell swelling, cell shrinkage and pycnotic nuclei as well as for cell injuries by Trypan blue dye exclusion technique. Subsequent subcultures were made at an interval of two weeks and the cells appeared healthy and proliferating. These subcultures survived ten subsequent passages and remained healthy and proliferated for more than four months.

The propagation and proliferation of sub cultured cells with every passage lead to establishment of a cell line. The cell line establishment was confirmed by going for sub culture of sub cultured cells every week till the tenth generation of the cells was obtained. However, technically sub culturing from the first sub cultured cell population that is propagating and proliferating, is ipso facto a development of a cell line. Further this secondarily sub cultured cell population proliferated and tolerated repeated sub culturing after a gap of one week. This is how a cell line was established and sub cultured for ten passages.

Table 3.1 Medium and the supplements that promoted cell migration, survivability of explant fragments of different tissues

Culture Medium tested for cell survivability				
Sr. No	**Medium**	**Diluents**	**Osmolality (mOsm /kg)**	**Maximal days of survival**
A. Gill cells.				
	2 x Leibovitz 15	ASW	1050	4-5 days
C. Heart cell				
	3 x Leibovitz 15 + FGF (10 ng /ml) + glucose (1.0 g^{-1})	MQW	902	6-8 days
D. Muscle fibroblasts.				
	3 x Leibovitz 15+ FGF (10 ng/ml) + glucose (1.0 g^{-1})	MQW	902	6-7 days
E. Neuronal cells				
	2xL15 medium + NGF (5 ng/ml)+ glucose (1.0 g^{-1})	ASW	1050	12 days
F. Ovarian cells				
	L15 + CS + EGF (20 ng/ml) + glucose (1.0 g^{-1})	MQW	1050	40 days
E. Testis				
	L15 + CS + EGF (20 ng/ml) + glucose (1.0 g^{-1})	MQW	1050	90 days

Table 3.2 Culture media tested for cell longevity of different dissociated tissues of *Scylla serrata*

Culture Medium tested for cell survivability				
Sr. No	**Media**	**Diluents**	**Osmolality (mOsm /kg)**	**Maximal days of survival**
A. Gill cells.				
1	1 x Leibovitz 15	ASW	1040	2-3 days
2	2 x Leibovitz 15	ASW	1050	4-5 days
3	3 x Leibovitz 15	MQW	902	2-3 days
4	L15 + crab saline (1:1- v/v) + glucose (1.0 g^{-1})	MQW	1082	3-4 days
5	2 x L15 + crab saline (1:1 – v/v) + glucose (1.0 g^{-1})	ASW	1088	2-3 days
6	ASW + glucose (1.0 g^{-1})	MQW	1050	2 -3days
7	F-12 Nutrient mixture + ASW + glucose (1.0 g^{-1})	MQW	1050	2-3 days
8	McCoy's Medium modified+ASW+glucose (1.0 g^{-1})	MQW	1040	1-2 days
B. Hemocytes				
1	1 x Leibovitz 15	ASW	1030	4-5 days
2	2 x Leibovitz 15	ASW	1050	11-14 days
3	3 x Leibovitz 15	MQW	902	2-3 days
4	L15 + crab saline (1:1- v/v) + glucose (1.0 g^{-1})	MQW	1050	8-9 days
5	2 x L15 + crab saline (1:1- v/v) + glucose (1.0 g^{-1})	ASW	1058	11-12 days
6	ASW + glucose (1.0 g^{-1})	MQW	1050	4-5 days
7	DMEM	ASW	1045	3-4 days
8	F-12 Nutrient mixture + ASW + glucose (1.0 g^{-1})	MQW	1050	4-5 days
9.	McCoy's Medium modified+ASW+glucose (1.0 g^{-1})	MQW	1045	2-3 days
C. Heart cell (Cardiomyocytes)				
1	1 x Leibovitz 15	ASW	1030	2-3 days
2	2 x Leibovitz 15	ASW	1050	5-6 days
3	3 x Leibovitz 15	MQW	902	6-8 days
4	L15 + crab saline (1:1- v/v) + glucose (1.0 g^{-1})	MQW	1050	2-3 days
5	ASW + glucose (1.0 g^{-1})	MQW	1058	2-3 days
6	M-199 + glucose (1.0 g^{-1})	ASW	1050	5-6 days
7	M-199 + salt mixture + glucose (1.0 g^{-1})	ASW	1053	5-6 days

Culture Media tested for cell survivability

Sr. No	Media	Diluents	Osmolality (mOsm /kg)	Maximal days of survival
D. Muscle fibroblast.				
1	1 x Leibovitz 15	ASW	1050	3-5 days
2	2 x Leibovitz 15	ASW	1050	3-4 days
3	3 x Leibovitz 15	MQW	902	4-6 days
4	L15 + crab saline (1:1 - v/v) + glucose (1.0 g^{-1})	MQW	1050	3-2 days
5	ASW + glucose (1.0 g^{-1})	MQW	1488	2 days
6	M-199 + glucose (1.0 g^{-1})	ASW	1050	3-5 days
7	M-199 + salt mixture (1:1 - v/v) + glucose (1.0 g^{-1})	ASW	1048	4-5 days
E. Neuronal cells				
1	1 x Leibovitz 15	ASW	1040	4-5 days
2	2 x Leibovitz 15	ASW	1050	8-10 days
3	3 x Leibovitz 15	MQW	902	7-8 days
3	3 x Leibovitz 15 + ASW	MQW	1050	8-10 days
4	L15 + crab saline (1:1- v/v) + glucose (1.0 g^{-1})	MQW	1050	7-8 days
5	2 x L15 + crab saline (1:1- v/v) + glucose (1.0 g^{-1})	ASW	1050	7-8 days
6	ASW + glucose (1.0 g^{-1})	MQW	1050	5-6 days
7	DMEM	ASW	1045	4-5 days
F. Ovarian cells				
1	1 x Leibovitz 15	ASW	1050	11-12 days
2	2 x Leibovitz 15	ASW	1050	15-16 days
3	3 x Leibovitz 15	MQW	902	16-17 days
4	L15+ crab saline (1:1- v/v) + glucose (1.0 g^{-1})	MQW	1050	20- 27 days
5	3 x Leibovitz 15 + Nutrient mixture F12 (HAM) + ASW (1:1:1 - v/v) + glucose (1.0 g^{-1})	MQW	1050	15-14 days
6	F-12 Nutrient mixture + glucose (1.0 g^{-1})	ASW	1050	8-9 days

Table 3.3 Culture medium, supplements tested for survivability of different tissues of *Scylla serrata*

a	Medium supplements with Growth Factors	
For gill tissue		
1.	2 X L15 + EGF (10 ng/ml) + glucose (1.0 g^{-1})	2-3 days
2.	2 X L15 + EGF (20 ng/ml) + glucose (1.0 g^{-1})	3-4 days
3.	2 X L15 + Insulin (0.06 IU/ml) + transferrin (5µm) + glucose (1.0 g^{-1})	2-3 days
4.	2 X L15 + 0.06 IU/ml Insulin + transferrin (5µm) + EGF (20 ng/ml) + glucose (1.0 g^{-1})	2-3 days
For Hemocytes		
1.	2 X L15 + EGF (10 ng/ml) + glucose (1.0 g^{-1})	16 days
2.	2 X L15 + EGF (20 ng/ml) + glucose (1.0 g^{-1})	12 days
3.	2 X L15 + Insulin (0.06 IU/ml) + transferrin (5µm) + glucose (1.0 g^{-1})	8 days
4.	2 X L15 + 0.06 IU/ml Insulin + transferrin (5µm) + EGF (20 ng/ml) + glucose (1.0 g^{-1})	11 days
For Heart		
1.	3 x Leibovitz 15 + FGF (5 ng/ml) + glucose (1.0 g^{-1})	11days
2.	3 x Leibovitz 15 + FGF (10 ng/ml) + glucose (1.0 g^{-1})	12 days
3.	3 x Leibovitz 15 + FGF (15 ng/ml) + glucose (1.0 g^{-1})	7-9 days
4.	3 x Leibovitz 15 + 0.06 IU/ml Insulin + transferrin (5µm) + EGF (10 ng/ml) +glucose(1.0g^{-1})	7-9 days
For Muscle		
1.	3 x Leibovitz 15 + FGF (5 ng/ml) + glucose (1.0 g^{-1})	5-7 days
2.	3 x Leibovitz 15 + FGF (10 ng/ml) + glucose (1.0 g^{-1})	6-7 days
3.	3 x Leibovitz 15 + FGF (15 ng/ml) + glucose (1.0 g^{-1})	4-5 days
4.	3 x Leibovitz 15 + 0.06 IU/ml Insulin + transferrin (5µm) + EGF(10 ng/ml) + glucose(1.0 g^{-1})	5-6 days
For Cerebral ganglion (Neurons)		
1.	2x Leibovitz 15 + NGF (1 ng/ml) + glucose (1.0 g^{-1})	6-7 days
2.	2x Leibovitz 15 + NGF (3 ng/ml) + glucose (1.0 g^{-1})	6-7 days
3.	2x Leibovitz 15 + NGF (5 ng/ml)+ glucose (1.0 g^{-1})	12 days
4.	2x Leibovitz 15 + 0.06 IU/ml Insulin + transferrin (5µm) + EGF(10 ng/ml) + glucose(1.0 g^{-1})	7-8 days
For Ovaries		
1.	Leibovitz 15 + Crab saline + EGF (10 ng/ml) + glucose (1.0 g^{-1})	32 days
2.	Leibovitz 15 + Crab saline + EGF (20 ng/ml) + glucose (1.0 g^{-1})	40 days
3.	Leibovitz 15 + Crab saline + Insulin (0.06 IU/ml) + transferrin (5µm) + glucose (1.0 g^{-1})	15 days
4.	Leibovitz 15 + Crab saline + 0.06 IU/ml Insulin + transferrin (5µm) + EGF (20 ng/ml) + glucose (1.0 g^{-1})	30 days

Table 3.4 Culture media and supplements used for primary culture of hepatopancreas of *Scylla serrata*

Culture No	Medium	Dilutent	Osmolality (mOsm /kg)	E		F		B		R		G	
A) Culture media				A	B	A	B	A	B	A	B	A	B
1	3 x Leibovitz 15	MOW	950	+++	10-15	++	9-10	+	4-5	++	30	+++	<153 days
2	3 x Leibovitz 15	ASW	1050	+++	10-15	++	8-14	+	5-9	++	30	+++	<160 days
3	Leibovitz 15 + Crab saline (1:1 - v/v) + glucose (1.0 g^{-1})	MQW	1030	+++	2-3	++	2-3	+	1-2	++	2-3	+++	<184 days
4	Leibovitz 15 + Crab saline (1:1 – v/v) + glucose (1.0 g^{-1})	ASW	1050	+++	5-6	++	2-3	+	1-2	++	3-4	+++	<182 days
5	3 x L15 + F-12 Nutrient mixture + ASW (1:1:1)	MQW	1050	+++	4--6	++	5-7	+	4-5	++	10-20	+++	<30-35 days
6	F-12 Nutrient mixture + glucose (1.0 g^{-1})	ASW	1040	+++	2-3	++	2-3	+	2-3	++	2-3	+++	<20-25 days
7	DMEM	ASW	1050	+++	2-3	++	1-2	+	1-2	++	2-3	+++	<10-13 days
B) Supplements added to the culture media													
1	3 x Leibovitz 15 + EGF (10 ng/ml) + glucose (1.0 g^{-1})	MQW	950	+++	10-15	++	8-10	+	7-9	++	33	+++	<186 days
2	3 x Leibovitz 15+ EGF (20 ng/ml) + glucose (1.0 g^{-1})	ASW	950	+++	10-15	++	8-10	+	8-9	++	37	+++	<192 days
3	L15 + CS (1:1- v/v) + EGF (10 ng/ml) + glucose (1.0 g^{-1})	MQW	1050	+++	3-4	++	2-3	+	3-4	++	5-6	+++	<140 days
4	L15 + CS (1:1 – v/v) + EGF (20 ng/ml) + glucose (1.0 g^{-1})	MQW	1050	+++	2-3	++	2-3	+	3-4	++	5-6	+++	<183 days

E : Embryonalzellen cell, F : Fibrenzellen cell, B : Blasenzellen cell, R : Restzellen cell, G : Granular cells, CS : Crab saline,
A : Attachment of cells, B : Maximum day of survival, + : Approximately 30-50% of cells adhere on the culture petridish,

++: Approximately 60-80% of cells adhere to the culture petridish, +++: Majority of cells adhere to the culture petridish. MQW : Mili Q water, ASW : Artificial sea water

Table 3.5 Culture media tested for longevity of testicular cell cultures of *Scylla serrata*

Culture Media tested for cell survivability							
Sr. No	Media	Diluents	Osmolality (mOsm /kg)	Substratum			**Maximum days of survival**
				A	B	C	
1	1 x Leibovitz 15	ASW	1050	+++	+++	+++	10 - 25 days
2	2 x Leibovitz 15	ASW	1050	+++	+++	+++	< 75 days
3	3 x Leibovitz 15	MQW	902	+++	+++	+++	< 90 days
4	L15 + CS (1:1-v/v)	MQW	1082	+++	+++	+++	< 150 days
5	3 x Leibovitz 15+ F12 Nutrient mixture + ASW (1:1:1)	MQW	1088	+++	+++	+++	< 123 days
6	F-12 (HAM) + glucose (1.0 g^{-1})	ASW	1050	+++	+++	+++	< 90 days
7	Sea water + glucose (1.0 g^{-1})	MQW	1050	+++	+++	+++	15 - 30 days

A : Collagen coated petri dishes.

B : Poly-L-lysine coated petri dishes.

C : Laminin coated petri dishes.

MQW : Mili Q water

ASW : Artificial Seawater

CS : Crab saline

+++ : Approximately 90–95 % of testicular cells adhere to the petri dish surface.

Table 3.6 Culture medium and supplements tested for survivability and proliferation of testicular cells of *Scylla serrata*. A) Medium supplemented with Growth Factors. B) Chemically Defined Medium supplemented with Sera

A	**Medium supplemented with Growth Factors**	
	Cell cultures tested for proliferation using MTT assay after 48 hr of Initiation of culture	O.D
1.	L15 + CS + glucose ($1.0\ g^{-1}$) (control culture medium).	0.30 ± 0.0024
2.	L15 + CS + EGF (10 ng/ml) + glucose ($1.0\ g^{-1}$)	0.34 ± 0.0005
3.	L15 + CS + EGF (20 ng/ml) + glucose ($1.0\ g^{-1}$)	0.43 ± 0.0037
4.	L15 + CS + Insulin (0.06 IU/ml) + transferrin (5µm) + glucose ($1.0\ g^{-1}$)	0.31 ± 0.0011
5.	L15 + CS + 0.06 IU/ml Insulin + transferrin (5µm) + EGF (20 ng/ml) + glucose ($1.0\ g^{-1}$)	0.39 ± 0.0003

B	**Chemically Defined Medium supplemented with Sera**	
	Cells tested for proliferation using MTT assay after 48 hr of Initiation of culture	O.D
1	L15 + CS + EGF 20 ng/ml + glucose ($1.0\ g^{-1}$) (control culture medium).	0.68 ± 0.0010
2	L15 + CS + EGF 20 ng/ml + glucose ($1.0\ g^{-1}$) + Horse Serum (5%)	0.43 ± 0.0045
3	L15 + CS + EGF 20 ng/ml + glucose ($1.0\ g^{-1}$) + Heat inactivated Horse Serum (5%).	0.40 ± 0.0050
4	L15 + CS + EGF 20 ng/ml + glucose ($1.0\ g^{-1}$) + Fetal Bovine Serum (5%).	0.37 ± 0.0015
5	L15 + CS + EGF 20 ng/ml + glucose ($1.0\ g^{-1}$) +Heat inactivated Fetal Bovine Serum (5%).	0.29 ± 0.0045

O.D : Indicates activity of mitochondrial dehydrogenase of live cells. Dead cells do no show +ve OD for MTT assay. Increase in OD indicates cell proliferation.

Table 3.7 Culture medium and supplements tested for cell longevity of different tissues of *Scylla serrata*

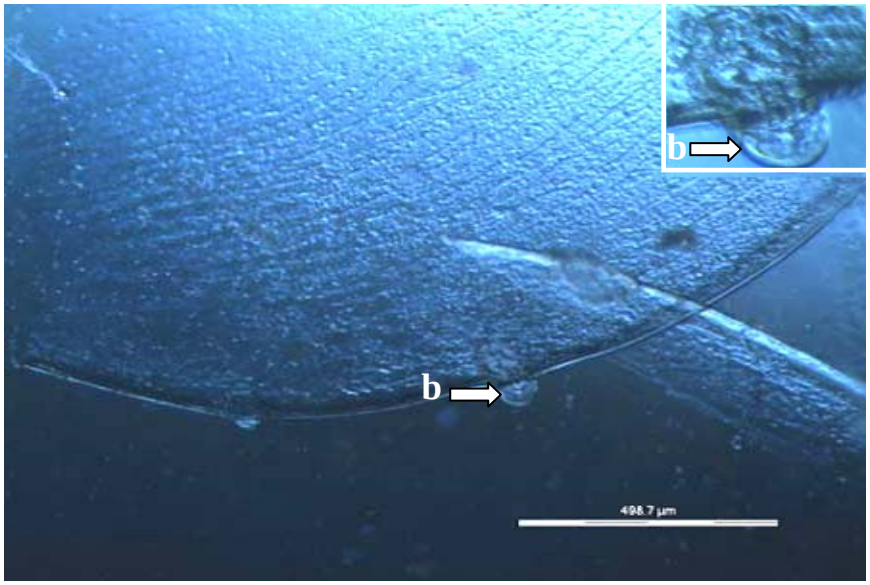
Tissue	Medium	Substrate			Maximum day of survival
		A	B	C	
Muscle	3 x Leibovitz 15 + FGF(10 ng /ml) + glucose(1.0 g^{-1})	+++	+++	+++	7 days
Gill	3 x Leibovitz 15	+++	+++	+++	5 days
Heart	3 x Leibovitz 15 + FGF (10 ng/ml) + glucose (1.0 g^{-1})	+++	+++	+++	12 days
Ovary	Leibovitz 15 + Crab saline + EGF (20 ng/ml) + glucose (1.0 g^{-1})	+++	+++	+++	40 days
Testis	L15 + CS (1:1- v/v) + glucose (1.0 g^{-1})	+++	+++	+++	Four months
Hepatopancreas	3 x Leibovitz 15+ EGF (20 ng/ml) + glucose (1.0 g^{-1})	+++	+++	+++	Embryonalzellen - 15 Fibrenzellen - 10 Blasenzellen - 9 Restzellen. - 37 Granular cells - 192
Neurons	2x Leibovitz 15 + NGF (5 ng/ml)+ glucose (1.0 g^{-1})	+++	+++	+++	12 days
Hemocytes	2 X L15 + EGF (10 ng/ml) + glucose (1.0 g^{-1})	_ _ _	_ _ _	_ _ _	16 days

A : Poly-L-Lysine
B : Collagen
C : Laminin.
FGF : Fibroblast growth factor
EGF : Epidermal growth factor
NGF : Nerve growth factor
CS : Crab saline
+++ : Approximately 90–95 % of testicular cells adhere on the petri dish surface
_ _ _ : No cell attachment

Table 3.8. Biochemical assay of cultured cells of different tissues

CELLS IN CULTURE	DNA	RNA	PROTEIN
Gill	2.1 pg/cell	0.6 pg/cell	1.82 ng/cell
Heaptopancreas	1.77 pg/cell	0.47 pg/cell	1.17 ng/cell
Hemocytes	0.4 pg/cell	0.1 pg/cell	1.3 ng/cell
Heart (cardiomyocytes)	1.3 pg/cell	0.3 pg/cell	1.23 ng/cell
Muscle	0.2 pg/cell	0.09 pg/cell	0.92 ng/cell
Neurons	0.8 pg/cell	0.14 pg/cell	1.91 ng/cell
Ovary	1.8 pg/cell	0.6 pg/cell	1.32 ng/cell
Testes	1.77 pg/cell	0.478 pg/cell	1.17 ng/ cell

A



B

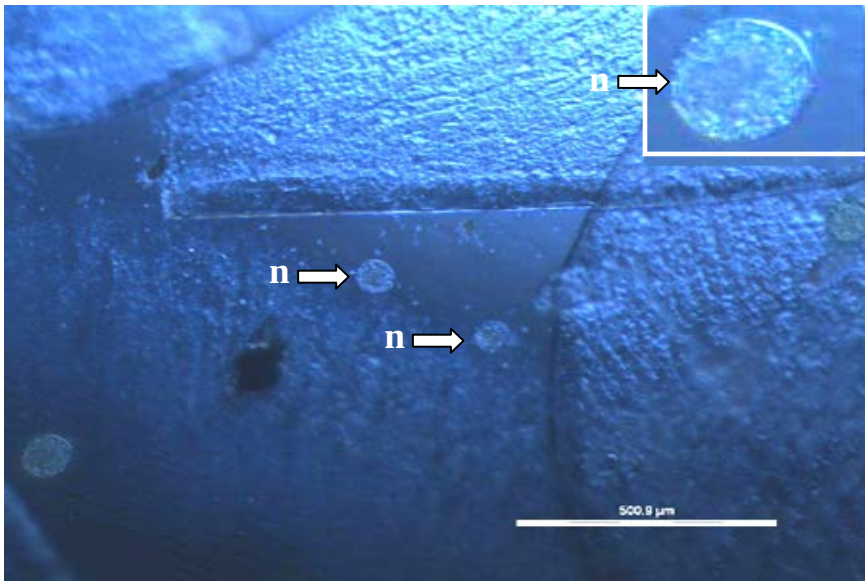


Figure 3.1 Explant culture of gills of crab *Scylla serrata*

A. Bleb (b) emerging from the gill fragment (M – 40 x), Inset: enlarged bleb (SB - 498.7 µm, M – 146 x)

B. Nephrocytes (n) migrating from the gill explant fragments (M – 40 x), Inset: enlarged nephrocytes (SB - 500.9 µm, M – 120 x)

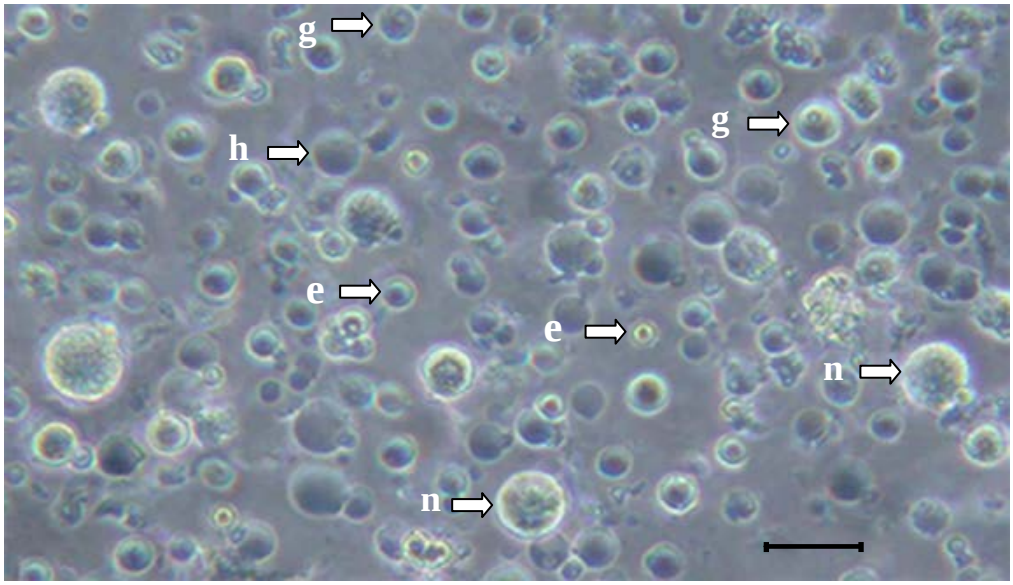


Figure 3.2 The figure shows different types of dissociated cells of the gills of *Scylla serrata* in primary culture. The cells marked with arrow and labeled (n) are nephrocytes while tiny cells labeled (e) are epithelial cells which have a distinct centrally located nucleus. Hemocyte (h) appears darker and looks like globule. Besides glycocytes (g) are also seen as cells larger than epithelial cells with larger dense depositions which many time appear like large nucleus. Scale bar 60 μm (M – 200 x)

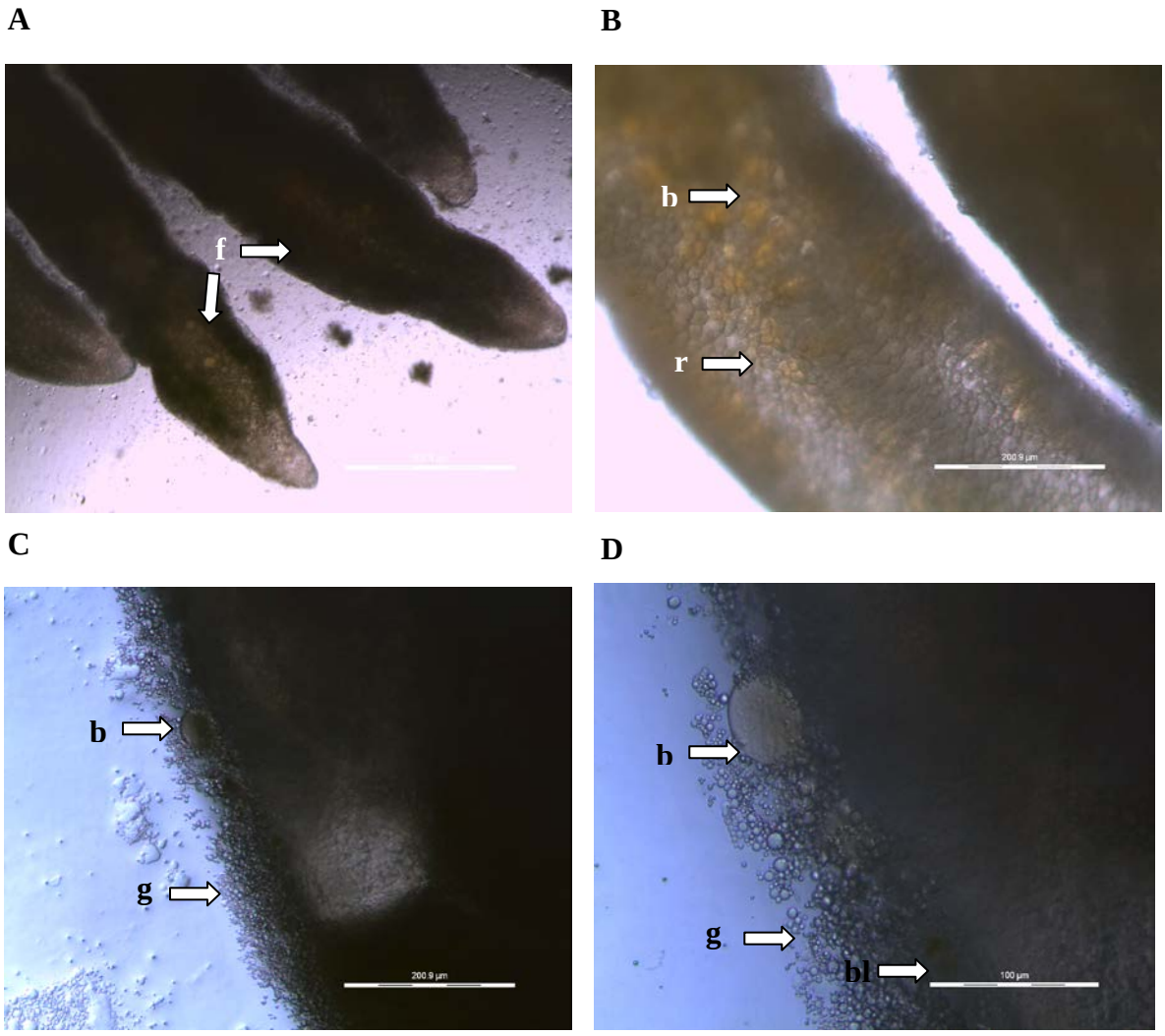


Figure 3.3 Explant culture of hepatopancreas

A. Finger like projections (f) (SB – 500.9 µm, M – 40 x)

B. Hepatopancreatic fragment showing embedded cells Blasenzellen (b) and Restzellen (r) (SB – 200.9 µm, M – 100 x)

C. Migratory cells from the fragment, Blasenzellen (b) and Granular (g) cells (SB – 200.9 µm, M – 100 x)

D. Enlarged picture showing cell migrations, Blasenzellen cell (b) and Granular cell (g), Bleb (bl) (SB – 100 µm, M – 200 x)

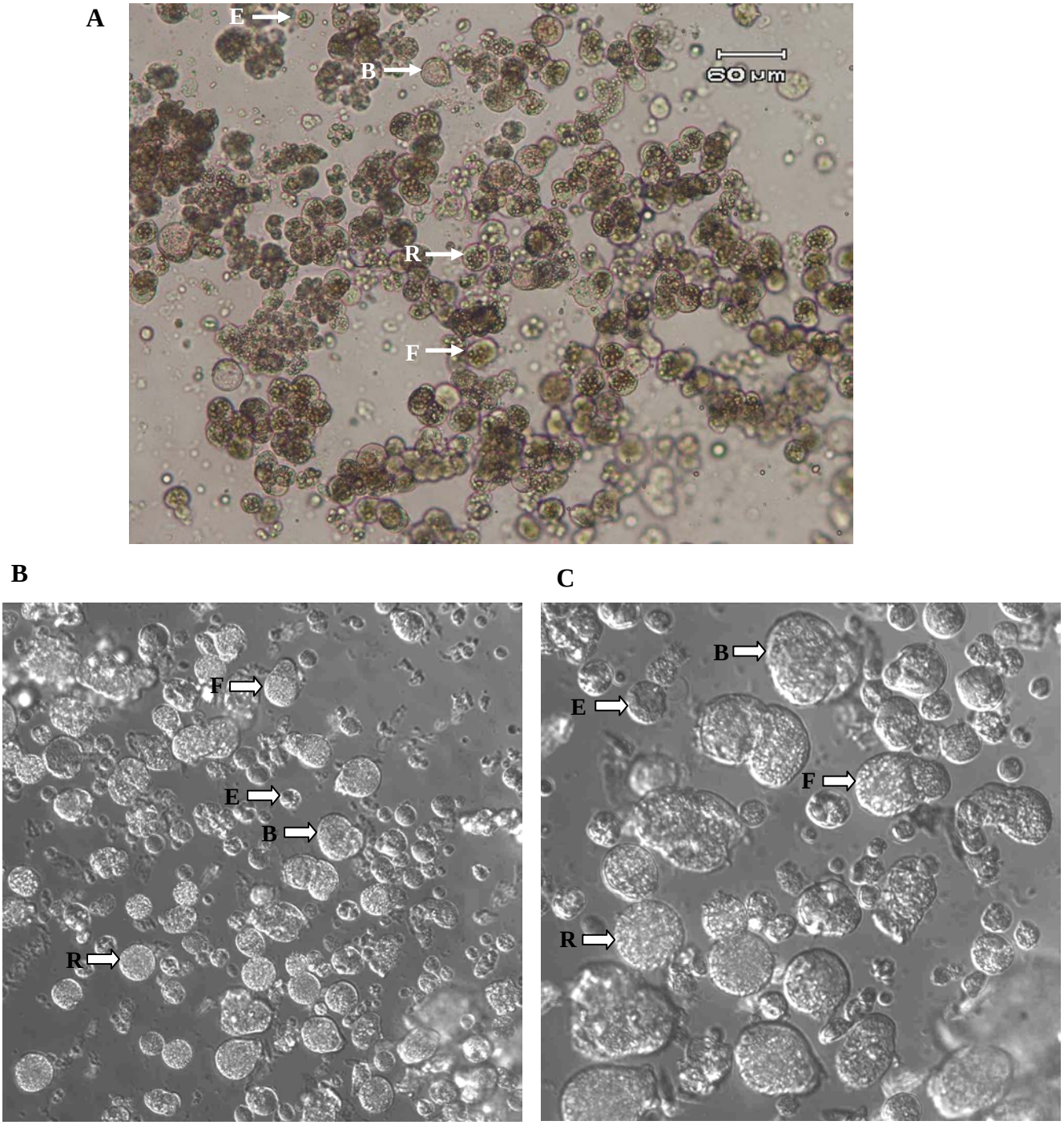


Figure 3.4 Culture of dissociated hepatopancreatic cells (Cells fixed in 2.0 % glutaraldehyde) of *Scylla serrata*

A. Light microscopic images showing hepatopancreatic cells such as (Embryonalzellen (E), Fibrenzellen (F), Blasenzellen (B), Restzellen (R) cells of *Scylla serrata* (M – 100 x)

B. Confocal images of hepatopancreatic cells (M – 200 x)

C. Enlarge picture of hepatopancreatic cells obtained using confocal microscopy (M – 400 x)

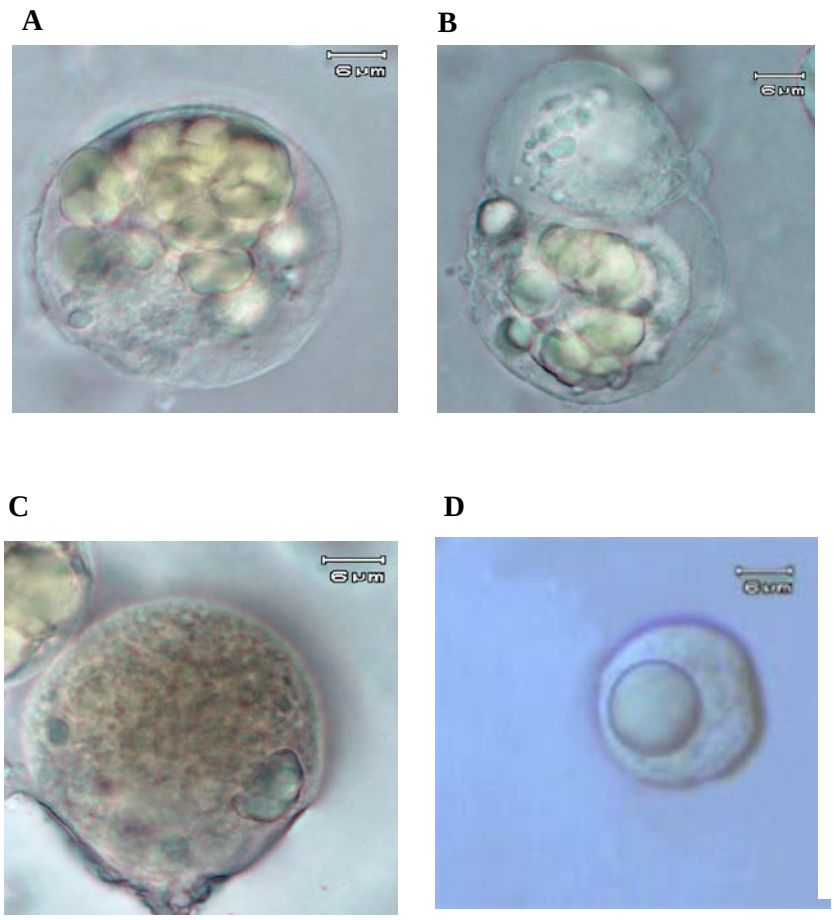


Figure 3.5 Different types of cells present in hepatopancreas of *Scylla serrata* (SB – 6.0 µm, M – 1000 x)

A. Restzellen cells – round or oblong cells having a size range of 21 – 48 µm

B. Fibrenzellen cells – oblong cells having a diameter 30 – 50 µm

C. Blasenzellen cells – round cells of 23 – 28 µm in diameter

D. Embryonalzellen cells – spheroid type cells having a diameter of 50 –57 µm

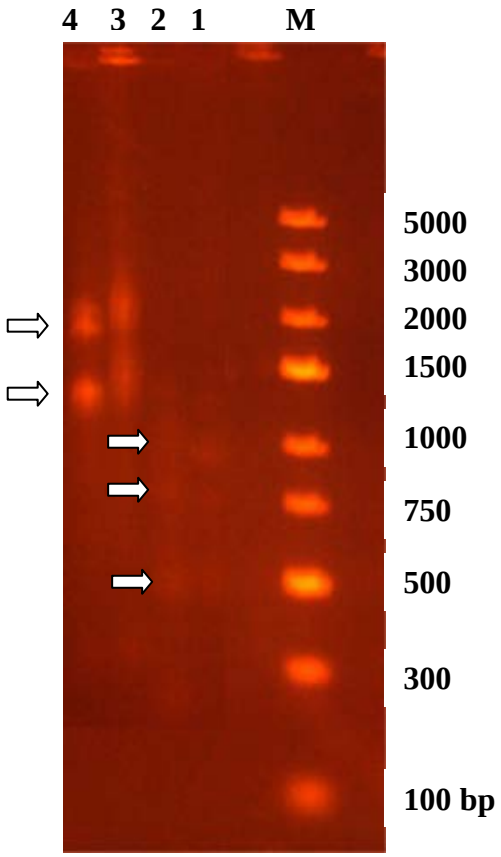
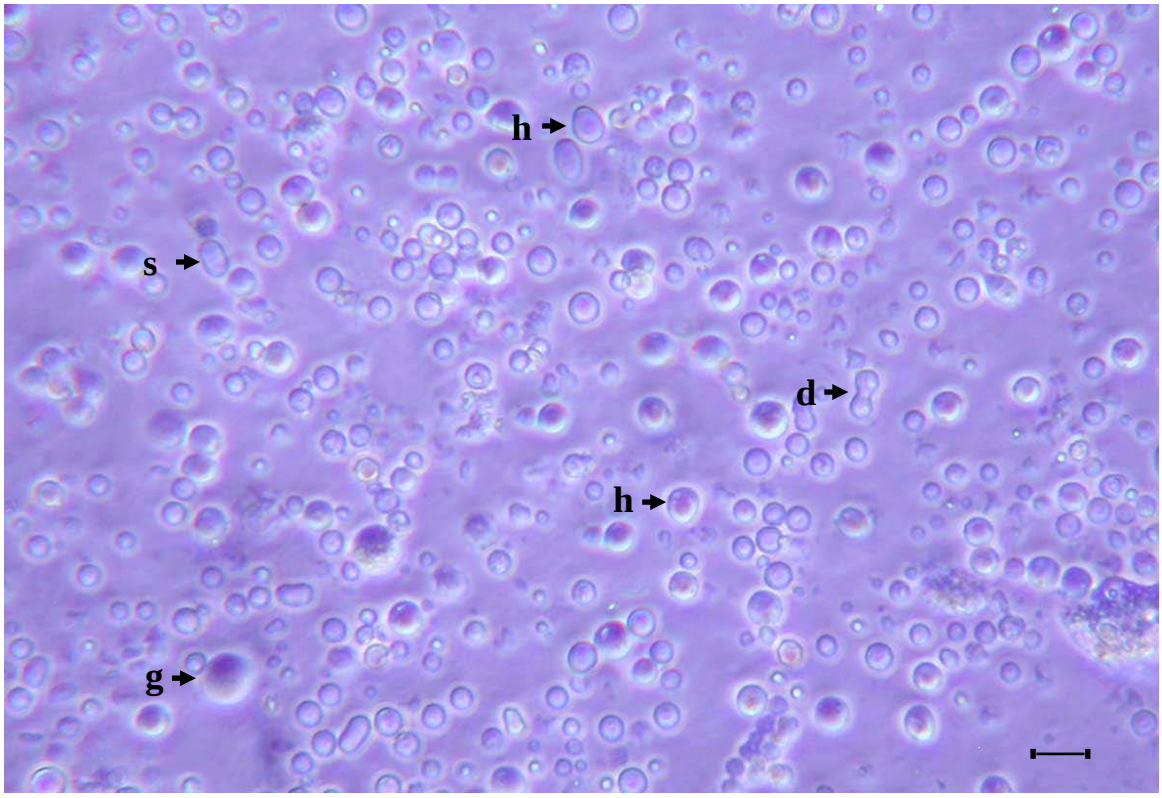


Figure 3.6 RAPD patterns of *Scylla serrata* using primers UBC457 and YNZ22, Lane M: DNA marker; Lane 1 - parental DNA amplified using primer UBC457, Lane 2 - cultured cell DNA amplified using primer UBC457, Lane 3 - parental DNA amplified using primer YNZ22, Lane 4 - cultured cell DNA amplified using primer YNZ22. Arrowheads indicate RAPD bands described in Table 3.9

Table 3.9 Sequences of UBC457 and YNZ22 primers and size ranges as well as RAPD bands obtained by using them

Primer	**Sequence**	**Parental DNA**	**Cultured cell DNA**
		Size range and (number of bands)	
UBC457	CGACGCCCTG	500 – 1000 (3)	500 – 1000 (3)
YNZ22	CTCTGGGTGTCGTGC	1400 – 2000 (2)	1400 – 2000 (2)

A



B

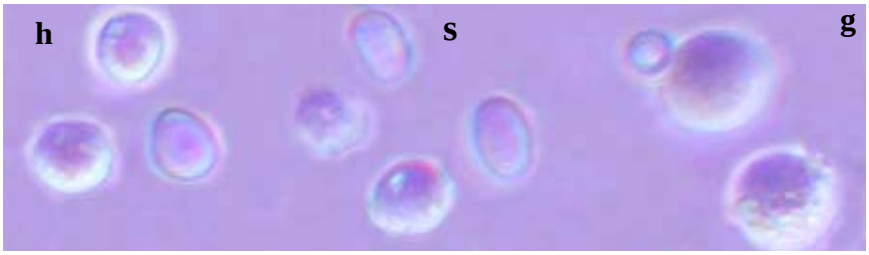


Figure 3.7 Culture of hemocytes of crab *Scylla serrata.*

A. The figure shows primary culture of different types of hemocytes, the cells marked with arrow and labelled (h) are hyalinocytes, (s) are semi-granulocytes and (g) granulocytes. Dividing cells are labelled as d (SB –6.0 µm, M –200 x)

B. Magnified view of different hemocytes picked up from figure. 3.7 for clarity of each cell type (M – 266 x)

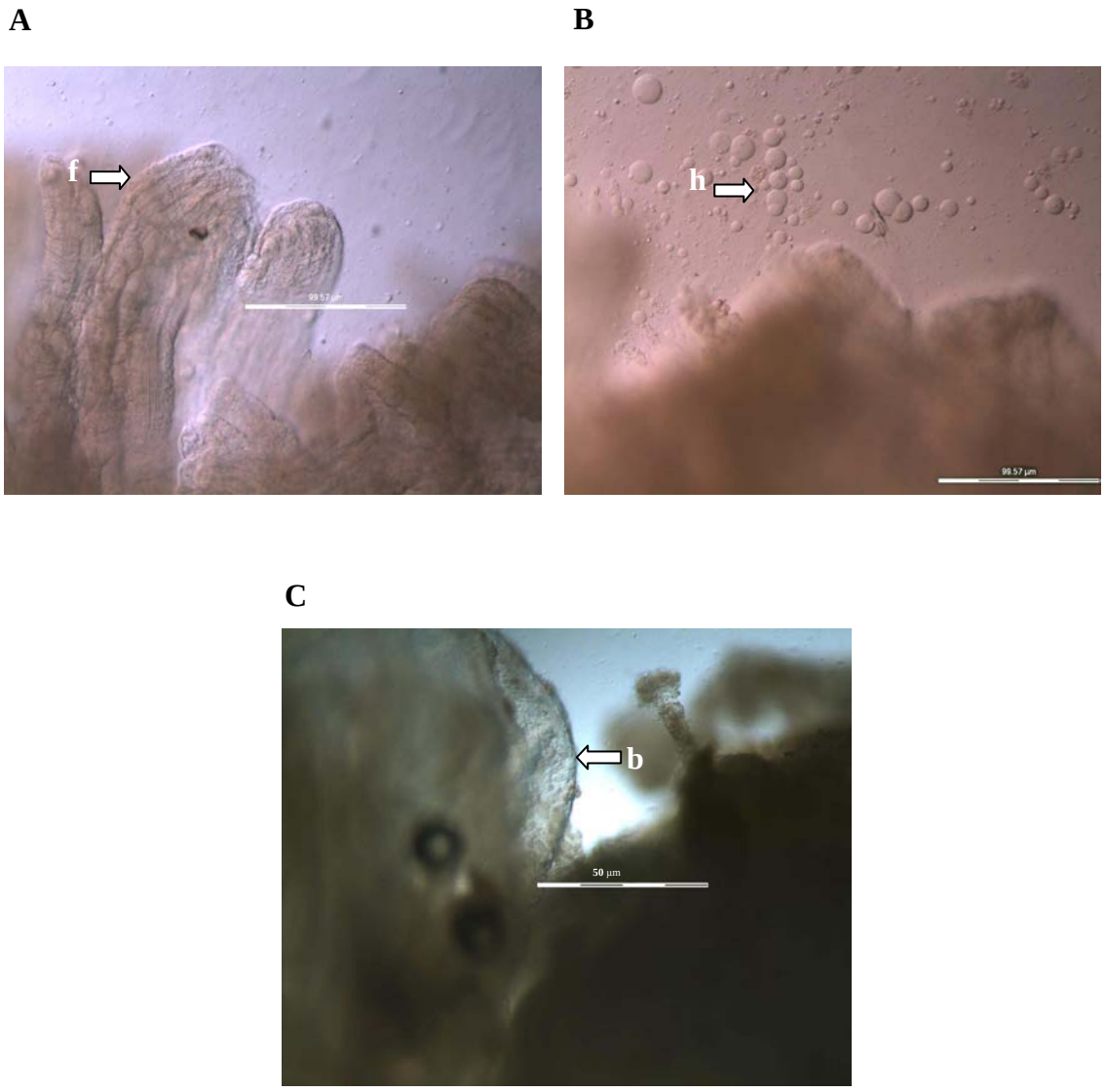


Figure 3.8 Explant culture of heart of *Scylla serrata*

A. Heart explant fragment (f) after 15 minutes of initiation of culture (SB – 99.57 µm, M – 200 x)

B. Hemocytes (h) migrating out from the heart explant fragments after one hour of tissue explant (SB – 99.57 µm, M – 200 x)

C. Bleb (b) projecting out from the explant heart fragment (SB – 50 µm, M – 400 x)

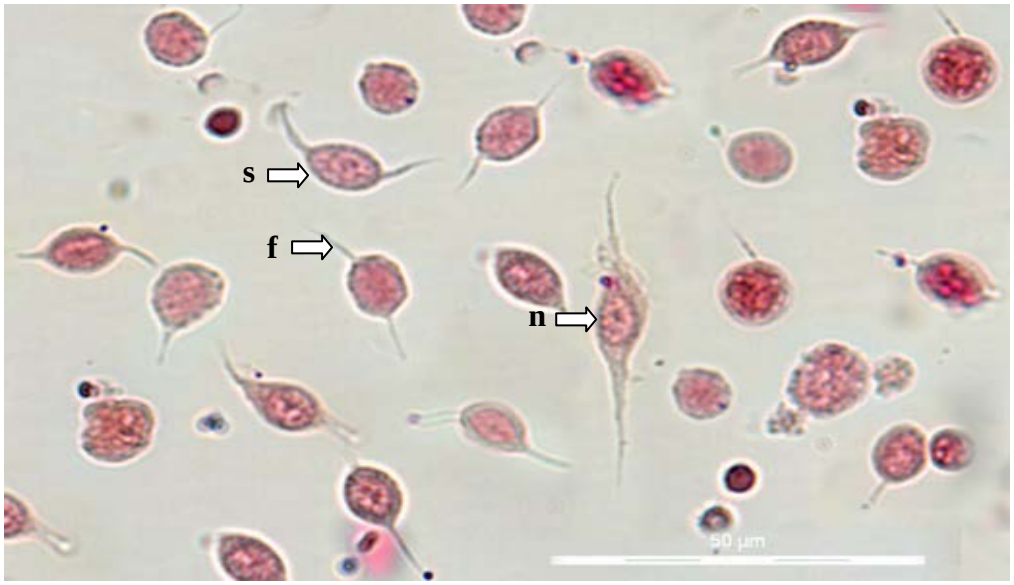
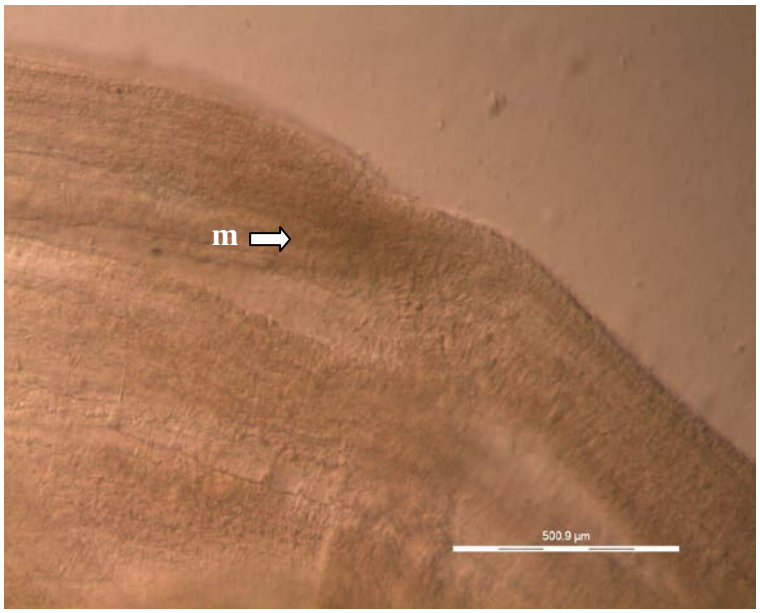


Figure 3.9 Culture of dissociated cardiomyocytes of *Scylla serrata*. The arrow head points, spindle shaped cardiomyocytes (s) with a prominent nucleus (n). These cells showed fiber (f) like extensions from the cell body (SB – 50 µm, M – 400 x)

A



C

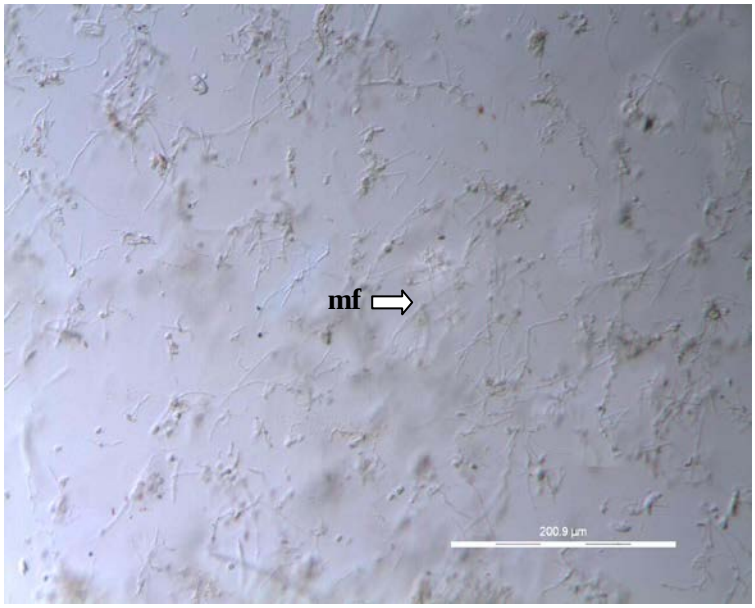
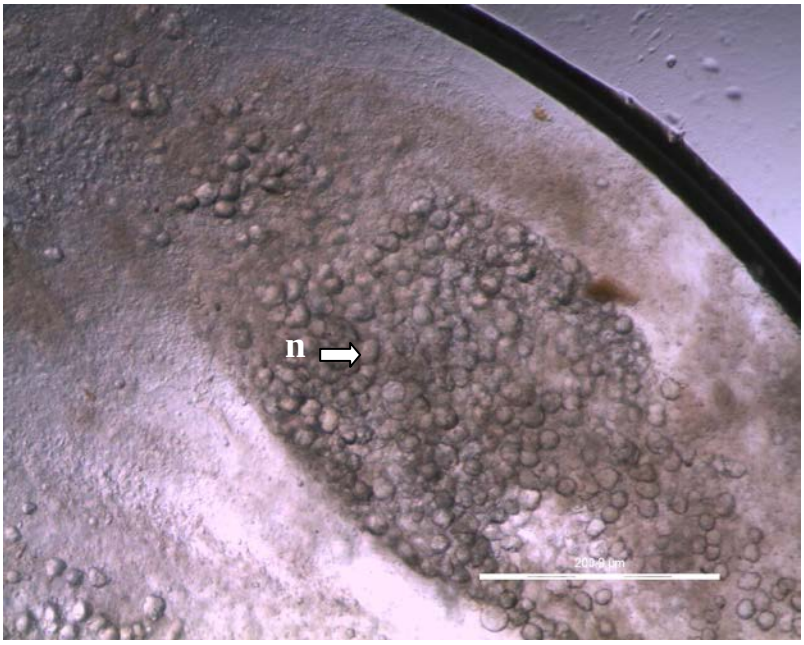


Figure 3.10 **A.** Explant of muscle fragments (m) of *Scylla serrata* (SB – 500.9 µm, M – 40 x)

B. Culture of dissociated muscle fibroblast (mf) (M – 100 x)

A



B

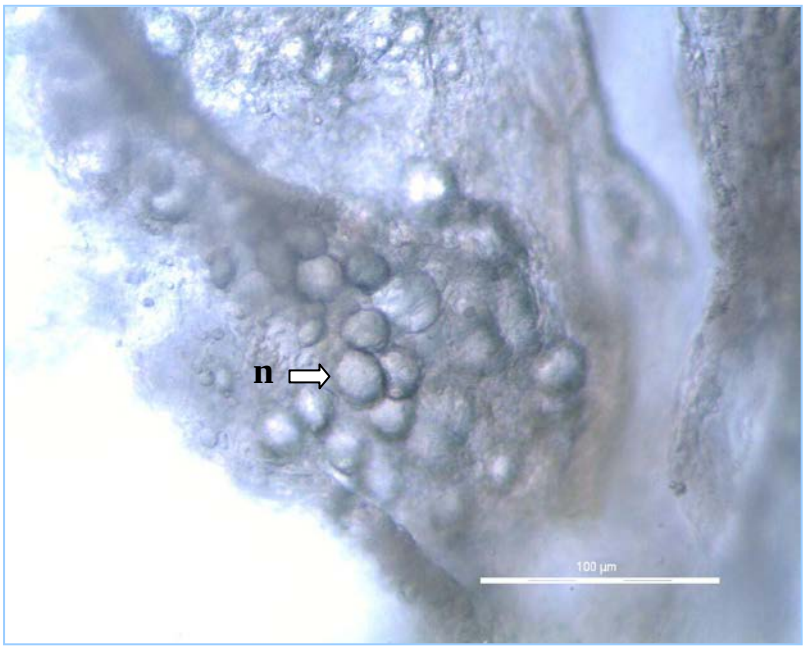


Figure 3.11 Explant of cerebral ganglion of *Scylla serrata*

A. Showing neurons in the explant fragment

(SB – 200.9 µm, M – 100 x)

B. Enlarged view of neurons (n) (SB – 100 µm, M – 200 x)

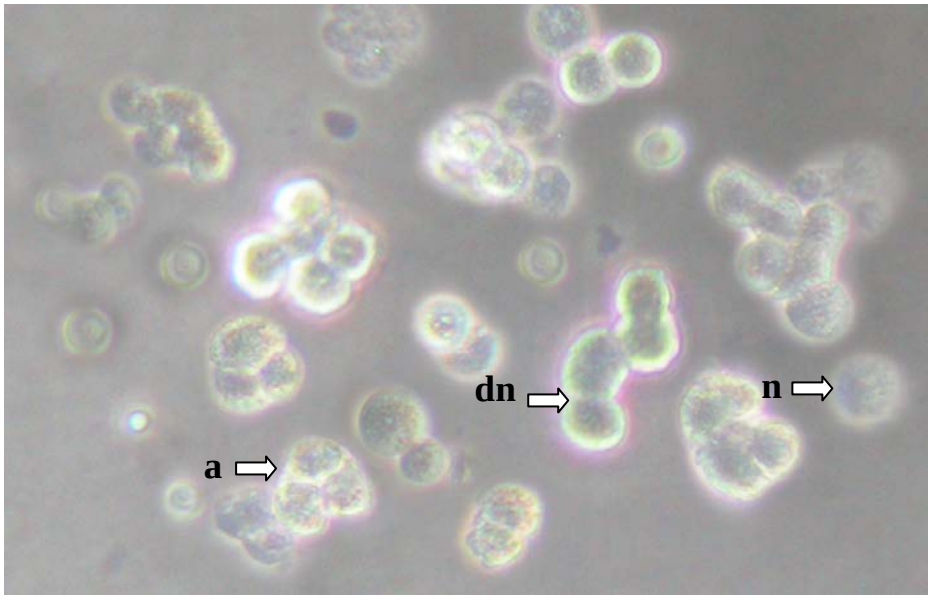


Figure 3.12 Culture of neurons of *Scylla serrata*. The arrow head points the neuron (n), dividing neuron (dn) and neuronal aggregation (a) (M – 200 x)

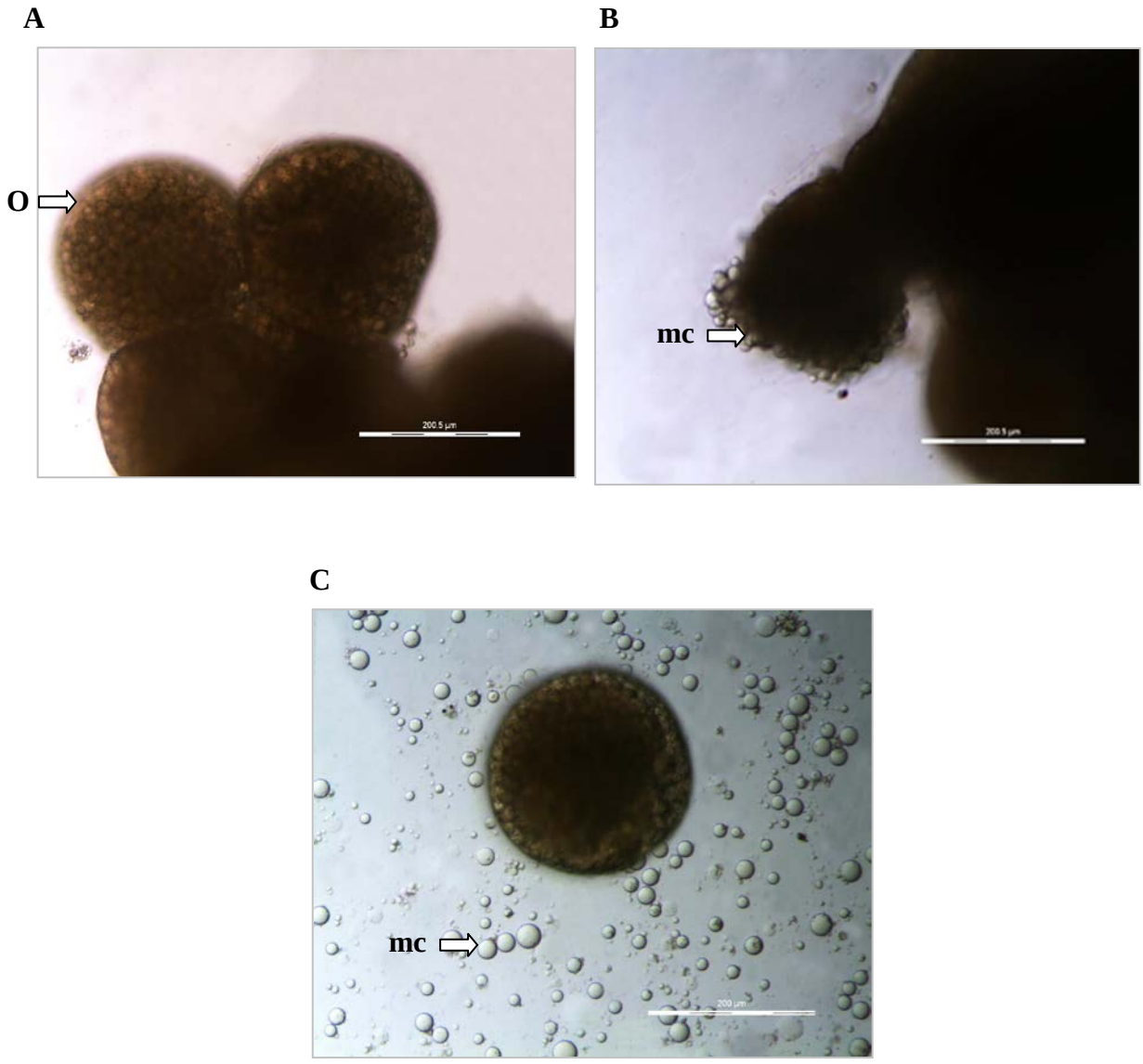


Figure 3.13 Explant cultures of ovary of *Scylla serrata.* (SB – 200 µm)

A. Explant of ovary (o). (M – 100 x)

B. Migratory cells (mc) of the explant of ovary (M – 100 x)

C. Migrated cells (mc) of the explant (M – 100 x)

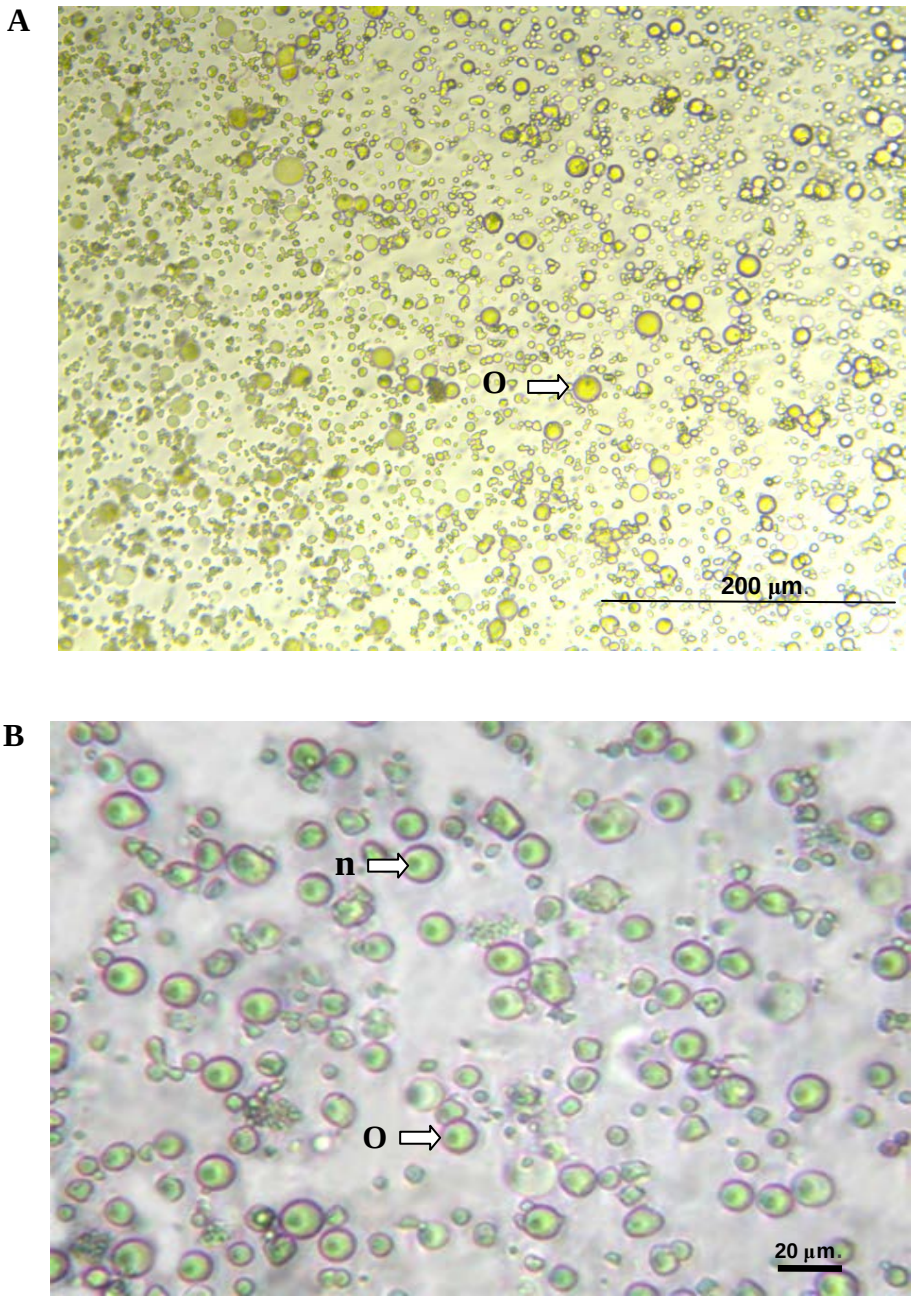
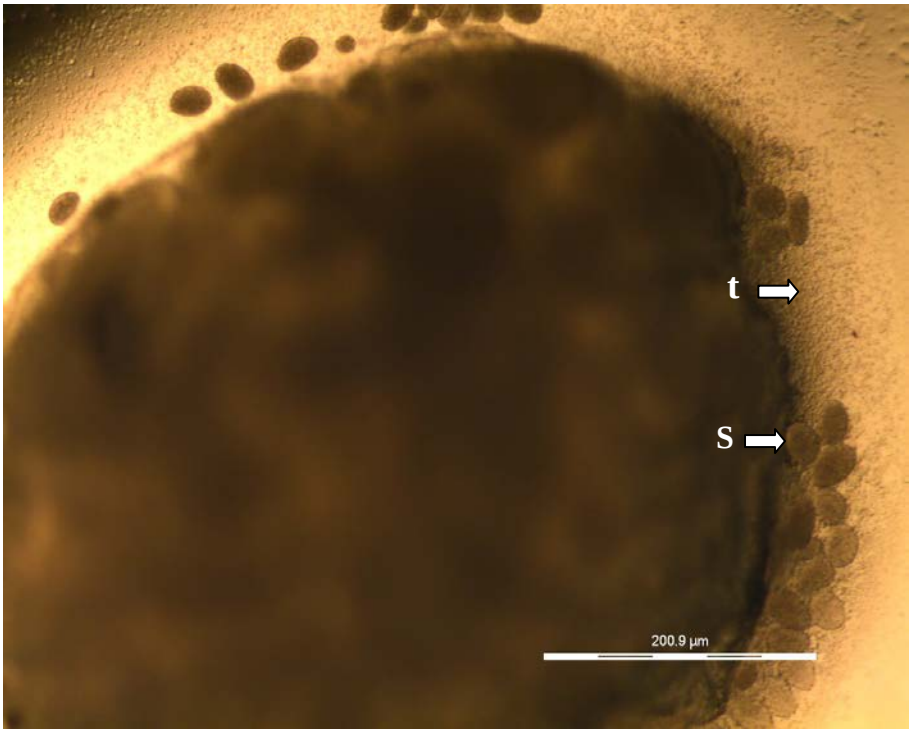


Figure 3.14 **A.** Culture of ovarian cells of *Scylla serrata* (SB - 200 µm, M – 100 x)

B. Enlarged picture of ovarian cells. Arrow points the round spheroid ovarian cells (o) with a prominent nucleus (n) (SB – 20 µm, M – 200 x)

A



B

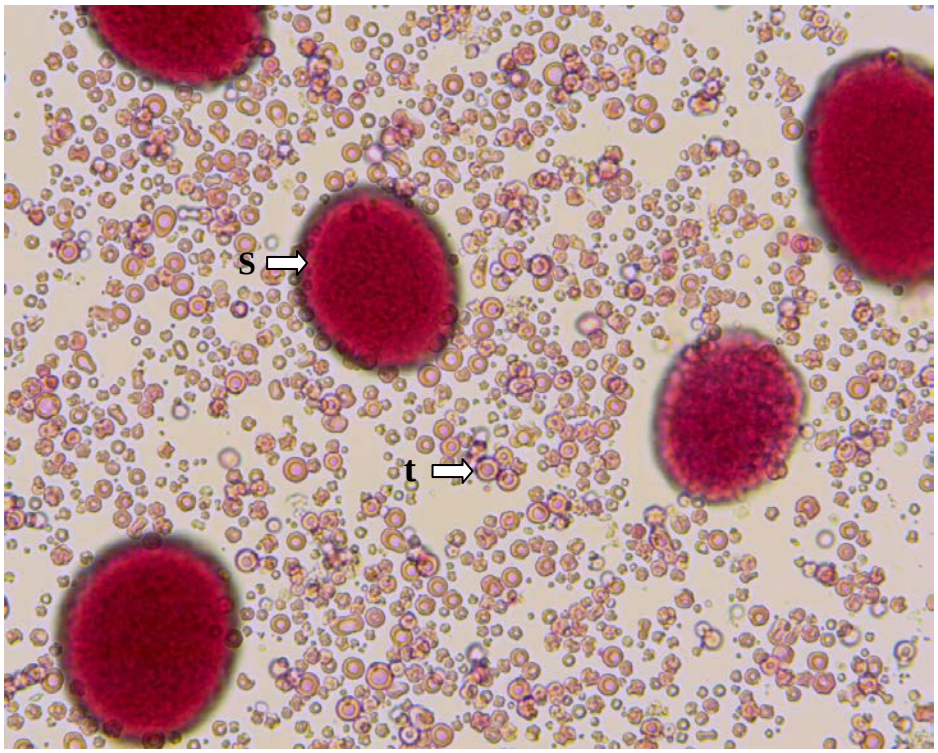
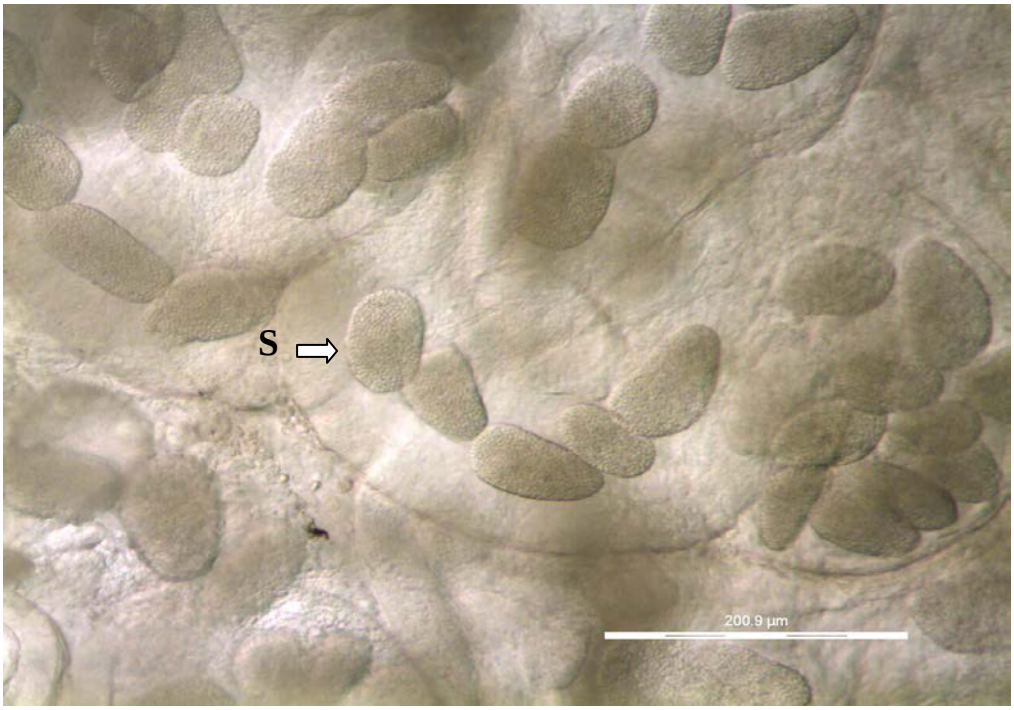


Figure 3.15 Explant culture of testis of *Scylla serrata*

A. Cell migration from the explant. Arrow points migrated spermatogonia (s) and testicular cells (t) (M – 100 x)

B. Enlarged view of spermatogonia (s) and testicular cells (t) stained with Acetocarmine (200 x)

A



B

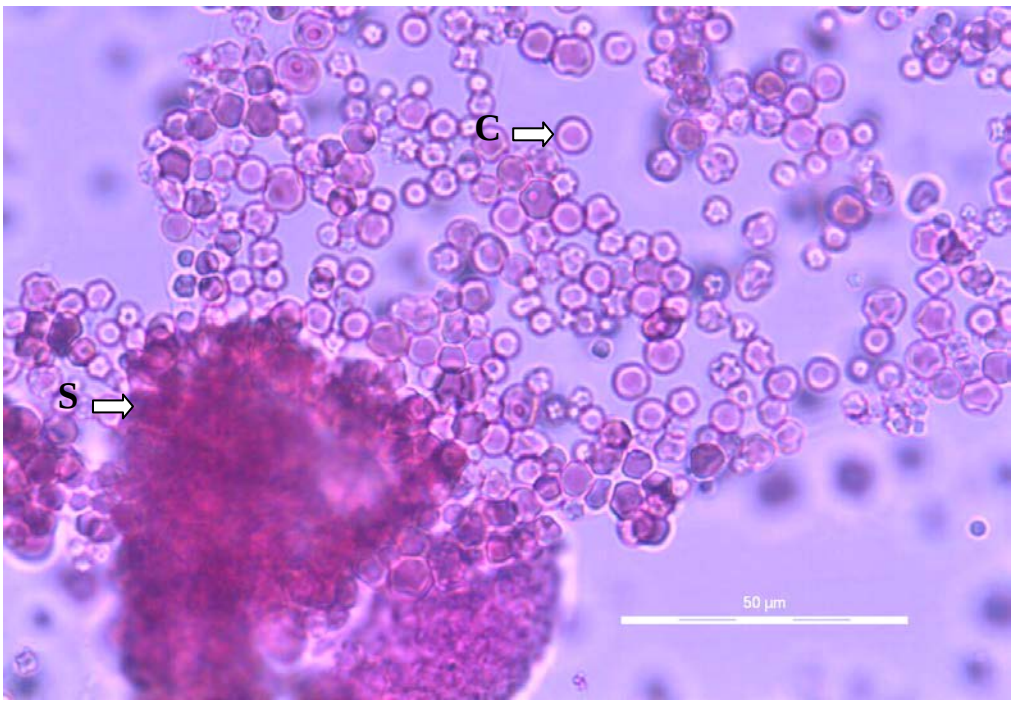


Figure 3.16 **A.** Vas defrence of crab showing spermatogonia (M - 100 x)

B. Liberation of cells from the spermatogonia. Arrow heads indicate Spermatogonia (s), cells (c) (M - 400 x)

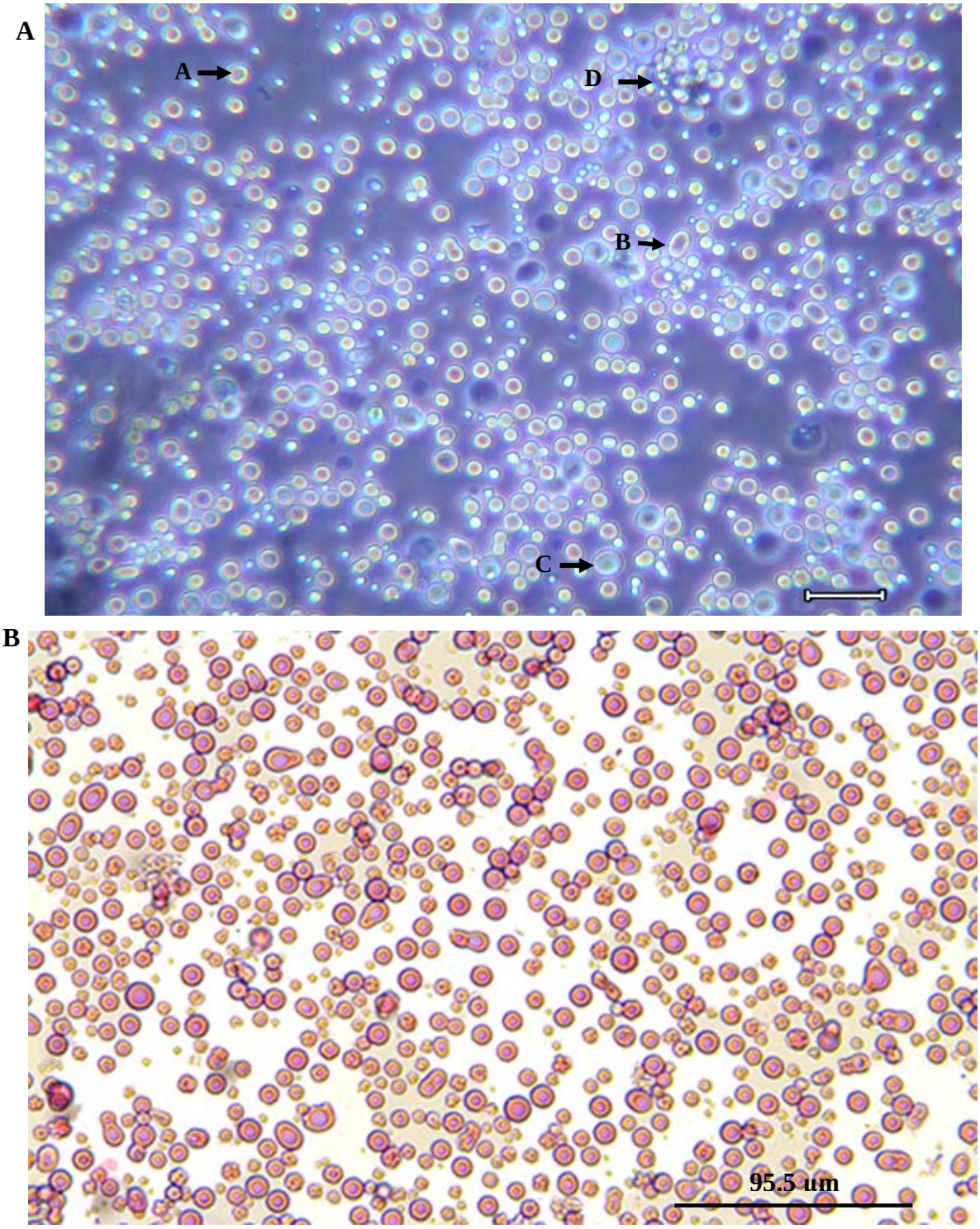


Figure 3.17 A. Cell culture from testis of *Scylla serrata.* a) round cells of ~ 10 –18 µm, b) cell of ~ 15–28 µm, c) large cells of ~ 21–38 µm, d) detached cell clumps (SB – 60 µm, M – 200 x)

B. Cells stained with acetocarmine (M – 200 x)

3.2 CHARACTERIZATION OF TESTICULAR CELL LINE

3.2.1 Biochemical characterization

3.2.1.a Methylthiazol Tetrazolium Assay (MTT) :- Cell proliferation and viability was assessed by MTT test. The assay is based on the intrinsic ability of mitochondrial dehydrogenases of viable cells to cleave MTT to purple-coloured formazan crystals which are dissolved in acidified iso-propanol and then the optical density is recorded spectrophotometrically at 570 nm wavelength.

The MTT assay revealed that the cells grown as controls using L15 medium supplemented with crab saline + glucose (1.0 g^{-1}) exhibited varying growth rates as depicted by the optical density changes at different growth periods. But the growth rates obtained with L15 medium containing crab saline + EGF + glucose were far better than controls (Figure 3.18). The prominent rise in OD on 7^{th} day of initiation of culture signified increase in cell density indicating elevated cell proliferations which clearly suggest that the cells have abandoned lag phase and entered into a log phase. The cells sub-cultured on the seventh day of initiation of culture could pass through ten passages while those sub cultured prior to this failed to pass through ten passages. If the cells were not sub-cultured and allowed to grow further they showed a decline in cell density by fourteenth day of culture but after a few days they showed a slight recovery as indicated by increase in cell density, thereafter the cultures enter into a plateau phase with retainment of few cell proliferations particularly when the cells were grown in culture medium containing EGF. However, such cultured cells remained in a plateau phase for a period of three to four months. In case the cells were grown in medium without EGF, the population declined gradually after two months period (Figure 3.18). Among the two media tested for cell

proliferation the media supplemented with growth factor showed 2 to 3 fold increase in cell proliferation. At the end of 24 hours, 7 days, 14 days, 21 days, 60 days and 120 days the growth rates with EGF supplemented medium were more than two folds for 7 to 14 days while for 60 to 120 days the increase was 3 fold (Table 3.10). This indicated that EGF has a significant influence on growth rate.

The testicular cell line proliferation test was executed after every seven days (Figure 3.19). A set number of cells (2×10^4) were withdrawn from the main population of a cell line for sub-culturing. For MTT test sub cultures were made at every seventh day for a period of one month and during this period cells underwent five passages, then next subculture was done after two months followed by another subculture after four months (Figure 3.19). The MTT tests were performed before every subculture after the first subculture. The MTT assay clearly indicated that the cells continued to grow and divide as long as over crowding and confluence of cells was avoided but such cell populations showed a slow decline in population up-to a period of four months indicating that though there is still proliferation of cells, the cells are losing the potential to proliferate at a higher rate and after four months period the cell population tends to decline further by virtue of onset of cell senescence.

3.2.1.b Lactate Dehydrogenase Assay :- LDH assay was performed to estimate the health status of the cells since LDH, a cytoplasmic enzyme, comes out into the medium only when cells get injured. LDH leakage was estimated by measuring LDH activities in culture medium and cell lysate using the formula given in methodology (protocol 2.3.1.b). The percentage of LDH release for testicular cell cultures grown in L15 medium supplemented with crab saline and glucose was always between 8 to 9.4 % while for those

cultures grown in the afore mentioned medium supplemented with EGF, the percentage of LDH release was in the range of 7.4 to 9 % only (Figure 3.20). This indicates that the cells are viable and healthy, as in any culture conditions less than 10 % LDH release is believed as a normal phenomenon.

3.2.1.c Cell Senescence Test :- The test is based on histochemical staining technique for β- galactosidase activity at pH 6.0. Under these conditions, β- galactosidase activity is easily detectable in senescent cells, but undetectable in quiescent, immortal and normal healthy cells. The cell senescence studies clearly show that the cell line does not show senescence up to a period of four months for cells grown in L15 medium supplemented with crab saline, EGF and glucose (Figure 3.21.B) but subsequently it enters into senescence as indicated by a light blue staining around the nucleus indicating the expression of β- galactosidase (Fig 3.21.A).

3.2.2. Mitosis

3.2.2.a. Mitotic stages :- When the cultured cells were processed for preparation of slides and slides observed under microscope as described in the protocol, the non dividing cells appeared round while the dividing cells exhibited changes in morphology depending upon the mitotic stages. Generally, non dividing cells appeared pinkish due to acetocarmine stain with a distinct staining of the nuclear membrane, while in the mitotic cells the chromatins looked darker. The Figure 3.22.A clearly shows the presence of non-dividing cells as well as mitotic cells, especially those in prophase, metaphase, anaphase and telophase stages. The prophase stage was clearly indicated by distinctly condensed chromosomes without nuclear membrane (Figure 3.22.B,p) in metaphase the chromosomes are equatorially

arranged (Figure 3.22.B,m) and the centromeres were located in the opposite poles, while in anaphase the chromosomes were aligned towards the polar end (Figure 3.22.B,a). The telophase was characterized by two distinct nuclei with partial cell separation (Figure 3.22.B,t). Few cells exhibited unequal division (Figure 3.22.A,u).

3.2.2.b Mitotic Index :- The cells cultured using plain culture medium exhibited mitotic index equivalent to 19 percent, while those cultured using CDM with supplements (L15 + crab saline + EGF + glucose) showed mitotic index equal to 26 percent. Even the sub-cultures or cell lines derived from the cells grown in CDM with supplements, exhibited the mitotic index equivalent to 26 %.

3.2.3 Genomic analysis

3.2.3.a RAPD Analysis using primer YNZ22 :- The RAPD profile of *Scylla serrata* generated using primer YNZ22 is presented in Figure 3.23, wherein the Lane L1 exhibits 100 bp ladder showing 10 bands representing DNA size in the range of 100 to 1000 bps. Lane 1 represents DNA bands obtained from the parental tissue wherein 5 bands are expressed in the range of 250 to 1300 bps. Lane 2 presents the DNA profile of the cultured cells which is identical to that of parental tissue seen in Lane 1. Lane L2 exhibits expression of 6 bands representing a range of 500 to 3000 bp Ladder. Table 3.11 shows the sequence of YNZ22 primer as well as the size range of parental and cultured cell DNA. Here the parental size range appears to match with the cultured cells DNA range. The RAPD profiles of both the parental tissue and cultured cells indicate that the culture cells were derived from parental tissue of *Scylla serrata*.

RAPD profile of a testicular cell line of *Scylla serrata* generated using primer OPD20 and OPD08 are presented in Figure 3.24.A. Lane L1 depicts expression of bands of 100 bp ladder in the range of 100 - 1000 bps, Lane 1 represents expression of RAPD bands of parental tissue while Lane 2 shows RAPD bands of testicular cell line. These RAPD bands were obtained using primer OPD20. Both Lane 1 and 2 share the identical bands (n = 07) with reference to the band size.

In Figure 3.24.B, Lane L1 represents 100 bp DNA ladder having a range of 100 to 1000 bps. Lane 3 represents expression of RAPD bands of parental tissue while Lane 4 presents RAPD profile of testicular cell line. A careful analysis of RAPD profile in Lane 3 and 4 reveals that the parental tissue and testicular cell line share the bands in the same range of base pairs though they differ partially with respect to band intensity. Table 3.12 shows the sequence of OPD20 and OPD08 primers along with size range and number of bands of parental tissue and cultured cells.

3.2.3.b 18S rRNA sequencing :- Genetic lineage of the cultured cells from the parental tissue as well as it's relatedness with its genus and other species was assessed by 18S rRNA sequence. The testicular cells' sequences showed 425 bases with a base count of 109 - adenine, 99 - thymine, 94 - cytosine, 123 - guanine (Figure 3.25). Further analysis of 18S rRNA sequence of testicular cells reveals that the frequency of occurrences of adenine in triplet and quadruplet form are three and four times respectively. Similarly thymine frequencies in triplet, quadruplet and septuplet forms have been expressed twice and once respectively. Cytosine triplet has occurred only once, while the most frequently occurring guanine base has occurred as triplet, quadruplet, pentuplet and octuplet sequences four, two, two times and once respectively. Besides further analysis of 18S rRNA sequencing

showed certain repeat base sequences particularly GAA has occurred eleven times, CGG and AGG have occurred eight time, ATT and GTG have been expressed six times, CAT, GGT and CTG have been expressed five times while TTG was repeated four times and CTC combination only three times.

These all analysis indicate clearly that *Scylla serrata's* 18S rRNA sequence has its own specific characteristics and the sequence when analyzed using BLAST (Table 3.13) exhibit 98 % phylogenetic similarity to *Scylla paramamosain's* 18S ribosomal RNA (gene accession no FJ774907) and 97 % similarity to *Scylla paramamosain's* hypothetical protein mRNA (gene accession no FJ774884) indicating that the cultured testicular cells were derived from parental tissue of *Scylla* (Table 9). The gene bank accession number for 18S rRNA sequence for *Scylla serrata* is HQ697253. The parental tissues' 18S rRNA sequence matched with cultured cells 18S rRNA sequence.

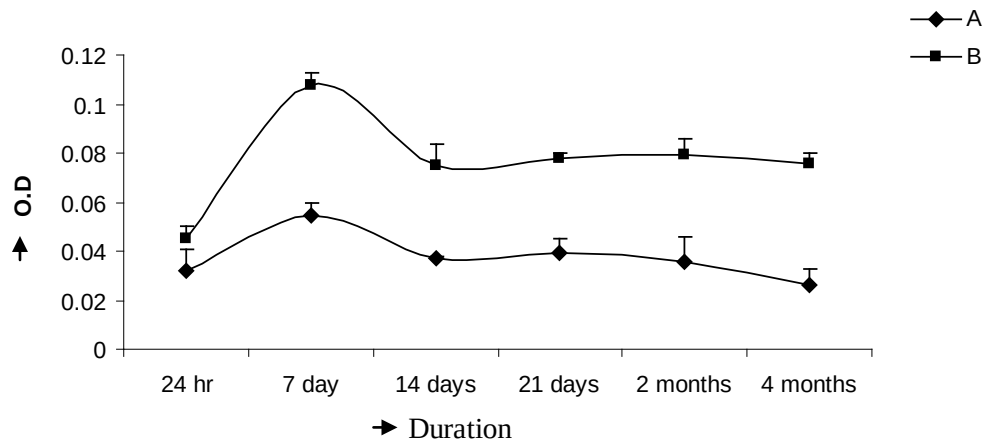


Figure 3.18 MTT assay of cultured testicular cells for cell proliferation: **A**. L15 prepared in crab saline + glucose (1.0 g^{-l}), **B**. L15 prepared in crab saline + EGF (20 ng/ml) + glucose (1.0 g^{-l}). All data represent means ± standard deviations

Table 3.10 Effects of media supplements on cell proliferation: **A**. L15 prepared in crab saline + glucose (1.0 g^{-l}), **B**. L15 medium prepared in crab saline + EGF (20 ng/ml) + glucose (1.0 g^{-l})

Cell Proliferation			
Days and months	**A**	**B**	**Fold Increase**
7^{th} day	100%	220%	2.2 fold increase
14^{th} day	80%	160%	2.0 fold increase
2 months	60%	180%	3.0 fold increase
4 months	52%	160%	3.0 fold increase

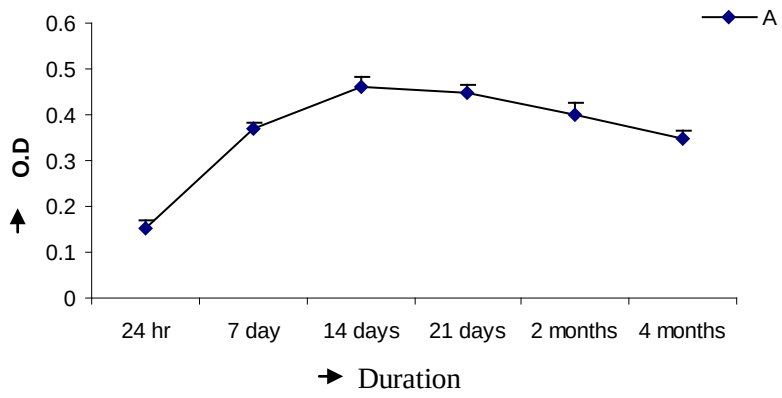


Figure 3.19 MTT assay of testicular cell line for testing proliferation. All data represent means, ± standard deviations

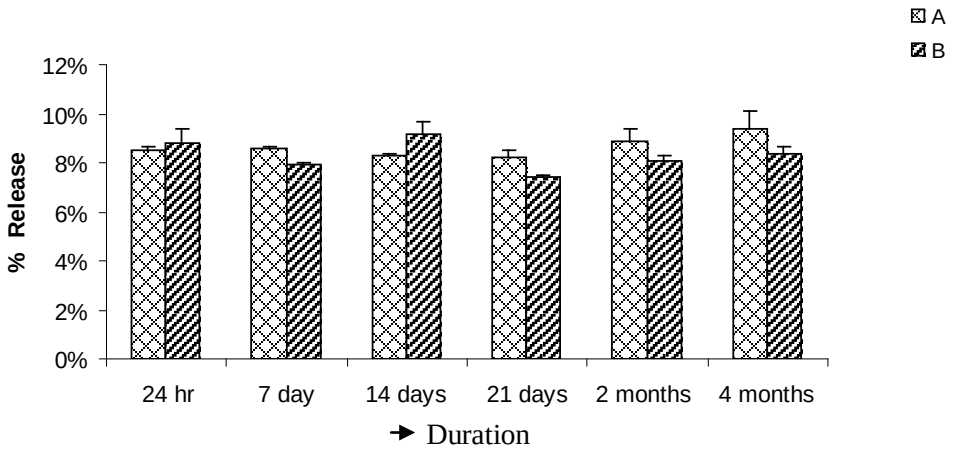
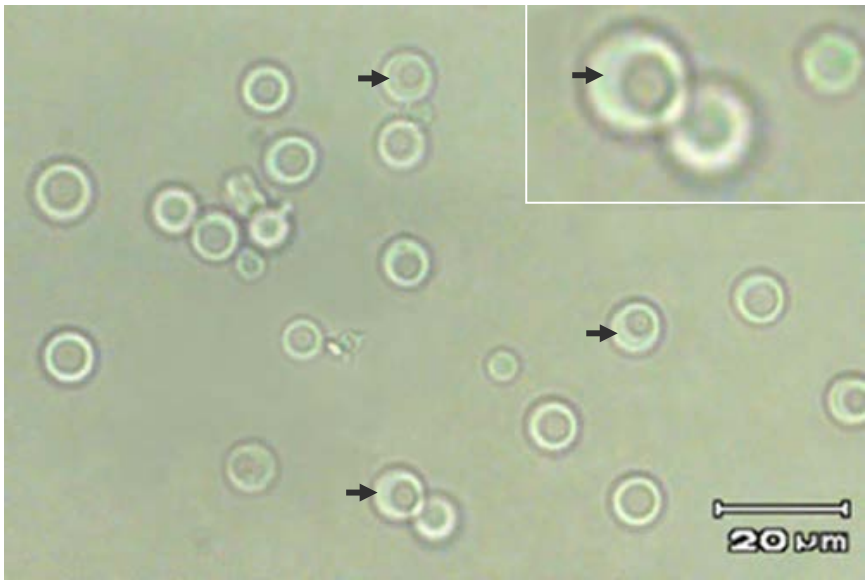


Figure 3.20 LDH release (%) by testicular cell cultures. All data represent means, ± standard deviations

A. L15 prepared in crab saline + glucose (1.0 g^{-l})

B. L15 prepared in crab saline + EGF (20 ng/ml) + glucose (1.0 g^{-l})

A



B

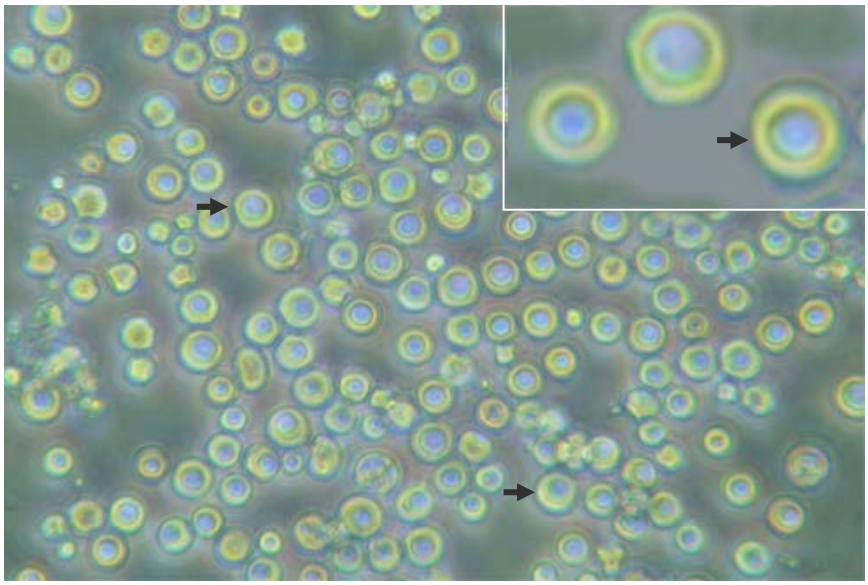


Figure 3.21 Cell senescence from a cell line

A. Blue colouration around the nucleus (➔) of cells indicating expression of β-galactosidase (cell senescence) (M – 300 x)

B. Non senescent cells (M – 300 x)

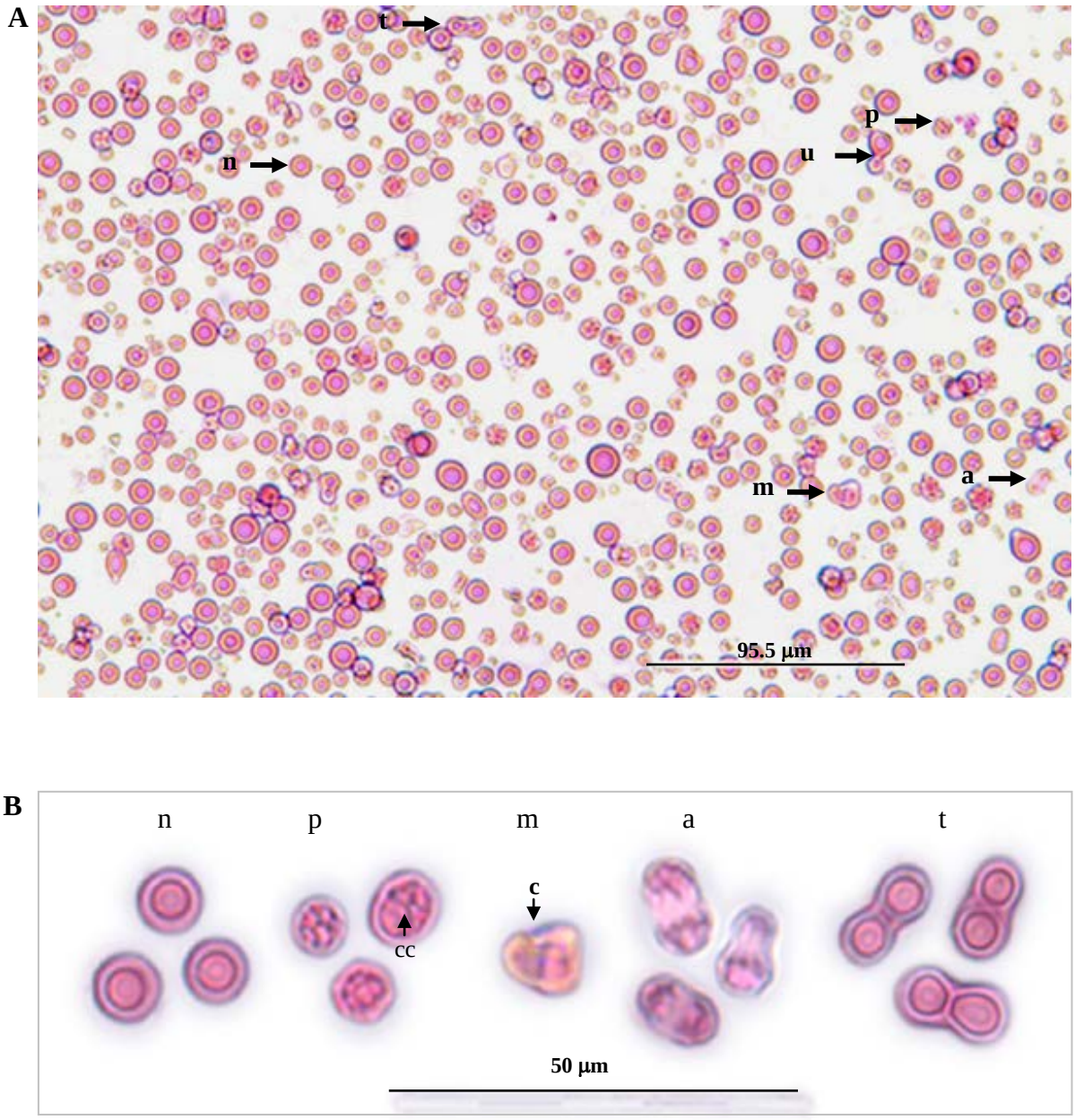


Figure 3.22 **A.** Testicular cell line of *Scylla serrata* stained with aceto-carmine, showing mitotic stages. n) non dividing mature cells, p) prophase, m) metaphase, a) anaphase, t) telophase, u) unequal division (M – 200 x)

B. Magnified mitotic stages picked up from figure 3.22 as well as from other slide preparations for clarity. cc) condensed chromosomes, c) chromosomes at equatorial plate (M – 400 x)

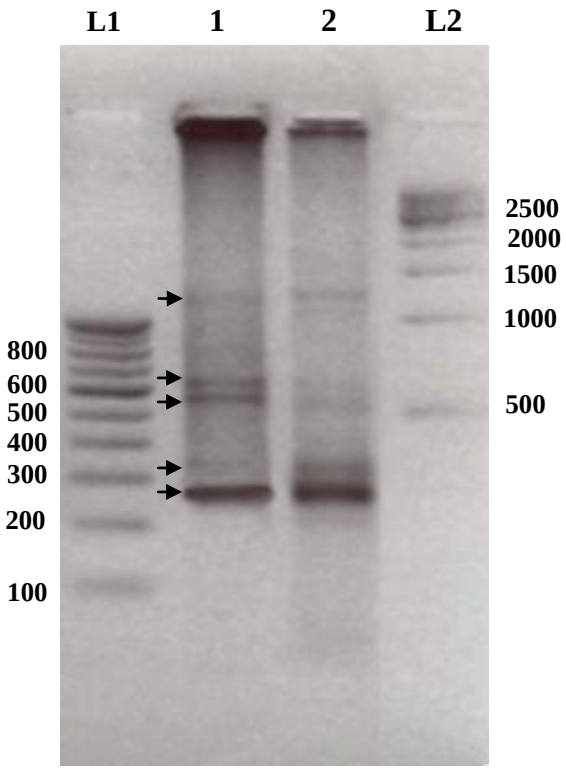


Figure 3.23 RAPD profile of *Scylla serrata* for testicular cells generated using RAPD primer YNZ22, L1- 100 bp Ladder; Lane 1 - parental tissue DNA, Lane 2 - Cultured cell DNA amplified using primer YNZ22, L2 – 500 bp Ladder. Arrows indicate RAPD bands (the band size range from 300 – 1300 bp)

Table 3.11 Sequence of primer YNZ22 and expression of size ranges as well as RAPD bands

Primer	**Sequence**	**Parental DNA**	**Cultured cell DNA**
		Size range and (number of bands)	
YNZ22	58-CTCTGGGTGTCGTGC-38	250-1300 (5)	300-1300 (5)

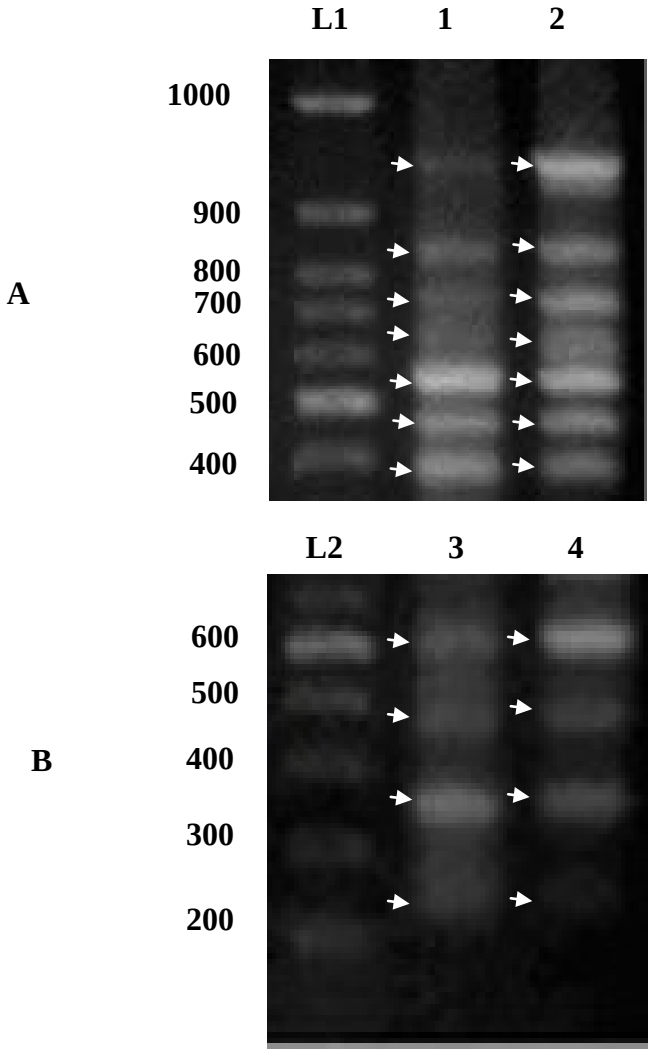


Figure 3.24 RAPD profile of *Scylla serrata* generated using RAPD primers such as OPD20 – **A.** Lane L1: 100 bp ladder; Lane 1: parental tissue DNA, Lane 2: testicular cell line DNA and Primer OPD08 – **B.** Lane L2: 100 bp ladder, Lane 3: parental tissue DNA, Lane 4: testicular cell line DNA). Arrows indicate RAPD bands

Table 3.12 Sequences of OPD20 and OPD08 primers with expression of size ranges and RAPD bands

Primer	Sequence	Parental DNA Size range and (number of bands)	Cultured cell DNA Size range and (number of bands)
OPD20	5’ ACCCGGTCAC’	400 – 950 (7)	400 – 950 (7)
OPD08	5’ GTGTGCCCC3’	200 – 600 (4)	200 – 600 (4)

GGGCTGTTGGTTAGCATGCATGTCTAGTACAGCCGATTAGGTGAACCGC
GAATGGCTCATTAAATCAGCTATGATTCATTGGATCTGTAGCCCACACTTACTT
GGATAACTGTGGTAATTCTAGAGCTAATACATGCACCACGTCTCTGACCGCAA
GGGAAGAGCGCTTTTATTAGTTCAAAACCGGTCGGGCCTCGGTCCGCCAACCA
CACCGTGTTGAATCTGAATAACTTGTGGCTGAGCGCACGGCCTCCAGTGCCGG
CGCCGCCTCTTTCAAGTGTCTGCCTTATCAGCTTTCGATTGTAGGCTATGCGCC
TACAATGGCTAAAACGGGGAACGGGGAATCATTTTTTTAAGCGGGAAAGAAG
CATGAGAGGGGGAAAAGGAGGAGGGGGGGGACAGGGGGACCAAAATTGGCG
AACAGA.

Figure 3.25 18S rRNA partial sequence from testicular cells of *Scylla serrata*

Table. 3.13 BLAST results showing phylogenic similarity (NCBI/BLAST/blastn)

Sr. No.	Gene Accession No.	Phylogenetic neighbours	% Similarity
1	*FJ774907*	*Scylla paramamosain isolate 2 18S ribosomal RNA, partial sequence*	98
2	*FJ774884*	*Scylla paramamosain hypothetical protein mRNA, partial cds*	97
3	*FJ774871*	*Scylla paramamosain hypothetical protein mRNA, partial cds*	97
4	*DQ079759*	*Necora puber voucher KC2161 18S ribosomal RNA gene, partial sequence*	95
5	*GU362670*	*Eriocheir sinensis 18S ribosomal RNA gene, complete sequence*	95
6	*EU934950*	*Tritodynamia horvathi voucher ULLZ 5585 18S ribosomal RNA gene, partial sequence*	95
7	*EU934946*	*Tumidotheres margarita voucher ULLZ 5533 18S ribosomal RNA gene, partial sequence*	95
8	*EU934945*	*Tumidotheres maculatus voucher ULLZ 5534 18S ribosomal RNA gene, partial sequence*	95
9	*EU934942*	*Dissodactylus crinitichelis voucher ULLZ 5561 18S ribosomal RNA gene, partial sequence*	95
10	*FM161989*	*Xantho poressa partial 18S rRNA gene*	95

3.3 CHARACTERIZATION OF HEPATOPANCREATIC GRANULAR CELLS

A new cell type (granular cell) was detected from the hepatopancreatic primary cell cultures. Earlier reports have shown that there exists electron dense granules' containing calcium, magnesium, phosphate and occasionally enzymes. Therefore, many ignored the granular cells, believing that these are electron dense granules only. The primary cultures of hepatopancreas revealed that these granular cells showed firm attachement to the substrate and could be maintained and be made to proliferate in the presence of EGF. These observations lead to attempt characterization in-order to investigate whether these are different cell type or the very granular break ups giving an impression of cell proliferation. Also attempts were made to know why they respond to EGF.

3.3.1 Morphology of granular cells :-

The granular cells appeared spheroid under light microscope with a prominent central nucleus (Figure 3.26.A). These cells are ~ 1-5 µm in diameter. The optical images clearly show the presence of a distinct nucleus at the centre (Figure 3.26.B). The confocal images of the granular cells show a central nucleus surrounded by the cytoplasm in the form of concentric rings (Figure 3.27). The scanning electron microscopy detected tennis ball like appearance of granular cells having occasional attachments to the adjacent cells (Figure 3.28). The presence of calcium in the cytoplasm of cells was demonstrated by exposing cells to Fura-2-AM a fluorescent dye. The Fura-2-AM treated cells when viewed under fluorescent microscope using 340 nm optical filters, exhibited calcium fluorescence that depended upon the calcium concentration in the cells (Figure 3.29 B). However, the cells loaded with Fura-2-AM when viewed under light microscope appeared tiny whitish granules (Figure 3.29.A)

3.3.2 Biochemical Analysis

3.3.2.a DNA, RNA, Proteins :- The biochemical assays of granular cells revealed the presence of DNA, RNA and proteins in concentrations equivalent to ~ 1.8 pg/cell, ~ 0.24 pg /cell and ~ 14.98 pg /cell respectively.

3.3.2.b Calcium concentration in Granular cells :- To estimate the calcium concentration per cell, an experiment was performed using Fura-2-AM (calcium indicator dye). The calibration curve of Fura-2-AM which showed an r^2 value of 0.996 was used to determine calcium concentration (Figure 3.31.A). The calcium concentration in these cells was equivalent to ~ 0.018 pmol/cell.

3.3.2.c Cell proliferation study using MTT assay :- MTT assays are employed to detect the cell proliferations. The granular cells proliferated significantly as indicated by the rise in optical density (OD) at the end of twenty four hours in L15 – crab saline medium supplemented with EGF in comparison to those grown in L15 - crab saline medium (Figure 3.30). However, the cells grown in L15 and crab saline showed a slight decline in the proliferation rate as indicated by decline in OD after five days but in contrast the cells grown in L15 crab saline medium supplemented with EGF exhibited significant enhancement in the proliferation rate.

3.3.2.d Ecdysterone mediated release of calcium by granular cells :- During ecdysis the calcium is released by the hepatopancreas for the formation of new exoskeleton. Granular cells (G) are the source of calcium. In the present study an attempt was made to know if the ecdysterone mobilises calcium from G cells. Calcium release by the granular cells

under the influence of ecdysterone at concentrations of 5, 10 20 µg/ ml was assayed. Ecdysterone at 10 µg/ ml concentration promoted optimum calcium release by the granular cells and therefore this concentration was used to determine ecdysterone mediated calcium release. It was observed that at the end of one hour the calcium released by the control (cell maintained in calcium free crab saline without ecdysterone) was equivalent to 0.368 mg per 4.0 ml of the medium and under the influence of ecdysterone the release of calcium elevated to 0.437 mg per 4.0 ml of the medium (Figure 3.31.B). At the end of two hours the controls exhibited insignificant increase in calcium release and it was equivalent to 0.370 mg per 4.0 ml of the medium but under the influence of the ecdysterone for the same period the calcium release elevated to 0.449 mg per 4.0 ml of the medium. The calcium release by the controls by the end of four hour was equivalent to 0.369 mg per 4.0 ml while under the influence of ecdysterone it exhibited maximum elevation in calcium release to the tune of 0.958 mg per 4.0 ml of the medium. Subsequently at the end of six hours the calcium release from the controls declined to 0.336 mg per 4.0 ml of the medium, even ecdysterone could not elevate much the calcium release from the cells though it was significantly more than that observed for the controls. However, this calcium released was almost half of that released under the influence of ecdysterone at the end of four hours. At the end of eight hours the controls exhibited significant rise in calcium release but the ecdysterone could not significantly elevate calcium release. Subsequently after 10 hours the controls exhibited drop in calcium release and relatively higher release of calcium was promoted by ecdysterone (Figure 3.31.B).

This experiment clearly indicated that ecdysterone at 10 µg/ ml concentration has a profound influence on the calcium release by the granular cells exclusively at the end of four hours.

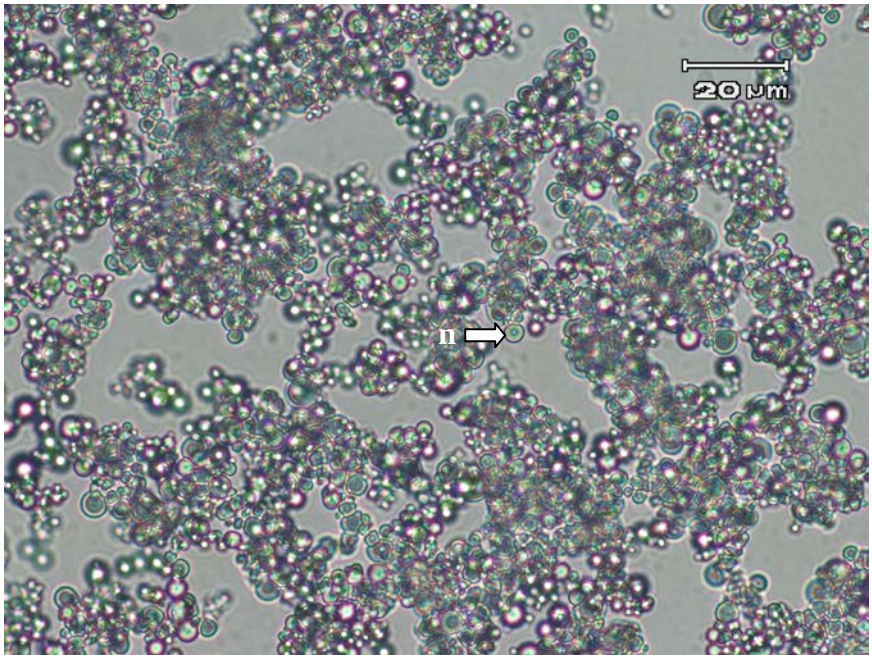
3.3.3 Genomic analysis

3.3.3.a RAPD :- RAPD profile was generated using primers (UBC457, YNZ22) specific for *Scylla serrata.* In Figure 3.32.A, Lane **L1** and **L2** represent the DNA ladders with a base pair range of 500 to 2000 and 100-900 respectively. Lane **1** represents control i.e. the parental DNA amplified using primer UBC457 wherein 9 bands are expressed in the range of 120 to 1200 base pairs, Lane **2** presents the DNA profile of cultured cells which when compared with the parental genome (Lane **1**) indicates that the cultured cells have descended from the parental tissue.

RAPD profile of parental tissue generated with Primer YNZ22 is represented in Lane **3**, while Lane **L3** represents DNA marker with a range of 100 bps to 900 bps. Lane **4** represents RAPD profile of granular cells and Lane **L4** shows DNA ladder having a range of 500 bp to 2000 bp. Careful analysis of Figure 3.32.B reveals that the parental tissue as well as cultured granular cells show expression of three bands in the range of 240 to 1400 bps indicating that the granular cells are truly originated from the parental tissue, since they share the same band viz-a-viz base pairs.

Table 3.14 shows the sequences of primers (UBC457, YNZ22). Besides, the table presents the size ranges and number of bands expressed by DNA of parental and cultured cells. The DNA of parental and cultured cells when amplified with each primer, exhibit expression of different bands in terms of numbers viz-a-viz base pairs. The RAPD profiles of both parental tissue and granular cells of *Scylla serrata* indicate that these cells have a specific genome which could be compared with the parental genome.

A



B

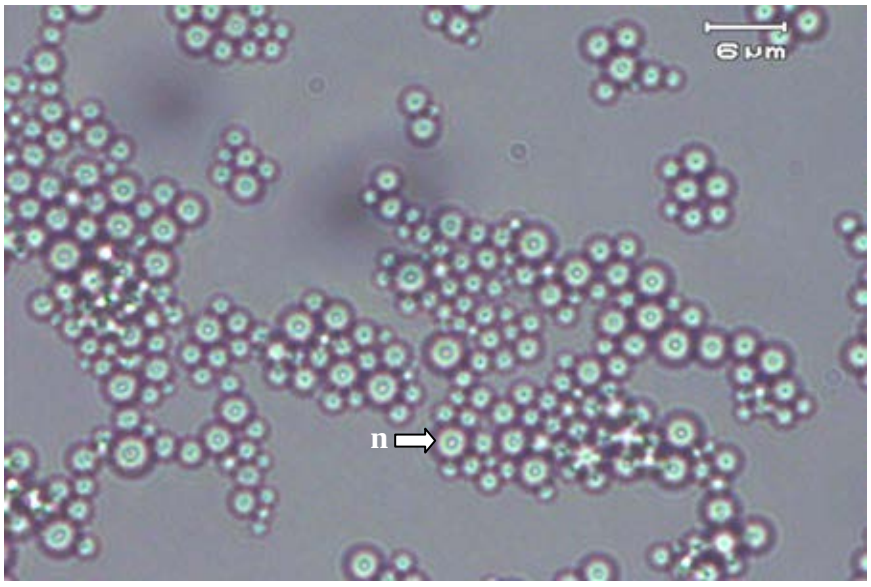


Figure 3.26 Granular cells of hepatopancreas fixed in 2.0 % glutaraldehyde. Digital images acquired on an Olympus Bx41 microscope

A. General picture of granular cells in a size range from 1 to 2 µm in diameter. Note: Distinct nucleus (n) also note the cells where nucleus is obliterated due to calcium depositions. (M – 200 x)

B. Phase contrast image of the select granular cells which show distinct nucleus. (M – 400 x)

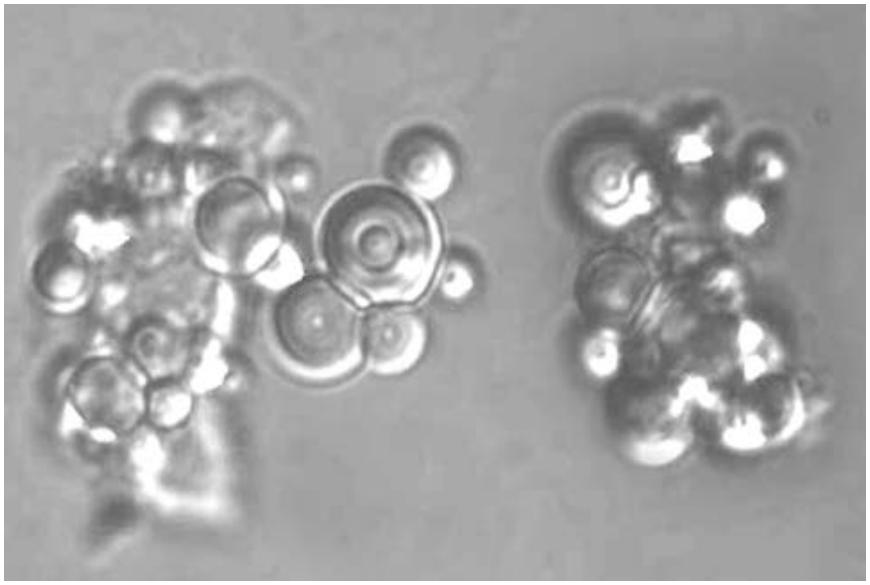


Figure 3.27 Confocal microscopic image of granular cells. Note distinct spheroidal granular cells with distinct nucleus at the centre. Also note concentric ring like calcium depositions in the cytoplasm around the nucleus (M – 600 x)

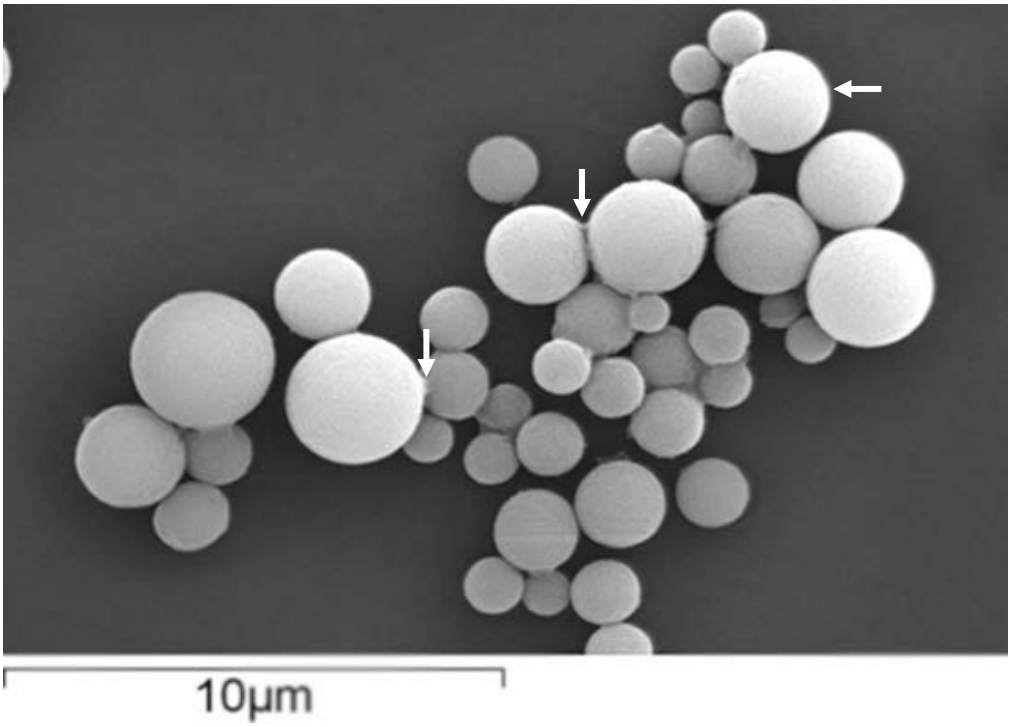
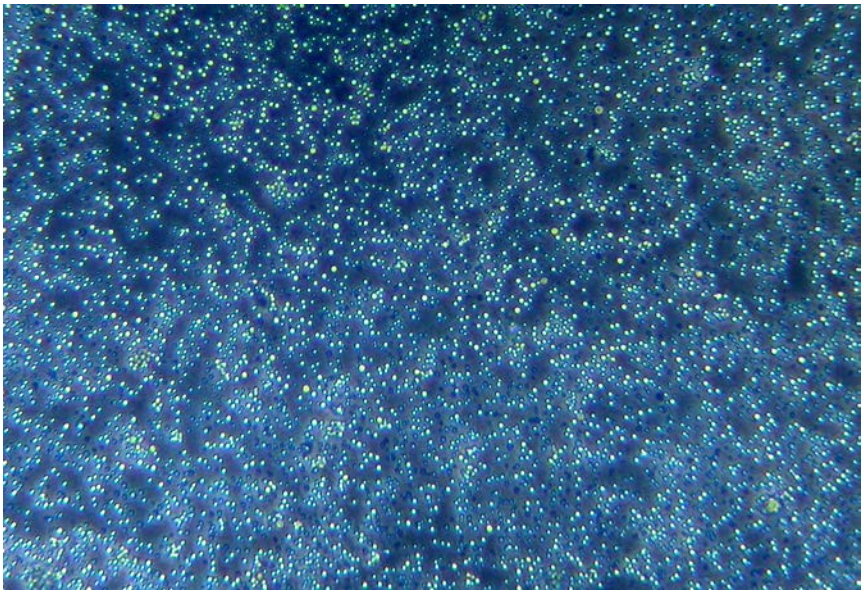


Figure 3.28 Scanning Electron Microscopic picture of granular cells from the hepatopancreas of *Scylla serrata*. Note tennis ball like appearance of granular cells of varying sizes. Arrow indicates membrane attachment between cells

A



B

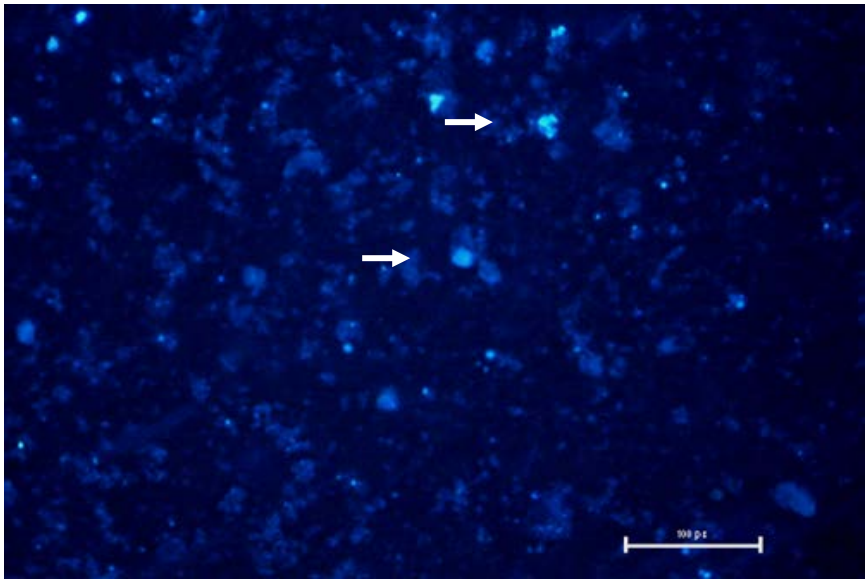


Figure 3.29 A. Light microscopic image of Granular cells loaded with Fura-2-AM. Note tiny whitish granular cells (M – 100 x)

B. Fluorescent image of granular cells loaded with Fura-2-AM. Note light emitted by the granular cells at a wavelength of 340 nm UV excitation (M – 200 x)

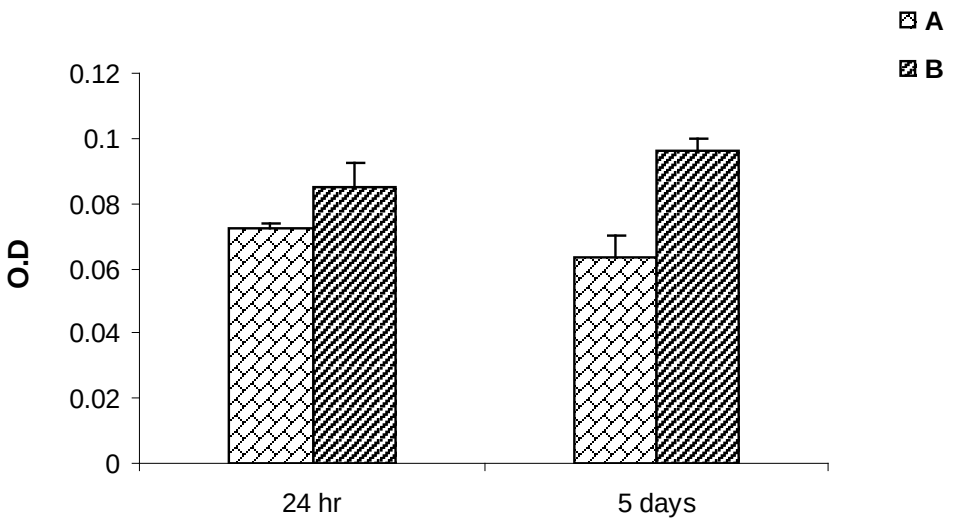
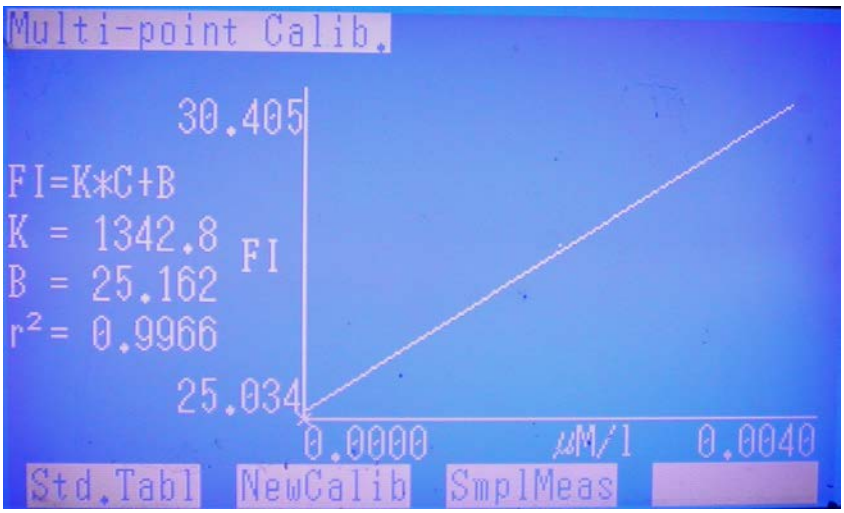


Figure 3.30 Proliferation assay of granular cells by MTT test: **A.** L15 prepared in crab saline + glucose (1g/l), **B.** L15 prepared in crab saline + EGF (10 ng/ml) + glucose (1g/l). All data represent means ± standard deviations

A.



B.

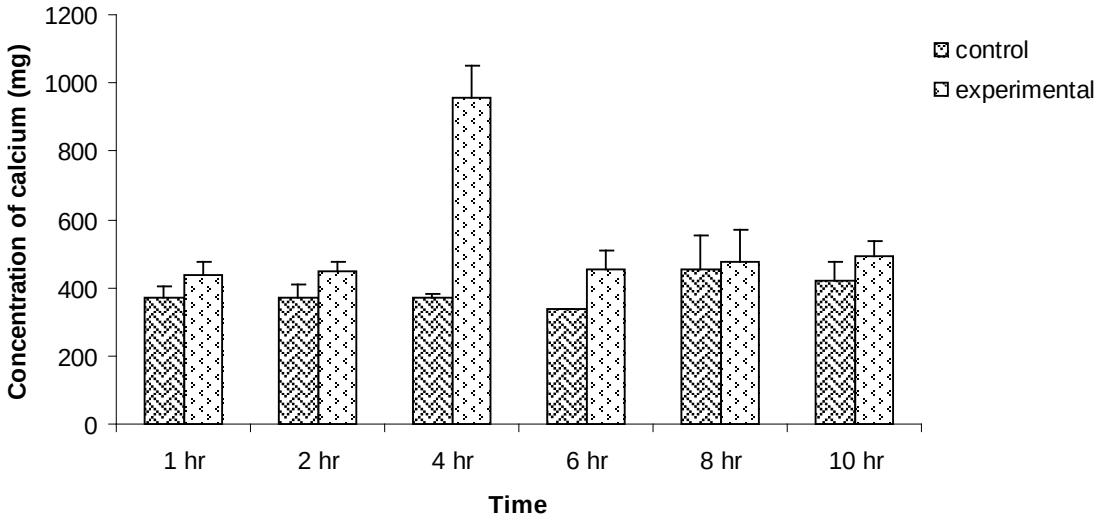


Figure 3.31 Ecdysterone mediated calcium release by the granular cells in culture

A. The calibration curve of Fura-2-AM

B. Ecdysterone mediated calcium release by the granular cells in culture

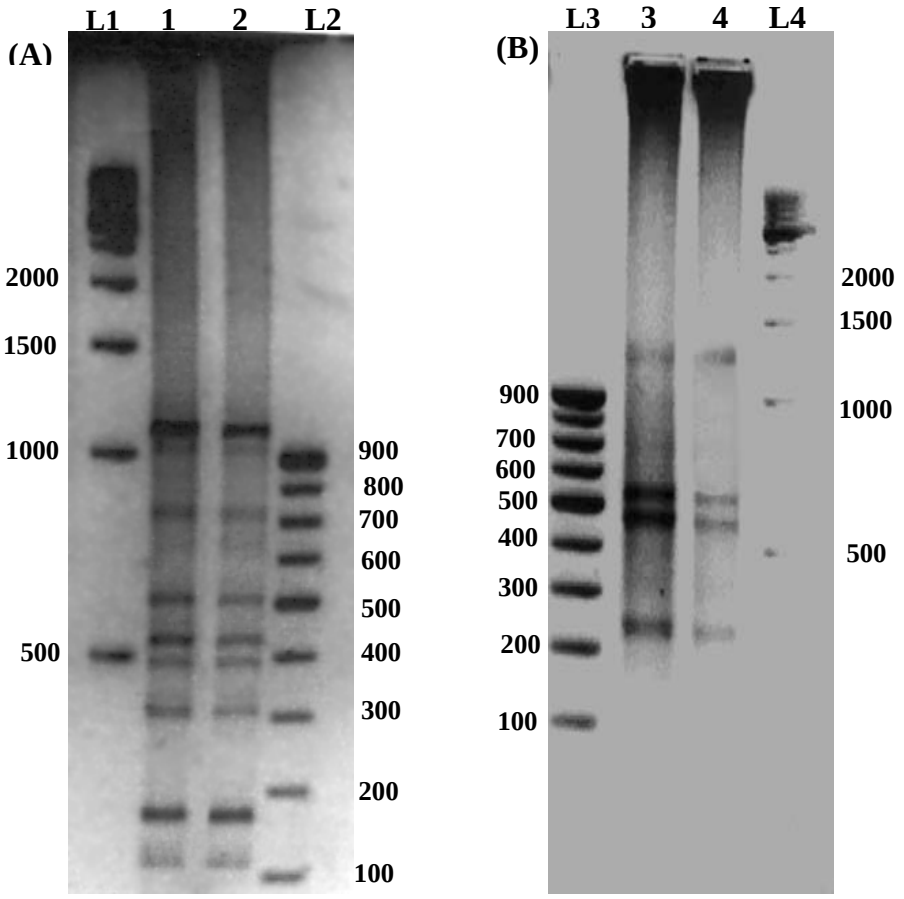


Figure 3.32 RAPD Profile of granular cells generated by PCR technique using primers UBC457 and YNZ22. **A:** Primer UBC457 [Lane L1: 500 bp ladder, Lane 1: Control-parental tissue, Lane 2: granular cells, Lane L2: 100 bp ladder], **B:** Primer YNZ22 [Lane L3: 100 bp ladder, Lane 3: Control-parental tissue, Lane 4: granular cell. Lane L4: 500 bp ladder]

Table 3.14 Sequences of UBC457 and YNZ22 primers along with expression of size ranges and RAPD bands

Primer	**Sequence**	**Parental DNA**	**Cultured cell DNA**
		Size range and (number of bands)	
UBC457	58-CGACGCCCTG-38	120 – 1200 (9)	100 – 1200 (9)
YNZ22	58-CTCTGGGTGTCGTGC-38	240-1400 (3)	240-1400 (3)

3.4 WSSV EFFECTS ON CULTURED TESTICULAR CELL LINE

3.4.1 Testicular cell line :- Once the testicular cell line was developed it was used for testing pathogenicity of WSSV. The testicular cell line tended to form a monolayer and confluence. This cell line survived at 25^0 C for more than four months when grown in L15 crab saline medium supplemented with EGF and glucose. All these cells anchored firmly to the substratum. Upon confirming that the cell line is healthy viable and stable it was exposed to WSSV as mentioned in the protocol (2.5.3)

3.4.2 WSSV effects :- Proliferating testicular cells exposed to WSSV showed cytopathic effects such as change in appearance, shrinkage, clumping, lyses and decline in cell density. The end point dilution assay indicated that 10^{-3} dilution of gill extracts and 10^{-1} dilution of cuticle extracts promoted 50 % cells' infections, indicating 10^{-3} and 10^{-1} dilutions are ID50 values respectively.

Table 3.15 shows the effects of WSSV inoculums on cultured testicular cells of *Scylla serrata* (Forskål) at different viral concentrations. On exposure for four hours to diluted viral suspension (10^{-1}) obtained from the gill extracts of infected *Penaeus monodon*, the cells appeared darker under the microscope (Figure 3.33.a,b) and then underwent shrinkage and lyses resulting into a decline in a population density within 24 hrs (Figure 3.34.d). Subsequently after 48 hrs of exposure to viral suspension, empty spaces developed in the cultures indicating cell losses At 10^{-3} dilution of V.S cell shrinkage was less prominent but as the time progressed, the infected cells gradually detached from the substratum and formed aggregates or clumps leading to cell lyses within five days. The cells exposed to 10^{-6} dilution of V.S appeared normal for about one month but

subsequently the cell cultures were interspersed with the cell debris (Figure 3.34.b). These results indicate that the viral toxicity was dependent on VS strength.

However, the cells exposed to 10^{-1} VS prepared from cuticles, showed cell aggregation at the end of 24 hrs (Figure 3.34.c) followed by cell lyses on fourth day, but the cells treated with cuticular VS of 10^{-3} and 10^{-6} appeared nearly normal for first twenty days and then exhibited accumulation of debris around the cell aggregates with a decline in cell population depending upon the strength of V.S dilution and exposure period. No WSSV effect was observed in the control cells exposed to heat inactivated V.S of any dilution. The controls that were not exposed to WSSV showed no cytopathic effects, including the one prepared form non infected (WSSV) prawn tissue (Figure 3.34.a).

3.4.3 MTT Test :- MTT test is a biochemical test for detecting changes in cell proliferation viz-a-viz cell toxicity. Cells infected with WSSV showed a significant decrease in cell viability as compared to controls. Viral suspension of 10^{-1} dilution prepared from gills and cuticles induced a prominent decrease in cell viability as indicated by drop in Optical density (O.D) after 24 hours of exposure (Figure 3.35), whereas with V.S of 10^{-3} and 10^{-6} dilution the toxicity effects were relatively less. The toxic effects of V.S of gills at 10^{-3} dilution was higher than that promoted by V.S prepared from cuticles of the same strength (Figure 3.35). The effects of V.S of gills and cuticles at 10^{-6} dilutions were significantly less than those exhibited by V.S of 10^{-3} dilutions (Figure 3.35). The MTT test was meaningful for first twenty four hours and subsequently was not useful as the cell lyses increased.

3.4.4 Nested PCR analysis :- Nested PCR technique was employed to detect WSSV in the testicular cell line after exposure to WSSV. The results of nested PCR are depicted in Figure 3.36. Lane M shows step up TM 100 bp ladder which ranges from 100 to 1000 bps, Lane 1 represents the control cell line which was not exposed to WSSV, while Lane 2 represents testicular cell line infected with WSSV. Lane 3 shows the results of positive control which shows expression of pure WSSV DNA sample. Lane 4 represents negative control having genetically engineered WSSV DNA. Figure 27 clearly show that there is no expression of any band in Lane 1 and 4, while Lane 2 and 3 shows expression of 275 bp bands. Thus Nested PCR technique clearly demonstrates the presence of viral DNA in the cells as evidenced by the presence of prominent bands at 275 bp for cells infected with WSSV and positive control (WSSV sample). However, the control cultured cells did not show any band.

3.4.4 RAPD analysis :- RAPD profile generated from RAPD polymerase chain reaction using 10 mere primers such as RPL 304, B2 and B9 is given in Figure 3.37. The legend of the figure gives information on expression of RAPD bands of infected and non infected testicular cell lines obtained by using different primers. The RAPD analysis indicates that the controls are expressing bands of host DNA (cell line) while Lane 2, 3,5,6,8 and 9 show additional bands of varying intensity along with their native DNA bands. Also it is evident that with a primer B 19 there is no expression of viral DNA band in addition to the host DNA band.

Table 3.15 Effects of WSSV inoculums of different concentrations on testicular cell line of *Scylla serrata*

Inoculation days.	**Gill V.S**				**Cuticle V.S**			
	10^{-1}	10^{-3}	10^{-6}	Control	10^{-1}	10^{-3}	10^{-6}	Control
2 hr	+	_	_	_	-	_	_	_
1	+++	+	_	_	+	_	_	_
2	+++	+	_	_	++	_	_	_
3	+++	++	_	_	+++	_	_	_
4	+++	++	_	_	+++	_	_	_
5	+++	+++	_	_	+++	_	_	_
6	+++	+++	_	_	+++	_	_	_
7	+++	+++	_	_	+++	_	_	_
8	+++	+++	_	_	+++	_	_	_
9	+++	+++	_	_	+++	_	_	_
10	+++	+++	_	_	+++	_	_	_
Survival days without obvious change			11-60	11-60		11-30	11-60	11-60

- : Cells remained without obvious change.

\+ : Cell clumped, cell shrinkage, few cell debris.

++ : The piles of cell debris were large.

+++ : Cell death.

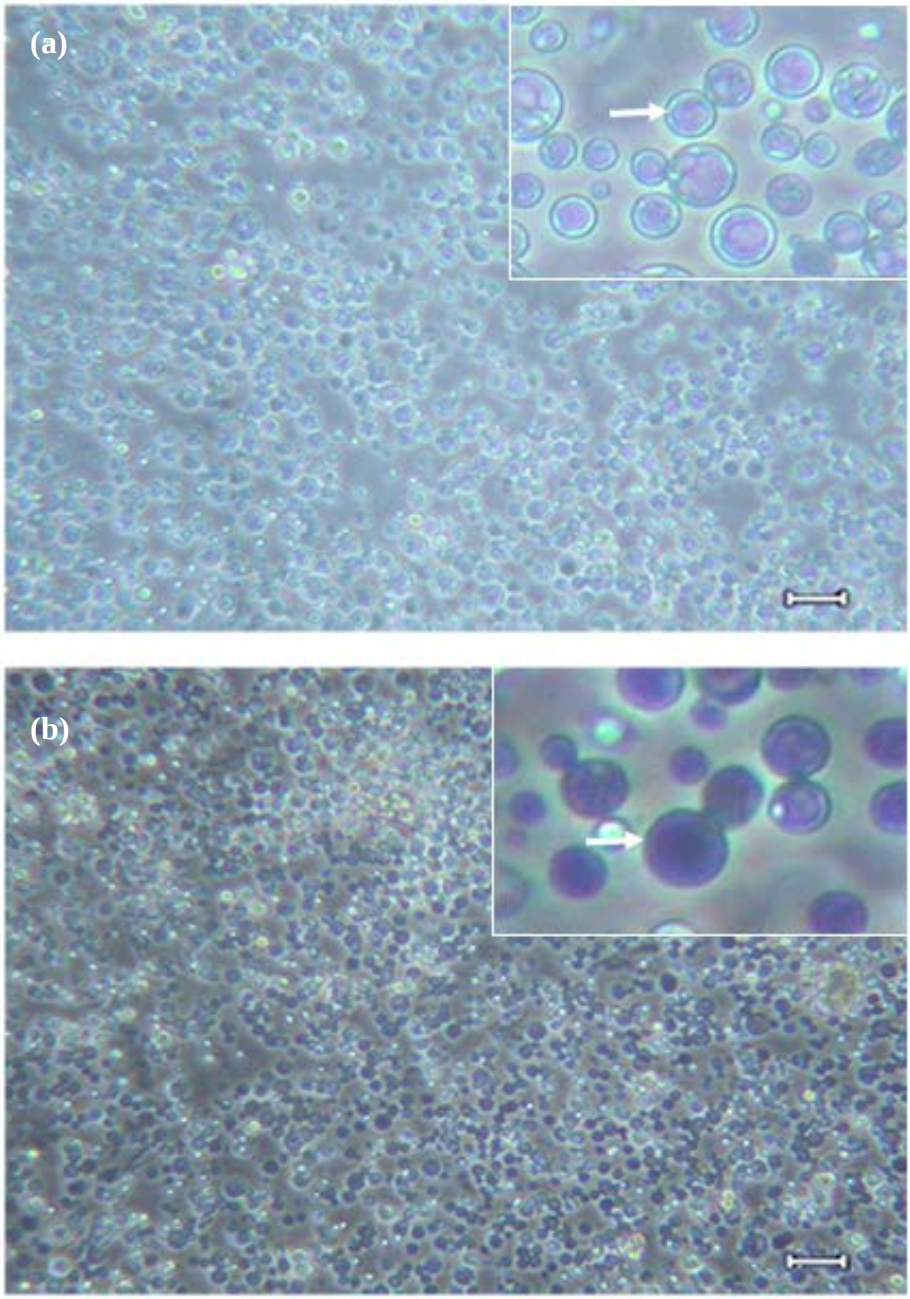


Figure 3.33 Cell culture of testis inoculated with WSSV suspension (10^{-1} dilution)

a. Control culture (SB – 20 µm, M – 100 x)

b. Experimental cells, showing change in cell appearance after four hours of exposure to viral suspension. (SB – 20 µm, M – 100 x)

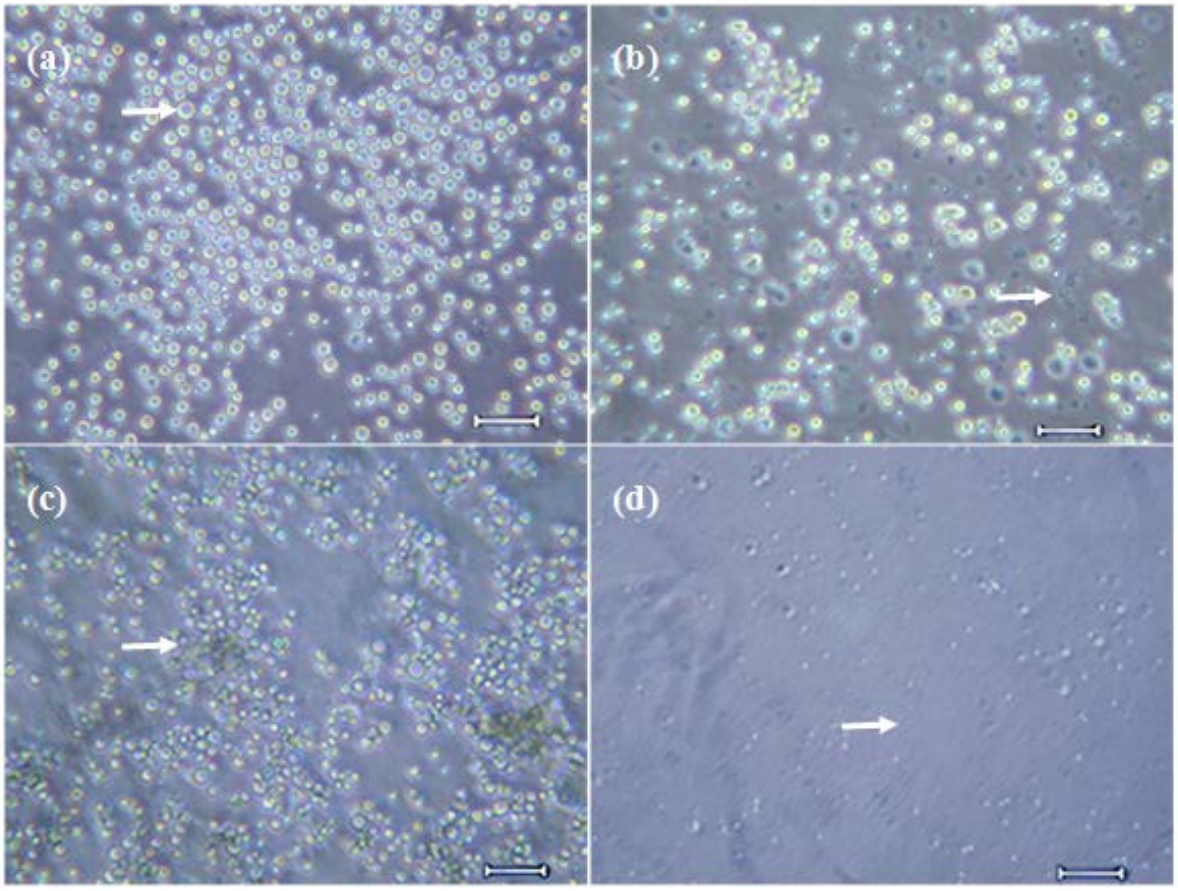


Figure 3.34 Cell culture of testis showing cytopathic effects on exposure to WSSV – V.S. **(a)** Control culture, **(b)** Experimental culture showing debris (Gill V.S 10^{-6} dilution), **(c)** Experimental culture showing cell aggregation (Cuticle V.S 10^{-1} dilution), **(d)** Experimental culture showing cell lysis (Gill V.S 10^{-1} dilution 24 hr exposure). Scale bar (a, c, d – 20 µm , M – 100 x), Scale bar (b – 10 µm, M – 100 x)

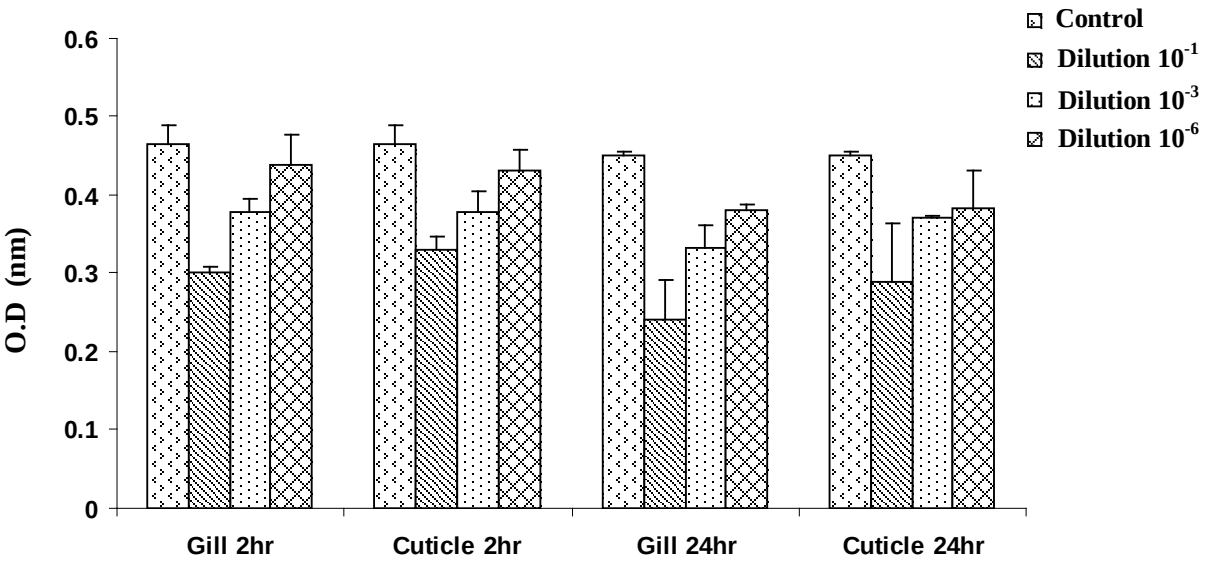


Figure 3.35 MTT test on cultured cells inoculated with viral suspension (Gill, cuticle) diluted to 10^{-1}, 10^{-3} and 10^{-6} respectively for 0-2, 2-48 hrs. All data represents mean ± standard error. Experiments were performed in triplicate

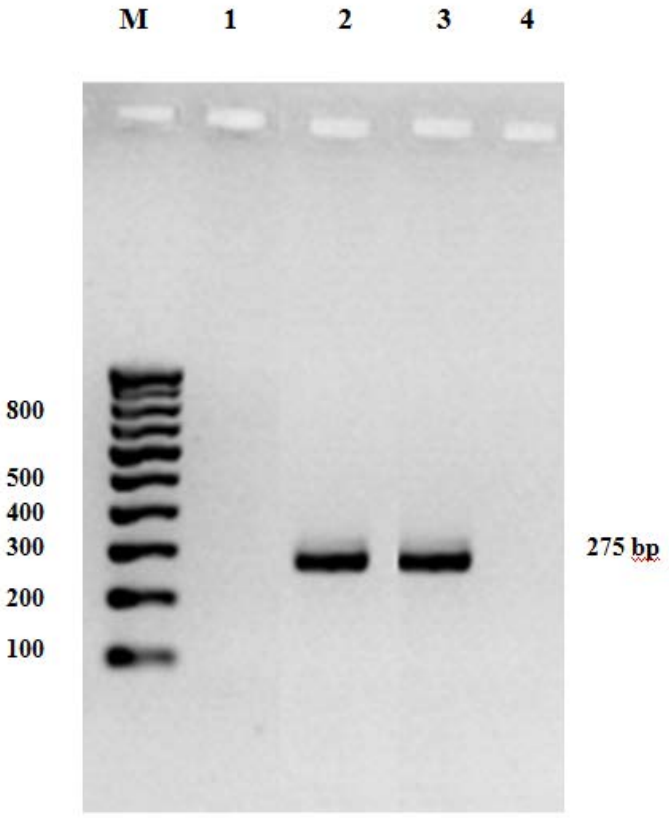


Figure 3.36 Detection of WSSV by Nested PCR technique in cultured testicular cells inoculated with V.S. Lane M: StepUpTM 100 bp Ladder; Lane 1: Control cultured cells, Lane 2: Cells experimentally infected with WSSV, Lane 3: Positive control (WSSV sample), Lane 4: Negative control (Sample without WSSV)

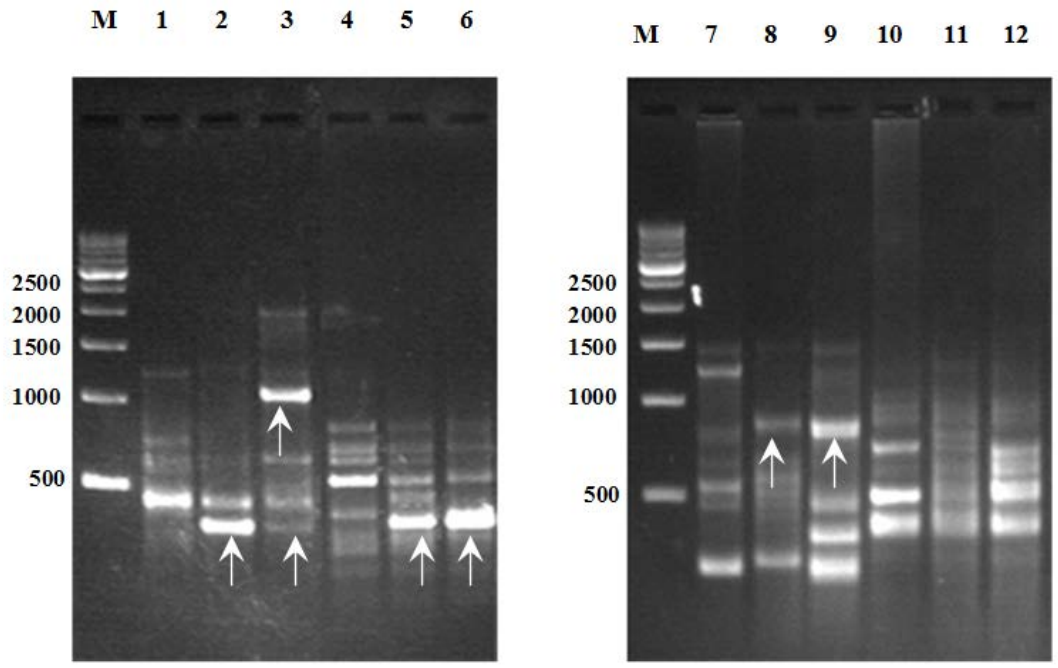


Figure 3.37 RAPD Profile was generated using 10-mers primers RPL 304, B2, B9 and B19. Lane M: StepUpTM 500 bp Ladder**, Primer RPL 304** [Lane 1: Control cultured cells, Lane 2: Cultured cells experimentally infected with WSSV (gill V.S), Lane 3: Cultured cells experimentally infected with WSSV (cuticle V.S)], **Primer B2** [Lane 4: Control cultured cells, Lane 5: Cultured cells experimentally infected with WSSV (gill V.S), Lane 6: Cultured cells experimentally infected with WSSV (cuticle V.S). **Primer B9** [Lane 7: Control cultured cell**,** Lane 8: Cultured cells experimentally infected with WSSV (gill V.S), Lane 9: Cultured cells experimentally infected with WSSV (cuticle V.S)] **Primer B19** [Lane 10: Control cultured cells**,** Lane 11: Cultured cells experimentally infected with WSSV (gill V.S)**,** Lane 12: Cultured cells experimentally infected with WSSV (cuticle V.S)]

CHAPTER - 4

Discussion

DISCUSSION

Crustacean fisheries and shrimp aquaculture have gained significant attention of the world aquaculturist owing to its economic values. The aquaculture industry has been growing leaps and bounds since the last decade. However, this growing aquaculture industry has started facing number of problems of which a phenomenal one is the disease of diverse origin. This disease not only harms the health of aquatic animals but also to the aquaculture industry players, workers and consumers at last leading to the adverse impact on the economy as well as the general society of many countries involved in aqua industry. The out breaks of disease, its progression, normally depend upon the host, their genetic background as well as on the pathogens, their toxicants and environmental factors (Snieszko, 1974., Bly et al 1997). Therefore, the researchers particularly fisheries researchers started paying more attention to not only disease producing pathogens but also the vectors and ultimate host. This led to the realization that we need to have authentic system by which one can understand host parasite relationship, the immunity of the host, genetic profile of the host as well as mode of pathogeneses in order to contain the disease outbreaks and their perils.

During the last decade a lot of attention was paid on identification, prevention and disease treatment rather than developing tools to understand the pathogens their ability to mutate and evolve rapidly in response to challenges faced by them in terms of preventive and curative chemicals (Catap et al 2003., Hsu et al 2000). Among the diagnostics these days PCR techniques have been employed to detect the pathogens of the modern world (Phromjai et al 2002). Crustacean cell culture gained momentum in the background of aquaculture diseases of commercially important species since crustaceans

are particularly vulnerable to viral diseases as their immune system is not effectively developed like modern vertebrate system (Sanchez-Paz, 2010). The lack of standardised system for isolation, identification, purification and characterization of viruses has become a major constrain in containing viruses that inflict heavy loses to aquaculture industries (Claydonet al 2010). This lead to realize the pressing need to develop a crustacean system particularly a cell culture system to understand the mode of viral infection, its propagation in the host cell, its identification and ultimately to develop the tools to control their spread in aquaculture system, through developing means to mitigate virus propagation from infected cells to the other proliferating cell cultures. This pressing demand and understanding of the need of developing an *in vitro* system to test pathogen efficacy, pathogen behaviours in the host cell and their propagation led many researchers to attempt establishment of primary cell cultures of many crustacean species with an aim to ultimately develop a cell line, Though many succeeded in developing various primary cell cultures, all of these cultures except a few survived for a short duration.

Rosenthal and Diamant (1990) developed long lasting cultures with 90 passages without a feeder layer and Hsu et al (1995a) developed a culture that could last for ten weeks to several months. But these researchers did not test these cultures for studying viral pathogenicity. Tapay et al (1995) and Claydon et al (2010) were successful in developing hybridised cell lines of crustacean tissues through cell fusion technique but failure of these cell lines to supportviral growth and viral replication along with their inadequacy for exploring molecular and cellular mechanism of virus pathogenicity became evident very soon. These failures were attributed to the absence of crustacean genes and proteins in the hybrid cells. Further the inability of the hybrid cells to support viral replication suggested that these hybrid cell lines lack key crustacean cell components required for the use as a

crustacean *in vitro* system (Clyadon et al 2010). Though in most cases the primary cell cultures survived well, their proliferation rate was conspicuously low with almost no cell proliferation on attempting the very first sub culture (Toullec et al 1996). In spite of these limitations in majority of cell cultures, the fisheries researchers realized that one has to keep on developing primary cell cultures of different tissues inorder to understand the reasons for limited survivability of cells in primary cultures and their inconspicuous proliferations in subcultures. The present attempt in this work is a step in this direction with an ultimate goal of finding ways to establish an *in vitro* cell system or a cell line suitable for testing pathogenicity of the virus like WSSV that has devastating capacity to damage crustacean aquacultures particularly of commercially important species.

Since the success of primary culture depends upon isolation of cells from the parental tissue with high viability, refining of the dissociation techniques is quite essential. It is well understood that soft tissues can be triturated mechanically either by stirring them with magnetic stirrer or by repeated aspirationusing a pipette. However, in the whole process one has to control the strokes of the magnetic stirrer to reduce cell injuries for that particular soft tissue. This requirement may vary from one soft tissue to another soft tissue. The greatest difficulty faced by the researchers for tissue handling and tissue disaggregation is the lack of regulatory systems to decide the number of strokes per minute with a magnetic stirrer. Therefore, in the present work the use of magnetic stirrer was avoided and this resulted in dependency on aspiration of tissue using a pipette. Here, in this aspiration by pipette the number of passages through pipette, the diameter of pipette bore and the suction generated by either the pipette bulbs or pipette pumps are the decisive factors for minimising cell injuries during aspirations. The technique of aspiration is found more suitable to obtain large number of viable cells. However, much depends upon how

one handles the cells viz-a-viz tissue and executes the process of mechanical cell dissociation.

Toullec (1999) has reported the suitability of mechanical dissociation for soft tissue but he is of opinion that mechanically dissociated cells from the tissues have reduced ability to attach to the substratum of the culture dish. In the present work the soft tissues like hepatopancreas, testis and ovaries were dissociated mechanically with cell viability over 90% but the use of enzymatic dissociation technique was found very effective for some specific tissues such as gills, heart, brain and muscles. Though, these are all soft tissue perse they did not give cell viability over 30% when dissociated mechanically. The phenomenal low viability of the dissociated cells of these tissues could be attributed to their extreme sensitivity to the variation in suction force applied during aspiration as well as to the forced passages of the fragments through tiny bore of the pipette mouth. The passages through a tiny bore or a mouth of a pipette could have inflicted laceration to the tissue fragments viz-a-viz to the cells of the tissue fragments. One need to note that these lacerations are not caused by the sharp edges of the pipette mouth as such sharp edges donot exists. Therefore, the injury must have occurred due to the friction of cells with the inner bore surfaces of the pipette coupled with the aspiratory force.

Upon contemplating these factors, employing a combination of enzymatic treatment and aspiration through a pipette with bare minimal aspiration force elevated cell viability to over 90%. Interestingly the well planned, regulated mechanical dissociation gave better viability for testis, hepatopancreasand ovaries. Toullec (1999) has reported that a few tissues have always attracted researchers especially for virology studies, among them

tissues such as hepatopancreas, ovary and hematopoietic tissues from crustaceans have been preferred.

4.1. Primary cell culture of gill epithelial cells

The explants of gills exhibit the formation of blebs which are nothing but cell mass projections from the tissue fragments. The size of the blebs depends upon number of cells attempting to migrate out of the fragments. The projecting blebs from the tissue fragments exhibited tendency to attach to the substratum. This could be attributed to a certain extent to the hemocytes present in the gills which might have migrated out well before the other cells from the blebs could migrate out. Since hemocytes or hemocyte like cells provide a natural attachment factor as reported by Toullec (1999), the attachment of blebs to the substratum could be considered as a result of the natural attachment factors released by the haemocytes.

The osmolality was deliberately maintained with a minor variation in an around 1050 mOsm/kg as the osmolality of the hemolymph was found to be 1050 mOsm/kg and even if there is a natural variation in the tissue fluid osmolality, it will always vary marginally not drastically. However, two extremes were tested such as 902 and 1088 osmolalities to know their effects with reference to cell survivability and cell migration in primary culture. Cells would tend to migrate, rather venture out of explant only when they judge that external environment that is milieu exterieur has same osmolality. When the cells sense the osmolality is either low or high they sense the stress and avoid migration and hence less number of migrations are observed though there are initial attempts of some cells to migrate out probably with hope of adaptation to the different osmolality.

The migrated cells from the explants of gills could not survive for more than two days and also the blebs did not promote migration of more cells. The low survivability of cells was definitely not due to osmolality stress or for the want of adequate nutrients or the media supplements like insulin, transferrin. Insulin and transferrin influence the nutrient metabolism particularly glucose and transport of molecules therefore they were expected to enhance the cell survivability. However, it appears that they have no influence on cell survivability. This indicates that there must be some factors other than the osmolality stress, nutrients or growth factors that are in operation to reduce cell survivability. Claydon and Owens (2008) are of opinion that primary cells have limited proliferating capacities in culture due to induction of cell senescence. In the present case the very low survivability of migrated cells could be attributed to this induction of cell senescence but it needs further investigation. Besides, the low survivability of migrated cells from explant could be partly attributed to a very low cell, population density. Since it is well established that the cells do not survive in isolation and cell aggregations are essential for cell anchorage which also decides the cell viability (Toullec 1999).

The mechanical dissociation of gills cells through pipette passages resulted in producing less viable cells and this could be attributed to injuries received by the tissue fragments due to probable factors like laceration, friction, aspiration pressure as described earlier. Besides, the very low survivability of the mechanically dissociated cells could also be attributed to the internal microscopic non visible injuries of the cells as well as to the pronounced expression of genes responsible for senescence (Claydon and Owens, 2008) as other factors such as nutrients, osmolality of media with supplements, pH of the media, and presence of contaminants were taken care of. However, the enzymatically dissociated cells also could not survive for more than two to three days, in-spite of provision of

adequate growth factors, nutrients or the media of osmolalities equivalent to the cells natural bathing media like tissue fluid and hemolymph. This indicates that there could be some factor present in the media that stimulate early senescence or not appreciated by crustacean cells. It needs further investigation to understand which of the factors pave way of expression of genes regulating senescence and cell death. Kerry (2009) reported that the dissociated gill tissues contain lot of floating debris with only a few cells attachment to the substrate. These could be contributing factors for the poor gill cell survivability as the toxicants released by the debris or injured cells could affect cell survivability.

4.2. Primary culture of hepatopancreas

The review of literature indicates that there are very few reports on the development of primary cell cultures of economically important crab species (*Scylla serrata*). As such the present results are comparable with studies on primary cultures of hepatopancreas of *Penaeus monodon* (Uma et al 2002), *Penaeu sorientalis* (Lipinget al 1990), *Palaemonte sargentinus* (Sousa et al 2003) and *Marscupenaeus japonicus* (Zilli et al 2003). Crustacean hepatopancreatic cell cultures were obtained by researchers following explant and mechanical dissociation techniques (Toullec et al 1996). However, enzymatic dissociation is always avoided for hepatopancreas owing to its fragile nature and enzyme sensitivity.

Explant techniques have been successfully used by Liping et al (1990), Cancer et al (1995), Toullec et al (1996) and Mulford and Austin (1998) for penaeid hepatopancreas. All these researchers are of the opinion that small types of cells (1 to 5 µm in size) always migrate from the explant and these cells tend to attach to the substratum, further they have added that round granular cells and refringent- type of cells remain around the explants.

The present work is in agreement with these reports, with reference to cell migrations from explants and its dependence on the medium used for the explant. Similar views have been expressed by Cancre et al (1995). The ability of the explant to give blebs resulting into quick migration of cells within four hours to form a confluence is a new finding since there is no such report of confluence formation from the migrated hepatopancreatic cells from explant. This could be attributed to the 3xL15 medium supplemented with EGF and glucose. The identification of cells such as embryonalzellen (E), fibrenzellen (F), blasenzellen (B) and restzellen (R) is well in agreement of the earlier classical reports of the presence of such cells in heaptopancreas (Hirisch and Jacobs, 1928., Hirisch and Buchman,1930). Subsequently few researchers have identified these cells in hepatopancreas (Jhonstonet al 1998., Zilli et al 2003., Sousa et al 2005). However, Al-Mohanna et al., (1985) described a fifth cell type identified as M-midget cell in hepatopancreas of *Penaeus semisulcatus* but such a type of cell was not found in the hepatopancreas of crab in the present work.

For the disaggregation of hepatopancreatic cells the mechanical trituration as described in the materials and method was employed in the present work. Upon the centrifugation of cell suspension obtained in the process of disaggregation, two layered pellets namely: yellow layer and a bottom white layer pellet were obtained but Zilli et al (2003) obtained three layers such as dark-black, yellow-white and pink-grey. Further they added that these layers represent Blasenzellen, Fibrenzellen and Restzellencells respectively. In the present work pink layer was not obtained for the hepatopancreas of *Scylla serrata*, instead, all the cells referred by Zilli et al (2003) were found in the yellow layer. Zilli et al (2003) did not obtain any layer exclusively representing granular cells which was obtained as a white layer in the present work.

Therefore, these findings are unique for *Scylla serrata* and at present it is not found in any other crustacean species. The presence of granular cells that survive for 192 days with limited proliferation in 3xL15 medium supplemented with EGF and glucose is a new finding for *Scylla serrata.* The ability of 3x L15 medium to increase longevity in presence of EGF indicates some role for EGF for inducing cell longevity of granular cells. Though some researchers have claimed usefulness of sera as supplements to the culture medium, the present work is in disagreement with the use of sera as arrest of cell growth and viability were observed when sera were added as supplement.

The present work is partly in agreement with the previous reports on the efficacy of L-15 medium for promoting cell longevity and growth of crustacean hepatopancreatic cells (Chen et al 1986). However, the present work demonstrates the suitability of 3xL-15 medium at 1050 mOsm/kg (osmolality) for primary cultures of hepatopancreatic cells, further it is also demonstrated that 3xL15 medium when supplemented with EGF (20 ng/ml) and glucose (1gm/l) enhance the survivability of hepatopancreatic cells. This indicates the role of EGF in promoting longevity of primary cultures. The use of EDTA-citrate buffer with phenyl methane sulfonyl fluoride (PMSF) at pH 7.1 was reported by Zilli et al (2003) for maintaining hepatopancreatic cells but in the present work PMSF was replaced by poly vinyl pyrrolidone (PVP) for adjusting osmolality to 860 mOsm/kg at pH 7.4 with equal success. Though there are reports of better growth of invertebrate cells at pH range of 7.0 - 7.4 (Toullec et al 1996., Owens and Smith, 1999) and 7.6 - 8.1 (Hsu et al 1995a,b) the present work shows that at pH 7.4 the hepatopancreatic cells of *Scylla serrata* survive well. Although the necessity of FBS or FCS at concentrations of 5-20% (v/v) as an ingredient in crustacean cell culture medium has been emphasized by some researchers (Tong and Miao, 1996., Mulford and Austin, 1998., Chen and Wang, 1999., Owens and

Smith, 1999., Mulford et al 2001., Uma et al 2002), Leudeman (1990) and Frerichs (1996) suggested that serum could be toxic for crustacean cells. The present study demonstrates that serum degrades the viability of hepatopancreatic cells of *Scylla serrata* and hence toxic.

The cell attachment observed in this study is partly in agreement (with reference to the loose attachment of fibrenzellen, blasenzellen and restzellen cells to the substratum) with that reported for *P. vannamei* by Toullec et al (1996) as they reported negligible attachment of hepatopancreatic cells. However, in the present work embryonalzellen and granular cells exhibited firm attachment to the substratum. This indicates that the embryonalzellen and granular cells behave differently in comparison to the other cells.

The general histomorphology of hepatopancreatic cells of *Scylla serrata* is similar to that observed in a few penaedon species (Gibson and Backer, 1979.,Al-Mohanna et al 1985., Al-Mohanna and Nott, 1987., Icely and Nott, 1992., Johnston et al 1998., Sousa et al 2005). The RAPD analysis of the hepatopancreatic cells taken together clearly indicates that the different cells of the hepatopancreas originate from the parental tissue as they share similar RAPD profiles. Further, the present work suggests that YNZ22 is a better primer for amplification and expression of hepatopancreatic cell DNA of *Scylla serrata*. In short, it is apparent that different types of hepatopancreatic cells of *Scylla serrata* could be separated, cultured / maintained and they did not proliferate much individually probably owing to the lack of specific growth factors or mitogenic agents in the culture media. However, this requires further investigation to develop ideal medium for culturing each type of hepatopancreatic cell of *Scylla serrata in vitro*.

4.3. Primary culture of hemocytes

The hemocytes exhibit anchorage to the substrate and showed a tendency to settle like the formed elements of mammalian blood. This observation is in agreement with that reported by Ellender et al (1992). In the present work the haemocytes survived for 14 to 16 days in 2xL15 medium having osmolality 1050 mOsm/kg. Li and Shields (2007) reported the survival of blue crab hemocytes for 15 days in Grace's modified medium. However, the haemocytes of *Penaeus monodon* in a primary culture have been reported to survive for 8 days by Jose et al (2010) while Ellender et al (1992) reported their survival by one month. But Li and Shields (2007a) reported survival of hemocytes of spiny lobster in primary culture for 18 days. Recently Liang et al (2012) reported the survival of crab (*Ericises sinensis*) hemocytes of primary cultures for more than 35 days. The demonstration of existence of three types of hemocytes in the present work is in agreement with that of Li and Shields (2007b). Therefore, it appears that present work on hemocytes with reference to survivability is in agreement with Ellender et al (1992), partially with Li and shields (2007b). But it must be noted that many of the researcher here worked on the crustaceans other than crab. However, researchers have used different media and supplements for the survival of hemocytes. There is no specific culture medium and supplements used universally for the primary culture of haemocytes and no exclusive attempts are made to enhance the longevity of the haemocytes. In general the morphology of the haemocytes of *Scylla serrata* is in agreement with the reports on hemocytes of blue crab and shrimps (Li and Shields, 2007b).

4.4. Primary culture of heart cells

The explant culture of heart cells exhibited migration of hemocytes but not of heart cells cardiomyocytes as such even after four days of initiation of culture. However,

migrated haemocytes tended to attach to the substratum and could have paved way for formation of a few blebs after four days of initiation of culture. From the blebs, few fibroblast like cells migrated into the culture medium which survived for six to eight days. The permutation combinations of culture media and supplements did not alter the longevity of the cells and could not promote cell proliferation. Peponnet and Quiol (1971) reported formation of layers of muscle fibre like cells emerging from the explants of marine crabs. Owen and smith (1999) observed 40 days survivability of heart cells of prawns in explant cultures using 2xL15, supplemented with 10% FBS and 10% prawn muscle extracts. Further, Owens and Smith (1999) observed that the explant culture derived dividing heart cells survive for 307 days in an *in-vitro* system. But Mulford and Austin (1998) reported survival of heart fibroblast of *Nephrops norvegicus* (prawn) for four weeks in 2xL15 + 20 % FBS having osmolality 740 mOsm/kg. These findings indicate that within crustacea the survivability of the heart cells or heart fibroblast like cells varies from genera to genera. The long survivability of heart cells observed by Owens and Smith (1999) for prawns has not been reported by any other researchers. The present work shows that the heart explants yield fibroblast like cells which survived for 6 to 8 days and do not respond to any changes in the culture media or their supplement combinations including various sera.

The present work indicates that collagenase is the most suitable enzyme for obtaining heart fibroblast cells. But even these cells though conspicuously distinct in their morphology, survived for short duration without any proliferation. The low survivability of these cells partly could be attributed to the action of collagenase on some of the cells or partly to the toxicants released by the injured cells in the cell suspension. Even though after centrifugation the cell suspension was rinsed thrice probably the effects of enzymes or toxicants could not be washed off. Also these heart cells could be sensitive to the handling

procedures indicating that further research is required in standardization of culturing and cell handling techniques in order to increase the survivability of such sensitive cells.

4.5. Primary culture of muscle fibroblast

The primary culture of muscle fibroblasts of *Scylla serrata* with explant techniques failed owing to the rigidity of the muscle explants in forming any blebs from the free ends of the fragments. Further, use of media of different concentrations with supplements proved to be ineffective and this could be attributed to the strong sheath that envelops the muscle fibres.

The dissociated muscle fibres of *Scylla serrata* survived for six to seven days in the culture and did not show change in longevity as well as proliferation in response to different media and supplement combinations. Therefore, it appears that the muscle fibres of the crabs have short survivability in *in-vitro* state. Though the crabs demonstrate expressive regeneration even of appendages, somehow this ability appeared to be lost in the culture. This could be attributed to the debris that accompanies the dissociated muscle fibres in the culture medium. Even though the dissociated muscle fibre suspensions obtained through enzymatic dissociation were well filtered through a nylon mesh however, it was difficult to separate out the toxicants form the debris. Not only this, along with the tiny debris particles some of the cell components of the injured cells or lysosomal enzymes present in the cells and activated during the injuries could be adversely affecting not only longevity and survivability but also the proliferation or regeneration. However it needs further investigation to analyse why muscle fibres do not survive for long and proliferate in *in-vitro* systems. It is also interesting to note that there is hardly any report on development

of primary culture of crab muscle fibres though researchers have attempted to culture fibroblasts of lymphoid or ovarian tissues (Rinkevich, 2005).

4.6. Primary culture of neurons

The explant cultures of neurons from the crab ganglia appear to be dependent upon the removal of ganglionic sheath and exposure of the underneath neurons to the culture media. The migration of neurons from the explants and their attachment to the substratum does not appear to be dependent on a specific type of substrate or a specific type of medium. However, the present experiment clearly indicates that the migrated neurons in explant have relatively better survivability in 2xL15 medium supplemented with nerve growth factor (NGF). The neurons appear to have limited life span in *in-vitro* state. This could be attributed to less understanding of the requirements of crab neurons and probably they may grow better when grown with feeder layer. However, it needs further investigation to explore the specific requirements of the crustacean ganglionic neurons, particularly with reference to cytokines and neurotransmitters and matrices.

The dissociated neurons exhibited anchorage to Poly-L-Lysine substrate but neuritogenesis which is indicator of cell growth was not observed in-spite of the presence of NGF in the culture medium. The limited survivability could be attributed to the induction of apoptosis or necrosis induced by the toxicants released from the injured cells. Owen and Smith (1999) cultured neurons of prawn with a longevity of just two days. Cooke et al (1989) reported cultures of peptidergic neurons in chemically defined medium as well as simple medium composed of physiological saline glucose and glutamine. However the growth was limited to outgrowth of the neurons in terms of cones. They reported that the crab eyes stock neurons survive for three weeks but subsequently the

growth gets retracted. The observation of neuronal survivability after trypsinization depends upon the reduction of trauma of separation of cells. In the present work relatively lesser survivability could be attributed to the trauma of cell handling and dissociation along with some specific factors. Grau and Crooke (1992) reported culture of peptidergic neurons of crab in defined culture media and observed that the different types of neurons preferred to grow in crab saline supplemented with glutamine and glucose. They further observed that osmolality changes cause transient varicosities in small cells. They are of opinion that the arrest of growth normally occurs when potassium concentration in the medium is eleven millimolar or more, particularly, when it reaches to 30 millimolar. However, in the present work the use of plain crab saline and glucose had no significant influence on neuronal growth with reference to neuritogenesis. Besides in the present work glutamine as supplement was not tested, therefore it needs further investigation to ascertain if glutamine and potassium combination could lead to better survivability and growth as observed by Grau and Cook (1992).

4.7. Primary culture of ovarian cells

The ovarian cell culture initiated by explant exhibited no media and substrate specificity for formation of blebs from the explant and the cell migration from the blebs of the explants began within a few hours of initiation of culture. The migrated cells had no substrate specificity for anchorage and within 48 hours they formed monolayer. The cell growth and cell migration rates in presence of media of different strength and different supplement combinations exhibited variations in survival rate even though the osmolality was constant. These behaviours of ovarian cells obtained from explant indicate that it is a particular composition or the components of the medium that might be deciding the cell growth and longevity of cells in culture. The provision of growth factors induced limited

cell proliferations indicating that growth factor has limited influence on the cell proliferation. The present work indicates that the better cell growth and proliferation of explant mediated cell cultures of ovary could be achieved when grown in L15 and crab saline (1:1 v/v) medium and the addition of supplement like EGF increases the cell longevity up to some extent. However, most of the researchers have tested different media for ovarian explant cultures of crab. Toullec et al (1996) reported better result with the use of M199 than using Grance's medium, and claimed that his observations are in agreement with those of Rosenthal and Diamant (1990) but further added that these observations conflict with the observations of Luedeman and Lighter (1992) and Nadala et al (1993a).

The success of ovarian cell culture in-terms of substantially higher longevity and proliferation rates would depend upon the state of ovaries. Toullec et al (1996) claimed that immature ovaries contain smaller oocytes with less vitelline which may be making them less fragile than the mature oocytes. Therefore, the longevity and proliferation of ovarian explant cells would also depend on the physiological stages as well as developmental stages of the ovary used for explant culture. In the present work the ovarian tissue used was mature and hence probably the migrated ovarian cells had less totipotency for proliferation and longevity in comparison to the penaeid ovarian cells used by Toullec et al (1996) with the longevity of three months. Chen and Wang (1999) observed that muscle extracts and ovarian extracts can prolong the survival of ovarian cell cultures to more than 15 days. In the present work the ovarian cells cultured through explants could survive healthily for 27 days with L15 and crab saline and their longevity could be enhanced upto 40 days by addition of EGF. This indicates that for the crab ovarian tissues the longevity depends upon the EGF to some extent. The cells of explant cultures might not be surviving beyond 40 days either due to induction of cell senescence or due to

confluence formation within a short period which could lead to crowding of cells due to increase in cell population of migratory cells and making them sensitive to the population density. Further the increasing nutrient demand of confluence of large number of cells would deprive them of the precious nutrients in adequate amount as well as oxygen. The metabolic product of glucose is always water and Co_2 gas. The excess of Co_2 liberated due to enhanced metabolic rate would alter the pH readily inspite of the presence of built in carbonate buffering system of the medium. The altered pH could eventually reduce the cell survivability. Therefore, the less longevity of ovarian cells could be attributed to many factors mentioned above.

The dissociated ovarian cells exhibited variation in survival rate and proliferation in response to media concentration, and media supplements. The higher concentration of media and nutrient mixtures didn't enhance the cell survivability indicating higher concentration of nutrients and nutrient mixtures are not only the factors that enhance cell survivability and cell proliferation of ovarian cells in culture. The enhancement of survivability of 27 days with plain L15 and crab saline mixture clearly demonstrate that high nutrient mixture and nutrients are not required for prolonging cell survivability. However, addition of EGF at 20ng/ml appears to have some influence on longevity and proliferation but additions of insulin and transferrin have no influence on cell survival.

4.8. Primary cell culture of testicular cells

There is hardly any report on the explant cultures of testes of crabs, but Mulford and Austin (1998) attempted gonadal explant culture of prawn, *Nephrops norvegicus*. This indicates that the present work on explant culture of crab testes is the first of its kind for *Scylla serrata*. Mulford and Austin (1998) have reported suitability of 1xL15 medium

supplemented with 20 % (w/v) FBS at total osmolality of 480 mOsm/kg. Further they observed that the monolayer of fibroblast like cells develop within 24 hrs of implantation without any mitosis of the cultured cells. These cells survived for 16 weeks and usage of 2xL15 medium resulted in rapid migration of spermatogonial cells from the explants of testes with moderate attachment but with no mitosis. They also have observed that after two weeks of explant culture some round cells with large nuclei emerge. In the present work the migration of cells occurred within half an hour of implanting the testis fragments and is in agreement with the observations of Mulford and Austin (1998) with respect to formation of confluence by migrated cells within 24 hours with a firm attachment but it differs with respect to the type of cells that migrate. In the present work no fibroblast like cells emerged from the explants of testes. Further the migration of spermatogonial cells from explants was observed in the present work in presence of L15 + crab saline supplemented with EGF and glucose. The permutation combinations of L15 with supplements and changes in the strength of the L15 promoted lesser migration of cells. This indicates that the crab explants behave differently and the migrated cells exhibited occasional mitosis which was not observed by any researcher earlier for crustacean explants of testes.

Brody and Chang (1989) reported the utility of 200 u/ml collagenase treatment for disaggregation of testicular cells with a survivability of eleven months, but in the present work it is observed that mechanical dissociation of testes in artificial sea water is the most effective technique for isolation of cells with 95% viability and no usage of any enzyme is required for obtaining viable cells that survival for four months with cell undergoing mitosis. Both the explants and segregated cells of testes yielded cells that could proliferate and grow. These cells ranged in size from 10 to 38 μm with distinct nuclei of varying

shapes. The testicular cells on exposure to different combinations and strengths of L-15 supplemented with growth factors such as EGF, insulin, transferrin glucose and different sera showed varying survivability and proliferation rates. The use of L15 as a suitable culture medium for crustaceans has been demonstrated by many researchers (Nadala et al 1993a,b., Hsu et al 1995a., Tong and Miao, 1996., Fraser and Hall, 1999., Owens and Smith 1999). However, the present study indicates that F-12, L-15 (X, 2X and 3X) are less suitable for proliferation of primary culture and subcultures of testicular cells while L-15 with crab saline (1:1) can promote five months survivability with limited cell proliferation. However, this combination requires additional supplements like EGF (20 ng/ml) and glucose (1 g/l) to promote good proliferation and extended survivability of cells. Nadala et al (1993a) has shown the efficacy of EGF on Oka cell proliferation from *Penaeus stylirostris* and *Penaeus vannamei*. Tapay et al (1995) reported benefit of 20 ng/ml of EGF in the medium formulation to culture lymphoid cells of *P. stylirostris*.

The addition of EGF promotes rapid cell proliferation (mitotic index = 26). The addition of insulin which enhances glucose metabolism had no influence on the cell proliferation, as indicated by MTT test and mitotic behaviours of cells in culture. Transferrin is an ion transport protein that is used in cell culture media to improve serum free cell growth and to replace iron chelating compound. Also in cell culture transferrinis added to increase the quality and quantity of cells by ensuring that the cells are adequately supplied with iron, transferrin also maintains cellular iron homeostasis by helping cells to regulate iron absorption from extra-cellular environment. However, one should note that oxidative reactions of excess iron in culture media leads to free radical molecules that can cause damage to cells. Several cell types including stem cells and primary cells require the use of transferrin because the use of iron chelators or other iron products cause cell death.

(Stafford and Belosevic, 2003., Ganz, 2008). However, in spite of wonderful utility of transferrin in a culture medium as referred above, the testicular cells particularly in disaggregated form did not respond to transferrin indicating that maintenance of iron homeostasis is not a requisite for testicular cell culture viability.

Mulford and Austin (1998) have shown the necessity of serum at a concentration of 20% (v/v) as an ingredient in *Nephrops norvegicus* testis cell culture. However, present results show that with 20% concentrations of different sera caused cell deterioration within a few days while use of 5% (v/v) sera extended cell survivability to some extent. Luedeman (1990) and Frerichs (1996) suggested that serum could be toxic for crustacean cells. Moreover Walton and Smith (1999) reported occasional clumping of cultured hemocytes of crabs (*Liocarcinus depurator* and *Carcinus maenas)* in 20 % (v/v) serum, while 10% serum significantly promoted the viability of culture. Perhaps this could explain why the higher level of sera i.e. 15-20% resulted in a shorter period of survival of testicular cells than that observed in five percent. Addition of horse serum in culture medium promoted relatively better cell proliferation and survivability than that promoted by fetal bovine serum supplement.

The very first subculture from the primary cell culture is development of a cell line (Freshney, 2000). Therefore, the subcultures of ten passages with proliferation and survivability for five months could definitely be considered as finite cell lines of testicular tissues of *Scylla serrata*. The cell proliferation and growth exhibited by the testicular cells only in presence of epidermal growth factor (EGF) suggests that they are epithelial types. The unequal mitotic cell division observed in a few testicular cells of crab gives an impression of budding yeast. However, there is no possibility of presence of budding yeast

in cell culture as the culture medium contained antibiotics like amphotericin-B and nystatin which are anti-yeast and anti-fungi.

4.9. Characterization of testicular cell line

Hardly any researcher has developed a testicular cell line of crustaceans and those who have developed long surviving primary cultures of testicular cells have not characterized them. The cell line and the primary cell cultures need to be characterised for their proliferation rate, their health, ability to enter into senescence, mitosis, mitotic index, RAPD analysis and 18SrRNA sequencing. Therefore, an attempt is made in the present study to characterize the testicular cell line.

The execution of MTT test after every seven days revealed that the cells were proliferating in primary culture state as well as when sub cultured for developing cell line. Further MTT test has also revealed that during cell passages there was rise in population density as indicated by increase in optical density in MTT test. When the cells were not sub-cultured on the seventh day and allowed to grow further their population density declined upto fourteenth day and this decline in the population could be attributed to the elimination of those cells that are sensitive to the crowding caused by increase in cell population density (Freshney, 2000). But after a decline in population once again the cells begin to proliferate and attain a plateau phase which indicates the end of log phase and onset of slow proliferating and maturating state of cell population. The MTT assays thus were successfully used in the present work to determine the rise in population as well as decline in population. The utility of the MTT test in crab cell cultures has been reported from the lab where the present work was carried out (Shashikumar and Desai, 2011, 2012).

It is well established that LDH is present in the cytoplasm of the cells and it leaks out into the culture medium only when the cells are injured. Therefore, the estimation of percent release of LDH in a given cell population signifies the viability viz-a-viz the health of the population. Since the LDH release by the testicular primary cultures as well as the cell line remained below 10% the cultures are believed to have more than 90% viable cells which is an accepted criterion of viability of cell population. Further cell population can be characterised on the basis of number of cells entering the senescence. Since β-galactosidase expression indicates the onset of senescence (Lee et al 2006), the appearance of the pale blue colour around the nucleus of the four to five months old cell cultures in the present work, suggests the development of cell senescence however, β- galactosidase expression was not observed in the cell cultures that survived upto fourth month. The present work clearly shows that the cell line was healthy and normal with an ability to proliferate.

Since crab testicular cells have 53 haploid chromosomes, they are not quite prominently seen in chromosomal preparations, but the mitotic stages are clearly indicated by distinctly condensed chromosomes in prophase, equatorially arranged chromosomes in metaphase and their polar alignment in anaphase. Telophase is characterized by two distinct nuclei with partial cell separation. The occurrence of mitosis as evidenced by the presence of mitotic stages in the cell cultures, clearly indicate that the cell cultures have capacity to proliferate and have proliferating cells. This indicates that the cultures are not static but a dynamic system. Freshney (2000) has observed that mitotic index is one of the criteria employed to characterise the cultured cells. Mitotic index clearly tells that at a given time in a given population how many cells of that population are in the state of mitosis. One can estimate the percentage of cells undergoing mitosis. In the present study

mitotic index depended upon the type of the culture medium used which is quite natural as mitosis depends upon the specific component/s of the medium which will trigger the synthesis of mitogenic factor/s or cytokines required for the cells to enter into mitosis. It may be that the media per-sewould not have any mitogenic agent or factor in it but can pave way through its key component/s to stimulate the synthesis of mitotic factor/s. It needs further investigation to investigate which component of the culture medium is responsible for inducing mitosis through expression of genes regulating synthesis of mitotic factors.

The RAPD profile of *Scylla serrata* generated using primer YNZ22 clearly indicates that the cultured cells were derived from the parental tissue of *Scylla serrata* since both the parental tissue and cultured cells' DNAs share the amplification and expression of bands representing nucleotide base pairs. Similarly RAPD profile of testicular cell line generated using primer OPD20 and OPD08 share the same banding pattern representing DNA amplification of parental and cultured cells' DNA indicating that the cultured cells are derived from the parental tissue only. The use of different primers helps in confirming that the results obtained with one primer are genuine and are not artefacts. Paul (2000) has reported RAPD analysis of marine penaeid prawns. Zeng et al (2010) used RAPD techniques to determine whether the DNA amplification of the cultured hepatopancreatic cells matches with that of parental tissue.

18S rRNA sequence is useful for assessing genetic lineage of the cultured cells from the parental tissue as well as its relatedness with its genus and other species. The 18S rRNA sequence in the present work is probably the only report available for *Scylla serrata* as this has been accepted by international gene bank at Maryland USA with an accession

number (HQ697253). The 18S rRNA of the testicular cell line though ipso-facto a partial it clearly allows one to determine adenine to thymine and cytosine to guanine ratios. This sequence speaks volume with reference to frequency of occurrence of specific nucleotides as triplets, quadruplets, pentuplatesetc which are unique to a particular species. This also helps in understanding how many repeat base sequences occur. The blast analysis of the 18S rRNA has clearly demonstrated that *Scylla serrata* has 98 % phylogenetic similarity to *Scylla paramamosain* and 97 % similarity to *S paramamosain* hypothetical protein mRNA. Recently Ismail and Sarijan (2011) reported phylogenetic inference from 18S rRNA Gene Sequences of Horseshoe Crab, *Tachypleus gigas*. They used 18S rRNA sequence to reveal the phylogenetic relationship among two populations, DNA divergence and nucleotides diversity value in the penaeid population. However, this report of Ismail and Sarijan (2011) is not with reference to cell cultures.

Recently, Claydon et al (2010) have attempted developing a hybridized *Penaeus monodon* cell line by cellular fusion for testing virus pathogenicity but it is reported that such cells lack crustacean components required for the use as an *in vitro* system for virus replication. Therefore, this testicular cell line could be a very useful tool for testing infection and replication of crustacean viruses such as WSSV, hypodermal and haematopoietic necrosis viruses that cause damages to the aquaculture industry. Nevertheless, the present study provides a technique that could be extended for developing other crustacean cell lines.

4.10. Characterization of hepatopancreatic granular cells

The granular cells (~ 1-5 µm in diameter) appear spheroid under light microscope with a prominent central nucleus. Becker et al (1974) reported the occurrence of refractile,

spherical mineral granules of similar size in hepatopancreas of blue crabs (*Callinectes sapidus*). The confocal images reveal the presence of concentric rings similar to that reported by Correa et al (2003) along with a distinct nucleus. These cells appeared as tennis ball like structures under scanning microscope, similar spherical granules were observed in the hepatopancreas of crustacean and pulmonate species (Correa et al 2003., Simkiss and Taylor, 1994).

Simkiss and Taylor (1994) are of opinion that large number of invertebrates has cells that produce intracellular granules of amorphous calcium and magnesium phosphate. According to Correa et al (2003) during moulting process, the crustacean exoskeletal calcium is stored in the internal organs such as hepatopancreas and are mobilized for the calcification of new carapace. In the present work it is observed that the granular cells from the hepatopancreas store calcium and release it under the influence of ecdysterone. The presence of calcium in the cytoplasm of cells was demonstrated by exposing cells to Fura-2-AM, a fluorescent dye.

The hepatopancreas of adult male blue crabs are known to contain mineral granules that store calcium and phosphate (Beckeret al 1974). Subsequently, Correa et al (2002, 2003) descried these granules as electron dense macro vesicle like structure with concentric spherical layers bearing calcium, magnesium phosphate along with phosphatases. However, none of the researchers have paid attention to the proliferative nature, cellular DNA, RNA and proteins concentrations and relied upon the storage activity with reference to minerals required during intermolt stages. The biochemical analysis of granular cells revealed the presence of DNA, RNA and proteins. The present observation

of presence of DNA, RNA is in agreement with earlier report of Becker et al (1974) who showed the presence of nucleotides in the granules of the hepatopancreas of blue crabs.

MTT assays are employed to detect the cell proliferations (Poncet et al 2000., Hatt et al 2001). The granular cells exhibited variations in growth rate in a plain medium (L15 - crab saline) and EGF supplemented medium. The cells grown in L15 – crab saline supplemented with EGF exhibited a prominent rise in OD (signifying increase in cell density due to proliferation) after both 24 hr and five days of initiation of culture in comparison to cells grown in medium without EGF, indicating that the granular cells are epithelial type that proliferate in response to EGF as indicated by elevated OD during MTT assays.

During the process of ecdysis the calcium has to be released for the formation of new exoskeleton. Granular cells are the source of calcium. In the present study an attempt is made to know if the ecdysterone mobilises calcium from G cells. It is observed that at about four hours of exposure to ecdysterone, maximum amount of calcium was released. The release of calcium in response to ecdysterone indicates that the cells are not mere passive stores and probably have the mechanism or receptors to recognise the ecdysterone.

RAPD profile generated using primers (UBC457, YNZ22) specific for *Scylla serrata* showed similarity in band size and numbers in both granular cells and tissue (control) of *Scylla serrata* indicating that these cells have a specific genome which could be compared with the parental genome.

Therefore, it's concluded on the basis of histomorphology, RAPD analysis and biochemical assay including MTT test that granular cells are a distinct cell type that proliferate in response to epidermal growth factor and exhibit ecdysterone mediated calcium release.

4.11. Utility of testicular cell line for WSSV infectivity

Cell cultures and established cell lines are regarded as better representatives of cells *in vivo* as they reflect the true activity and functions that they display in their natural environment (Morgan and Darling 1993). Primary cell cultures from different shrimp tissues such as lymphoid organ (Chen and Kou, 1989., Chen and Wang, 1999., Wang et al 2000), Oka organ (Chen and Wang, 1999); heart (Chen and Wang, 1999., Owen and Smith 1999), ovaries (Maeda et al2004) have been used to study various viral infections and replications but long surviving and proliferating cell cultures were not then available for evaluating viral infections. Though, few researchers have attempted primary cell cultures of crabs (Ballard et al 1993., Walton and Smith, 1999., Li and Shields, 2007b., Sashikumar and Desai, 2008., Zeng et al 2010) none have used crabs' cell culture for studying WSSV pathogenicity.

The present study, probably the first of its kind, shows WSSV pathogenicity to the testicular cell line of crab (*Scylla serrata*). Microphotographs of infected cells clearly show: change in appearance, shrinkage, clumping, detachment from the substratum, cell lyses and declined cell population. But the control cells (normal, those treated with heat inactivated WSSV with various dilutions and the extracts of non infected *Penaeus monodon*) appeared normal and unaffected. Since the cell cultures exposed to WSSV were rinsed with culture medium (L15 – crab saline) after 4 hrs to avoid further viral exposure

of cells, the cytopathic effects obtained are due to the viruses which entered the cells. These results are similar to those reported for crayfish haematopoietic stem cells (Jiravanichpaisal et al 2006b., Uma et al 2002). Maeda et al (2004) reported rounding of primary cell cultures exposed to WSSV but in the present study WSSV infection did not promote rounding of cells.

Mosmann (1983) reported utility of MTT test to assess antiviral compounds' efficacy against a variety of viruses. However, this assay cannot be utilized for viruses that do not replicate well in cell cultures or for cells with low mitochondrial enzyme activity (Watanabe et al 1995). The present study clearly demonstrates the utility of MTT test in detecting WSSV mediated cell damage and possibility of replication of WSSV in testicular cells as it does not show proper results for viruses that do not replicate in cell cultures, (Watanabe et al 1995). Besides, the present work reports the limitations of MTT test in case of very low infection of viruses in the cultured cells as those exposed to virus suspension of 10^{-3} and 10^{-6} dilution didn't significantly respond to MTT test. Nevertheless, MTT test significantly demonstrated the ability of WSSV (in 10^{-1} dilution) to infect the testicular cells causing cell lyses.

The very fact that virus suspension of strength 10^{-6} or 10^{-3} did not promote cytopathy for a period less than twenty days, indicate that for induction of cytopathy a higher concentration is required. The lower concentration of V.S was not effective probably the viruses required a specific strength to attain by their replication and when their strength is low the natural immunity of the cells prevented expression of virus effects. However, it is not clear why the virulent nature of cuticular V.S is lesser than that exhibited by gill V.S.

Boucard et al (2010) have shown WSSV infection in oysters using PCR based molecular probes. PCR is the most sensitive molecular technique employed for the detection of WSSV infectivity. Currently, PCR techniques for WSSV detection use either a conventional amplification with a single sense/antisense primer set (Kanchanaphum et al 1998., Kiatpathomchai et al 2001) or a nested amplification (Lo et al. 1996). Nested PCR provides an increased level of sensitivity compared with conventional single primer-pair PCR. The present work reports here WSSV infection in testicular cell cultures of *Scylla serrata* using PCR based molecular probes. The Nested PCR test and RAPD techniques clearly indicate that the morphological changes of testicular cells and subsequent decline in population density are due to WSSV infection.

Nested PCR is an effective tool in detecting early infection of WSSV and prognosis of it has been reported earlier by Otta et al (1999), Hameed et al (2003) and Uma et al (2007). Chakraborty et al (2002) successfully employed Nested-PCR for screening 89 tissue samples obtained from shrimps, crabs and squilla. The use of Nested PCR technique in the present work is in conformity of the reports of aforementioned scientists with reference to detection of WSSV infection and pathogenesis.

In addition to Nested-PCR technique RAPD technique using RPL 304, B2, B9 and B19 primers was used in the present work for ascertaining the infection. The RAPD technique has shown that primer RPL 304, primer B2 and B9 are suitable for detection of WSSV virus as the cultured cells infected with the WSSV distinctly showed bands which were not reflected in controls.

Since the cultured cells were rinsed repeatedly (N=5) after removal of virus containing medium, there was no chance for the virus to remain in the replenished medium or even attached to the cells. Had the toxicity been due to toxic substances released by the virus the results of the Nested PCR and RAPD analysis would have been negative, in the absence of the viral DNA. As the Nested PCR results are positive it clearly indicates the presence of viral DNA in the cells. Therefore, when the DNA was extracted from the cells the viral DNA accompanied the host DNA. The Nested PCR is known to be a highly sensitive test used in the detection of viral infections. The current observations based on the positive Nested PCR test of infected cell line of crab, indicates the utility of cultured cells of crab for testing pathogenicity of WSSV.

There is hardly any report on establishment of a cell line from the crustacean species. This has resulted in lack of diagnostic techniques for detection of WSSV. Besides, non-availability of either cell lines or long lasting viable primary cell cultures has posed difficulties in studying the mechanism of viral infection, its replication and treatment as well as development of new drugs to contain WSSV. The present technique of culturing testicular cells has opened new vistas for development of drugs for WSSV and other crustacean diseases. As the testicular cells can be maintained in a viable and proliferating state for four months with susceptibility to WSSV, they could be a good diagnostic tool.

Summary

SUMMARY

The thesis presents the results of the work carried out on the development and characterization of cell cultures from a few tissues of mud crab *Scylla Serrata.*

The present study has established a technique to isolate cells from different tissues of crab with higher viability. Among the two dissociation techniques employed (mechanical and enzymatic), mechanical dissociation technique was more effective for tissues such as hepatopancreas, testis and ovary while enzymatic dissociation technique was effective for gills, brain, neurons and muscles. The cultured cells from different tissues exhibited variation in survival pattern. Among the various tissues tested (Gills, Muscles, Heart, Ovaries, Testes, Brain (neurons), Hepatopancreas, and Hemocytes) the most suitable tissue for longer survival was testes followed by hepatopancreas with reference to granular cells and ovaries.

Gill: Explant cell cultures of gills didn't survive for more than five days. However, the cells isolated by enzymatic dissociation technique by using 0.1 % Trypsin survived for five days in 2 x L15 medium prepared in artificial sea water with an osmolality of 1050 mOsm/kg. Though supplements were added to the media cell division / cell proliferation was not observed. These cells remained viable for a period of five days and thereafter as time progressed the cell viability deteriorated. The non anchored cells died within 1-2 days

Hepatopancreas: Five different cell types were observed in the hepatopancreatic tissue, E (Embryonalzellen), F (Fibrenzellen), B (Blasenzellen), R (Restzellen) and G (Granular cells). The explant culture of hepatopancreas resulted in cell migration however, among the

migrated cells the granular and embryonalzellen cells showed longer survivability in comparison to R, F and B cells. For disaggregation of hepatopancreatic cells mechanical dissociation technique using citrate buffer was found to be effective with 90 % cell survivability. Upon seeding, the hepatopancreatic E, F, B, and R cells, showed different survival pattern than granular cells. A modified 3 x L15 medium supplemented with EGF and glucose supported the best survival of hepatopancreatic E, F, B, and R cells in in-vitro culture. Use of sera such as Fetal bovine serum and Fetal calf serum as additives was not productive since they hampered cell viability.

In the present study a new cell type (Granular cells) was detected. Earlier reports have shown that there exist electron dense granules containing calcium, magnesium, phosphate and occasionally enzymes. The primary cultures of hepatopancreas revealed that granular cells could be maintained and be made to proliferate in the presence of EGF. These observations led to attempt characterization of granular cells in-order to investigate whether these are a different cell type or the granular break ups giving an impression of cell proliferation. Optical and confocal microscopy revealed them as spheroid epitheloid cell type having a prominent centrally located nucleus while scanning electron microscope showed them as occasionally interconnected round tennis ball like structures. Under fluorescence microscope the Fura-2-AM treated cells displayed calcium fluorescence. The biochemical analyses disclosed the presence of DNA (~ 1.8 pg/cell), RNA (~ 0.24 pg /cell) and protein (~ 14.98 pg /cell) while RAPD analyses confirmed the existence of genome. The MTT assay indicated that the granular cells proliferated in response to the epidermal growth factors (20 ng/ml). Besides, these cells released calcium on exposure to ecdysterone (10 µg/ml). These characteristics clearly indicate that the granular cells are true living structures and not mere storage entities.

Hemocytes: The culture of hemocytes from the hemolymph of the crab revealed the presence of three types of cells namely hyalinocytes, semi-granulocytes and granulocytes. These cells showed best survivability for a period of 14 days in 2 x Leibovitz's medium having an osmolality of 1050 mOsm/kg. Hemocytes exhibited anchorage to the substratum and showed a tendency to settle like the formed elements of mammalian blood.

Heart: The explant cultures of heart exhibited migration of hemocytes but not of heart cells (cardiomyocytes) as such even after four days of initiation of culture. Enzymatic dissociation using collagenase at a concentration of 0.3 mg/ml was more suitable for isolating cardiomyocytes. The cells exhibited maximum survivability for a period of eleven days in 3 x Leibovitz's medium supplemented with FGF (10 ng/ ml) + glucose (1.0 gm/ml) in comparison to other media tested. The cardiomyocytes were spindle shaped with a size ranging from ~11 to 13 μm in diameter particularly with reference to main cell body.

Fibroblasts: For fibroblasts tissue explant technique didn't promote cell migration owing to the rigidity of the muscle explants. However, treatment of tissue with collagenase (0.3 mg/ml) solution prepared in 3 x Leibovitz's resulted in obtaining viable fibroblast cells. These cells when cultured in 3 x Leibovitz's medium supplemented with FGF (10 ng/ml) and glucose (1.0 mg/ml) with an osmolality of 902 mOsm/kg showed maximum survivability of 6 to 7 days.

Ovary: The ovarian cell culture by explant technique exhibited no specific media and substrate requirements for cell migration from the explants blebs. For isolating cells of ovarian tissue mechanical dissociation technique was found to be effective. These cells

showed best survivability in Leibovitz's + Crab saline (1:1 v/v) medium supplemented with EGF (20 ng/ ml) and 1.0 mg/ml glucose at osmolality equivalent to 1050 mOsm/kg. In this combination of medium and supplements, the cells remained viable with proliferative for a period of 40 days. Nearly round and some slightly elongated cells with a size range from ~ 7.0 to 14 μm in diameter were observed.

Testis: Both the explant and segregated tissues of testes yielded cells that could proliferate and grow. These cells ranged in size from 10 to 38 μm with distinct nuclei of varying shapes. The testicular cells survived and proliferated best in L-15-crab saline medium supplemented with epidermal growth factor (20 ng/ml) and glucose (1.0 mg/ml). The cell proliferation rate was assessed by Methyl tetrazolium (MTT) assay in terms of changes in optical density which clearly indicated a prominent increase in cell density. The sub cultures of testicular cells were attempted on sixth day at a requisite density of 2×10^5 cells / ml and thereafter at an interval of two weeks. These sub cultured cells remained healthy and proliferated for four months with a minimum of ten subsequent passages. The finite cell line was characterized in terms of morphology, growth rate by using MTT assay, lactate dehydrogenase release (for detecting health status), RAPD and 18S rRNA sequencing.

Utility of cultured testicular cells for testing WSSV infectivity: Testicular cell line of crab, *Scylla serrata* (Forskal) was used to study the effects of White spot syndrome virus (WSSV). The proliferative cell line inoculated with different concentrations of virus titers showed distinct cytopathic effects such as change in cell appearance, shrinkage and cell lysis. WSSV infection of cultured cells was confirmed by Nested PCR technique. The incorporation of viral DNA in cultured cells was shown by RAPD profile generated using

10-mer primers. The controls that were not exposed to WSSV did not show cytopathic effects. This work shows the usefulness of proliferating testicular cell culture for studying WSSV infection using molecular tools. Thus, this report gains significance as it opens new vistas for diagnostics and drugs for WSSV.

Presentation and Publications

Oral Presentation.

- Shashikumar, A., Desai, P.V. 2010. Characterization of primary cultured granular cells from hepatopancreatic tissue of crab *Scylla serrata*. National seminar on Zoology, Life processing and Nanotechnology, Dept of Zoology, Goa University.
- Shashikumar, A., Desai, P.V. 2012. Characterization of finite cells line from testicular cells of crab *Scylla serrata*. Diversity and Physiological processes. Department of Zoology, Goa University.

Publications.

- Sasikumar, A., Desai, P. V. 2008. Development of primary cell culture from *Scylla serrata*: Primary cell cultures from *Scylla serrata. Cytotechnology.* 56(3): 161–169.
- Shashikumar, A., Desai, P. V. 2011. Development of cell line from *Scylla serrata. Cytotechnology*. 63(5):473-80.
- Shashikumar, A., Desai, P. V. 2012. Susceptibility of testicular cell cultures of crab, *Scylla serrata* (Forskal) to White Spot Syndrome Virus. *Cytotechnology.* DOI:10.1007/s10616-012-9482-x.
- Shashikumar, A., Desai, P.V. 2012. Characterization of finite cells line from testicular cells of crab *Scylla serrata.* In: Diversity and physiological processes. (ed) Desai, P.V., Roy, R. Publ: Goa University. ISBN: 978-81-908791-3-2. pp: 38-45.

References

REFERENCES

Al-Mohanna, S.Y., Nott, J.A. 1987. R-cells and the digestive cycle in *Penaeus semisulcatus* (Crustacea, Decapoda). *Mar Biol*, 95: 129-137.

Al-Mohanna, S.Y., Nott, J.A., Lane, D.J.W. 1985. Mitotic E-and secretory F-cells in the hepatopancreas of the shrimp *Penaeus semisulcatus* (Crustacea, Decapoda). *J Mar Biol ASS UK*, 65: 901-910.

Auzoux, S., Domart-Coulon, I., Doumenc, D. 1993. Gill cell cultures of the butterfish clam *Ruditapes decussates*. *J Mar Biotechnol*, 1: 79-81.

Awaji, M., Suzuki, T. 1998. Monolayer formation and DNA synthesis of the outer epithelial cells from pearl oyster mantle in coculture with amebocytes. *In Vitro Cell Dev Biol Anim*, 34: 486–491.

Ballard, T.A., Roer, R.D., Dillaman, R.M. 1993. Long-term culture of integumental explants from premolt blue crabs, *Callinectes sapidus*. *J Tissue Culture Methods*, 15: 11-14.

Battaglia, M., Davoli, C.1997. Long-term tissue culture of the earthworm *Eisenia foetida* (Annelida, Oligochaeta). In: Maramorosc., Mitsuhashi, J (eds). Invertebrate Cell Culture: Novel Directions and Biotechnology Applications. Science Publishers, Enfield. pp : 261-268.

Becker, G.L., Chen, C.H., Greenawalt, J.W., Lehninger, A.L. 1974. Calcium phosphate granules in the hepatopancreas of blue crab *Callinectes sapidus*. *J Cell Biol*, 61:316-326.

Bly, J.E., Quiniou, S.M., Clem, L.W. 1997. Environmental effects on fish immune mechanisms. *Dev Biol Stand*, 90: 33-43.

Boonyaratpalin, S., Supamattaya, K., Kasornchandra, J., Direk-busaracom, S., Aekpanithanpong, U., Chantanachooklin, C. 1993. Non-occluded baculo-like virus, the causative agent of yellow-head disease in the black tiger shrimp *Penaeus monodon*. *Fish Pathol*, 28: 103-109.

Boucard, C.V., Ruiz, P.A., Fregoso, C.E., Vega, G.A., Avelar, M.D.J.D., Pinto, V.S., Bonilla, C.M.E. 2010. Detection of white spot syndrome virus (WSSV) in the pacific oyster *Crassostrea gigas*. *J Invertebr Pathol*, 104: 245-7.

Boulo, V., Cadoret,J.P. Le, M.F., Dorange, G., Miahle, E. 1996. Transient expression of luciferase reporter gene after lipofection in oyster (*Crassostrea gigas*) primary cell cultures. *Mol Mar Biol Biotechnol*, 5: 167-174.

Braasch, D.A., Ellender, R.D., Middlebrooks, B.L. 1999. Cell cycle components and their potential impact on the development of continuous *in vitro* penaeid cell replication. *Methods Cell Sci*, 21: 255–261.

Brody, M.D., Chang, E.S. 1989. Development and utilization of crustacean long-term primary cell cultures: Ecdysteroid effects *in vitro*. *Invert Reprod Dev*, 16: 141-147.

Burch, J.B., Cuadros, C. 1965. A culture medium for snail cells and tissues. *Nature*, 206: 637-638.

Burnett, A. L., Ruffing, F. E., Zongker, J., Necco, A. 1968. Growth and differentiation of tubularia cells in a chemically defined physiological medium. *J Embryol Exp Morpho*, 20: 73-80.

Burrows, M.T. 1910. The cultivation of tissues of the chick-embryo outside the body. *J Am Med Assoc,* 55: 2057-2058.

Cancre, I., Van-Wormhoudt, A., Legal, Y. 1995. Effects of cellular growth factors on crustacean hepatopancreas cell suspension. *J Marine Biotechnol*, 2: 83-87.

Carr, T., Evans, P., Campbell, S., Bass, P., Albano, J. 1999. Culture of human renal tubular cells: positive selection of kallikrein-containing cells. *Immunopharmacology*, 44: 161–167.

Carrel, A. 1913. Contributions to the study of the mechanism of the growth of connective tissue. *J Exp Med,* 18: 287-299.

Carrel, A., Rivers, T. M. 1927. La fabrication du vaccin *in vitro*. *Compt Rend Soci Biol,* 96: 848-850.

Catap, E.S., Lavilla-Pitogo, C.R., Maeno, Y., Travina, R.D. 2003. Occurrence, histopathology and experimental transmission of hepatopancreatic parvovirus infection in *Penaeus monodon* postlarvae. *Dis Aquat Org*, 57:11-17.

Cecil, J.T. 1969. Mitoses in cell culture from cardiac tissue of the surf clam, *Spisula solidissima. J Invert Pathol*, 14: 407-410.

Chakraborty, A., Otta, S.K., Joseph, B., Kumar, S., Hossain, Md.S., Karunasagar, I., Veugopal, M.N., Karunasagar, I. 2002. Prevalence of white spot syndrome virus in wild crustaceans along the coast of India. *Curr Sci,* 82: 1392–1397.

Chan, W., Lin, K.C., Lin, P.S., Trachtman, H., Chan, J.C.M. 1999. Growth inhibition of rat mesangial cells by high glucose: effect of vitamin E. *Clin Exp Nephrol*, 3: 268-271.

Chang, E.S., O'Connor, J.D. 1977. Secretion of alpha-ecdystone by crabs Y-organ *in vitro*. *Proc Natl Acad Sci USA*, 74: 615-618.

Chang, P.S., Chen, H.C., Wang, Y.C. 1998. Detection of white spot syndrome associated baculovirus in experimentally infected wild shrimp, crab and lobsters by *in situ* hybridization. *Aquaculture,* 164: 233–242.

Chantanachookin, C., Boonyaratpalin, S., Kasornchandra, J., Direkbusarakom, S., Ekpanithanpong, U., Supamataya, K., Sriurairatana, S., Flegel, T.W. 1993. Histology and ultra- structure reveal a new granulosis-like virus in *Penaeus monodon* affected by yellow-head disease. *Dis Aquat Org*, 17: 145-157.

Chen, L.L., Lo, C.F., Chiu, Y.L., Chang, C.F., Kou, G.H. 2000. Natural and experimental infection of white spot syndrome virus WSSV in benthic larvae of mud crab *Scylla serrata*. *Dis Aquat Org*, 40:157–161.

Chen, S.N., Chi, S.C., Kou, G.H., Liao, I.C. 1986. Cell cultures from tissues of grass prawn, *Penaeus monodo*n. *Fish Pathol*, 21: 161–166.

Chen, S.N., Jong, K.J., Kou, G.H. 1988. Cell cultures from hematopoietic tissue and ovary of penaeid shrimp, *Penaeus monodon*. In: Kuroda. Y., Kurstak, E., Maramorosch, K (eds). Invertebrate and Fish Tissue Culture, Springer Verlag, Berlin. pp: 195-198.

Chen, S.N., Kou, G.H. 1989. Infection of cultured cells from the lymphoid organ of *Penaeus monodon* Fabricius by monodon- type baculovirus (MBV). *J Fish Dis,* 12:73–76.

Chen, S.N., Shih, H.H., Kou, G.H. 1995. Primary cell cultures from tissues of penaeid shrimps and their susceptibilities to monodon-type baculovirus (MBV). *Rep Fish Dis Res,* 16: 1–14.

Chen, S.N., Wang, C.S. 1999. Establishment of cell culture systems from penaeid shrimp and their susceptibility to white spot disease and yellow head viruses. *Methods Cell Sci*, 21: 199-206.

Chernin, E.1963. Observations on hearts explanted *in vitro* from the snail *Australorbis glabratus*. *J Parasitol*, 49: 353-364.

Chomczynski, P., Mackey, K., Drews, R. Wilfinger, W. 1997. DNAzol: A reagent for rapid isolation of genomic DNA. *Bio Techniques*, 22: 550-553.

Chomczynski, P., Sacchi. N. 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*, 162: 156-159.

Chun, L.G., Jin-sheng, S., Jian-Hai, X. 2003. Primary culture and characteristic morphologies of medulla terminalis neurons in the eyestalks of Chinese shrimp, *Fenneropenaeus chinensis*. *J Exp Mar Biol Ecol*, 290: 71-80.

Claydon, K., Owens, L. 2008. Attempt at immortalization of crustacean primary cell cultures using human cancer genes. *In Vitro Cell Dev Biol Anim*, 44: 451– 457.

Claydon, K., Roper, K.G., Owens, L. 2010. Attempt at producing a hybridised *Penaeus monodon* cell line by cellular fusion. *Fish Shellfish Immunol*, 29: 539-543.

Cooke, I., Graf, R., Graw, S., Haylett, B., Meyers, D., Ruben, P. 1989. Crustacean peptidergic neurons in culture show immediate outgrowth in simple medium. *Proc Natn Acad Sci U.S.A*, 86: 402–406.

Corbel, V., Zuprizal, Z., Shi, C., Huang, I., Sumartono., Arcier, J.M., Bonami, J.R. 2001. Experimental infection of European crustaceans with white spot syndrome virus (WSSV). *J Fish Dis*, 24: 377–382.

Correa, .J.D.Jr., Allodi, S., Farina, M. 2003. Enzymatic, analystical and structural aspects of electron dense granules in cells of *Ucides cordatus* (Crustacea, Decapoda) hepatopancreas. *Cell Tissue Res,* 311: 107-116.

Correa, J.D.Jr., Farina, M., Allodi, S. 2002. Cytoarchitectural features of *Ucides cordatus* (Crustacea, Decapoda) hepatopancreas: structural and elemental composition of electron-dense granules. *Tissue Cell,* 34: 315-325.

Cory, J., Yunker, C. E. 1971. Primary cultures of tick hemocytes as systems for arbovirus growth. *Ann Entomol Soc Am,* 64: 1249-1254.

Couch. J.A. 1974. Free and occluded virus, similar to *Baculovirus*, in hepatopancreas of pink shrimp. *Nature*, 247: 229–231.

Curtis, A. S. G. 1962. Pattern and mechanism in the reaggregation of sponges. *Nature*, 196: 245-248.

Decker, T., Lohmann, M.M.L. 1988. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J Immunol Method*, 115: 61-69.

Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., Peacocke, M., Campisi, J. 1995. A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. *Proc Natl Acad Sci USA*, 92: 9363–9367.

Domart-Coulon, I., Doumenc, D., Auzoux-Bordenave, S., Le-Fichant, Y. 1994. Identification of media supplements that improve the viability of primarily cell culture of *Crassostra gigas* oyster. *Cytotechnology,* 16: 109-120.

Durand, S., Lightner, D.V., Nunan, L.M., Redman, R.M., Mari, J., Bonami, J.R. 1996. Application of gene probes as diagnostic tools for white spot baculovirus (WSBV) of penaeid shrimp. *Dis Aquat Org*, 27:59–66.

Durand, S., Lightner, D.V., Redman, R.M., Bonami, J.R. 1997. Ultrastructure and morphogenesis of white spot syndrome baculovirus (WSSV). *Dis Aquat Org*, 29: 205–211.

Ellender, R.D., Najafabadi, A.K., Middlebrooks, B.L. 1992. Observations on the primary culture of hemocytes of penaeus. *J Crustacean Biol*, 12: 178-185.

Fan, T.J., Wang, X.F. 2002. *In vitro* culture of embryonic cells from the shrimp *Penaeus chinensis*. *J Exp Mar Biol Ecol*, 267: 175-184.

FAO. 2006. Fisheries Department. State of world aquaculture. 2006. FAO Fisheries Technical Paper. No. 500. Food and Agriculture Organization, Rome. pp :134.

Fischer-Piette, E. 1931. Culture de tissus de Crustaces, la glande Iymphatique du Homard. Arch *Zool Exp Gen*, 74: 33-52.

Flegel, T.W. 1997. Special topic review: major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. *World J Microbiol Biotech*, 13: 433-442.

Flegel, T.W., Fegan, D.F., Kongsom, S., Vuthikornudomkit, S., Boonyaraptalin, S., Chantanachookhin, C.H., Vickers, J.E., MacDonald, O.D. 1992. Occurrence, diagnosis and treatment of shrimp diseases in Thailand. Diseases of penaeid shrimp. In: Fulks, W., Main, K.L (eds). Diseases of cultured penaeid shrimp in Asia and the United States. The Oceanic Institute, Honolulu. pp: 57–112.

Frank, U., Rabinowitz, C., Rinkevich, B. 1994. Occurrence, diagnosis and treatment of shrimp diseases in Thailand. Diseases of penaeid shrimp. *Mar Biol*, 120: 491-499.

Fraser, C.A., Hall, M.R. 1999. Studies on primary cell cultures derived from ovarian tissue of *Penaeus monodon*. *Methods Cell Sci*, 21: 213-218.

Frerichs, G.N. 1996. *In vitro* culture of embryonic cells from the freshwater prawn *Macrobrachium rosenbergii*. *Aquaculture*, 143: 227–232.

Freshney, R.I. 2000. Culture of animal cells. In: John, W (ed). A manual of basic techniques, 4th ed. Inc publication, New York. pp: 177-193.

Freshney, R.I. 2010. Culture of Animal Cells, a Manual of Basic Technique, 6th ed. Published In: John Wiley and Sons (ed). Hoboken, New Jersey. pp: 163-204

Fyhn, U. E. H., Fyhn, H.J., Costlow, J.D. 1977. Cirriped vitellogenesis: effect of ecdysterone *in vitro*. *Gen Comp Endocrinol*, 32: 266-271.

Fyhn, U.E., Costlow, J.D. 1975. Tissue culture of criripeds. *Biol Bull*, 149: 316- 330.

Ganz, T. 2008. Iron homeostasis: fitting the puzzle pieces together. *Cell Metab*, 7: 288-290.

Ghosh, D., Ray, A.K., Damahapatra, A.K. 1995. Primary culture of prawn hepatocytes in serum free media. *In Vitro Cell Dev Biol Anim*, 31: 811-813.

Gibson, R., Barker, P.L. 1979. The decapod hepatopancreas. *Oceanogr Mar Biol Ann Rev*, 17: 285-346.

Goldschmidt, R. 1915. Some experiments on spermatogenesis *in vitro*. *Proc Natl Acad Sci USA*, 1: 220-222.

Gong, T., Jimjem, K., Manning, J.R.S., Georgis, R., Montgomery, T.J. 1997. *In vitro* production of *Anagrapha falcifera* multiple nuclear polyhedrosis virus (AfMNPV) in

two insect cell lines. In: K. Maramorosch., J. Mitsuhashi (eds). Invertebrate Cell Culture, Science Publishers. pp: 149-156

Grace, T.D.C. 1962. Establishment of four strains of cells from insect tissue grown *in vitro. Nature*, 195: 788-789.

Gratzner, H.G. 1982. Monoclonal antibody to 5-bromo and 5- iododeoxyuridine: A new reagent for detection of DNA replication. *Science*, 218: 474 - 475.

Grau, S.M., Cooke, I.M. 1992. Peptidergic neurons of the crab, *Cardisoma carnifex*, in defined culture maintain characteristic morphologies under a variety of conditions. *Cell Tissue Res*, 270: 303-317.

Hameed, A.S., Balasubramanian, G., Musthaq, S.S., Yoganandhan, K. 2003. Experimental infection of twenty species of Indian marine crabs with white spot syndrome virus (WSSV). *Dis Aquat Org*, 57: 157–161.

Hameed, A.S.S., AnilKumar, M., Stephen-Raj, M.L., Kunthala, J. 1998. Studies on the pathogenicity of systemic ectodermal and mesodermal baculovirus and its detection in shrimp by immunological methods. *Aquaculture*, 160: 31- 45.

Harrison, R.G. 1907. Observation on the living developing nerve fiber. *Proc Soc Exp Biol Med*, 4: 140-143.

Hatt, P.J., Liebon, C., Moriniere, M., Oberlander, H., Porcheron, P. 2001. Activity of insulin growth factors and shrimp neurosecretory organ extracts on a lepidopteran cell line. *Arch Insect Biochem Physiol*, 34: 313-328.

Hirsch, G. C., Jacobs, W. 1928. Der Arbeitsrhythmus der Mitteldarmdrüse von Astacus leptodactylus. In: Harrison, F. W (ed). Microscopic Anatomy of Invertebrates. New York, Wiley-Liss, Volume 10. pp: 147-201.

Hirsch, G.C., Buchman, W. 1930. Der Arbeitsrhythmus der Mitteldarmdrüse von *Astacus leptodactylus.* II. Wachstum als primärer Faktor des Rhythmus eines polyphasichen organischen Sekretionssystems. *Z Vgl Physiol*, 12: 524–557.

Hsu, Y.L., Wang, K.H., Yang, Y.H., Tung, M.C., Hu, C.H., Lo, C.F., Wang, C.H., Hsu, T. 2000. Diagnosis of *Penaeus monodon*-type baculovirus by PCR and by ELISA of occlusion bodies. *Dis Aquat Org,* 40: 93-99.

Hsu, Y.L., Yang, Y.H., Chen, M.C., Tung, J.L.Wu., Engelking, M.H., Leong, J.C. 1995b. Development of an *in vitro* subculture system for the oka organ (Lymphoid tissue) of *Penaeus monodon*. Aquaculture, 136:43-55.

Hsu, Y.L., Yang, Y.H., Chen, Y.C., Tung, M.C., Wu, J.L., Engleking, M.H., Leong, J.C. 1995a. Development of an *in vitro* subculture system for the Oka organ (lymphoid tissue) of *Penaeus monodon*. *Aquaculture*, 136: 43-55.

Huang, J., Song, X.L., Yu, J., Kong, J., Yang, C.H. 1995. Baculoviral hypodermal and hematopoietic necrosis-study on the pathogen and pathology of the explosive epidemic disease of shrimp. *Mar Fish Res*, 16: 1-10.

Humphrey, T., Humphrey, S., Moscona, A. A. 1960. A procedure for obtaining completely dissociated sponge cells. *Biol Bull*, 119 - 294.

Icely, J.D., Nott, J.A. 1992. Digestion and absorption: digestive system and associated organs. In-Microscopic Anatomy of Invertebrates: Decapod, Crustacea. F.W. Harrison and A.G. Humes (eds). Wily-Liss Inc., New York, Volume 10. pp: 147-201.

Inouye, K., Miwa, S., Oseko, N., Nakano, H., Kimura, T., Momoyama, K., Hiraoka, M. 1994. Mass mortalities of cultured kuruma shrimp, *Penaeus japonicus*, in Japan in 1993: electron microscopic evidence of the causative agent. *Fish Pathol*, 29: 141–148.

Ismail, N., Sarijan, S. 2011. Phylogenetic inference from 18S rRNA gene sequences of horseshoe crabs, *Tachypleus gigas* between Tanjung Dawai, Kedah and Cherating, Pahang, Peninsular Malaysia. *World Acad Sci Engi and Tec*. 56: 947-950.

Itami, T., Aoki, Y., Hayashi, K.I., Yu, Y., Takahashi, Y. 1989. *In vitro* maintenance of cells of lymphoid organ in kuruma shrimp *Penaeus japonicas*. *Nippon Suisan Gakkaishi*, 55: 2205.

Itami, T., Maeda, M., Kondo, M., Takahashi, Y. 1999. Primary culture of lymphoid organ cells and haemocytes of kuruma shrimp, *Penaeus japonicus*. *Methods Cell Sci,* 21: 237 - 244.

Ivic, L., Nesic, O., Kartelija, G., Pasic, M. 1995. Maintenance of *Helix pomatia* neurons in simplified cell culture system. *Comp Biochem Physiol*, 111: 413-421.

Iwanaga, Y., Tsuji, M., Oka, S. 1985. Primary cultivation of tissues from *Oncomelania hupensis*. *Venus*, 44: 103-109.

Jiang, G., Yu, R., Zhou, M. 2004. Modulatory effects of ammonia-N on the immune system of *Penaeus japonicus* to virulence of white spot syndrome virus. *Aquaculture*, 241: 61-75.

Jiang, Y.S., Zhan, W.B., Wang, S.B., Xing, J. 2006. Development of primary shrimp hemocyte cultures of *Penaeus chinensis* to study white spot syndrome virus (WSSV) infection. *Aquaculture,* 253:114–119.

Jiravanichpaisal, P., Bangyeekhun, E., Soderhall, K., Soderhall, I. 2001. Experimental infection of white spot syndrome virus in freshwater crayfish *Pacifastacus leniusculus*. *Dis Aquat Org*, 47: 151–157.

Jiravanichpaisal, P., Lee, B.L., Soderhall, K. 2006a. Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. *Immunobiology*, 211: 213– 236.

Jiravanichpaisal, P., Soderhall, K., Soderhall, I. 2006b. Characterization of white spot syndrome virus replication in *in vitro*-cultured haematopoietic stem cells of freshwater crayfish, *Pacifastacus leniusculus*. *J Genl Virol*, 87: 847–854.

Johnson, P.T. 1969. The coelomic elements of sea urchin (*Strongylocentrotus*). I. The normal coelomocytes; their morphology and dynamics in hanging drops. *J Invert Pathol,* 13: 25-41.

Johnson, P.T., Beeson, R.J. 1966. *In vitro* studies on *Patiria miniata* (Brandt) coelomocytes, with remarks on revolving cysts. *Life Sci*, 5: 1641-1666.

Johnson, P.T., Lightner, D.V. 1988. Rod-shaped nuclear viruses of crustaceans: gut–infecting species. *Dis Aquat Org,* 5: 123-141.

Johnston, D.J., Alexander, C.G., Yellowhees, D. 1998. Epithelial cytology and function in the digestive gland of *Thenus orientalis* (Decapoda, scyllaridae). *J Crust Biol*, 18: 271-278.

Jose, S., Mohandas, A., Philip, R., Bright Singh, I.S. 2010. Primary hemocyte culture of *Penaeus monodon* as an *in vitro* model for white spot syndrome virus titration, viral and immune related gene expression and cytotoxicity assays. *J Invert Pathol*, 105: 312-321.

Kanchanaphum, P., Wongteerasupaya, C., Sitidilokratana, N., Boonsaeng, V., Panyim, S., Tassanakajon, A., Withyachumnarnkul, B., Flegel, T.W. 1998. Experimental transmission of White Spot Syndrome (WSSV) from crabs to shrimp, *Penaeus monodon*. *Dis Aquat Org*, 34: 1-7.

Kaneko, H., Kawahara, Y., Dan-Sohkawa, M. 1995. Primary culture of mesodermal and endodermal cells of the starfish embryo. *Zool Sci*, 12: 551 - 558.

Kaneko. J., Ozawa, T., Tomita, T., Kamio, Y. 1997. Sequential binding of staphylococcal gamma-hemolysin to human erythrocytes and complex formation of hemolysin on the cell surface. *Biosci Biotechol Biochem*, 61: 846 - 851.

Karunasagar, I., Otta, S.K., Karunasagar, I. 1997. Histopathological and bacteriological study of white spot syndrome of *Penaeus monodon* along the west coast of India. *Aquaculture*, 153: 9-13.

Kasornchandra, J., Khongpradit, R., Ekpanithanpong, U., Boonyaratpalin, S. 1999. Progress in the development of shrimp cell cultures in Thailand. *Methods Cell Sci*, 21: 231–235.

Ke, H., Liping, W., Yumei, D., Shuji, Z. 1990. Studies on a cell culture from the hepatopancreas of the oriental shrimp, *Penaeus orientalis* Kishinouye. *Asian Fish Sci*, 3: 299-307.

Kerry, C. 2009. Advance in crustacean cell culture. PhD thesis, James Cook University.

Kiatpathomchai, W., Boonsaeng, V., Tassanakajon, A., Wongteerasupaya, C., Jitrapakdee, S., Panyim, S. 2001. A non-stop, single-tube, semi-nested PCR technique for grading the severity of white spot syndrome virus infections in *Penaeus monodon*. *Dis Aquat organ*, 47: 235–239.

Klautau, M.,Custodio., Borojevic, R.M.R. 1993. Cell cultures of sponges *Clathrina* and *Polymastia*. *In Vitro Cell Dev Biol*, 29: 97-99.

Klinbunga, S., Boonyapakdee, A., Pratoomchat, B. 2000. Genetic diversity and species-diagnostic markers of mud crabs (Genus *Scylla*) in Eastern Thailand determined by RAPD analysis. *Mar Biotechnol*, 2: 180–187.

Krontowsky, A., Ruumianzew, A. 1922. Zur Technik der gewebs-kulturen von regenwurmern *in vitro*. *Pflugers Arch Gesamte Physiol Menschen Und Der Tiere*, 195: 291-299.

Kshirsagar,S.G., Patole, M.S., Shouche, Y.S. 1997. Characterization of insect cell lines: heteroduplex analysis employing a mitochondrial 16S ribosomal RNA gene fragment. *Anal Biochem*, 253: 65-69.

Kumar, G.S., Singh, I.S.B., Philip, R. 2001. A cell culture system from the eye stalk of *Penaeus indicus*. In: Linder- Olsson, E. et al (eds). Animal Cell Technology: From Target to Market. Kluwer Academic Publishers, Netherlands. pp: 261- 265.

Lang, G.H., Nomura, N., Matsumura, M. 2002a. Growth by cell division in shrimp (*Penaeus japonicus*) cell culture. *Aquaculture*, 213: 73–83.

Lang, G.H., Nomura, N., Wang, B.Z., Matsumura, M. 2002b. Penaeid (*Penaeus japonicus*) lymphoid cells replicate by cell division *in vitro*. *In vitro Cell Dev Biol Anim*, 38: 142–145.

Lang, G.H., Wang, Y., Nomura, N., Matsumura, M. 2004. Detection of telomerase activity in tissues and primary cultured lymphoid cells of *Penaeus japonicus*. *Mar Biotechnol*, 6: 347–354.

Lebel, J.M., Giard, W., Favrel, P., Boucaud-camou, E. 1996. Effects of different vertebrate growth factors on primary cultures of hemocytes from the gastropod mollusc, *Haliotis tuberculat*a. *Biol Cell*, 86: 67-72.

Lebel, J.M., Giard, W., Favrel, P., Eve Boucaud-Camou, E. 1996. Effects of different vertebrate growth factors on primary cultures of hemocytes from the gastropod mollusc, *Haliotis tuberculata*. *Biol Cell*, 86: 67-72.

Lee, B.Y., Han, J.A., Im, J.S., Morrone, A., Johung, K., Goodwin, E.C., Kleijer, W.J., Di-Maio, D., Hwang, E.S. 2006. Senescence-associated beta-galactosidase is lysosomal beta-galactosidase. *Aging Cell*, 5: 187–95.

Le-Groumellec, M., Martin, C., Haffner, P., Martin, B. 1995. Aquacop cell culture from tropical shrimps. *J Aquacult Tropic*, 10: 277-286.

Leudeman, R.A. 1990. Development of *in vitro* primary cell cultures from the penaeid shrimp *Penaeus stylirostris* and *Penaeus vannamei* and evaluation of a potential application. Master Thesis. Tucson AZ: The University of Arizona.

Leudeman, R.A., Lightner, D.V. 1992. Development of an *in vitro* primary cell culture from the penaeid shrimp *Penaeus stylirostris* and *Penaeus vannamei*. *Aquaculture*, 101: 205-211.

Levine, A.J. 1997. The cellular gatekeeper for growth and division. *Cell*, 88: 323-331.

Li, C., Shields, J.D. 2007a. Primary culture of hemocytes from the caribbean spiny lobster, *Panulirus argus* and their susceptibility to *Panulirus argus* virus1 (PaV1). *J Invertebr Pathol*, 94: 48-55.

Li, C., Shields, J.D. 2007b. Characterization and primary culture of hemocytes from the blue crab, *Callinectes Sapidus*. In: Cai, S.L. (ed), 5th world chinese symposium for the crustacean aquaculture, Ocean Press, Beijing, China. Transactions of the Chinese Crustacean Society. pp: 25-35.

Li, M.F., Stewart, J.E., Drinnan, R.E.1966. *In vitro* cultivation of cells of the oyster, *Crassostrea virginica*. *J Fish Res Board Can*, 23: 595-599.

Li, Y.Y., Xia, X.A., Wu, Q.Y., Liu, W.H., Lin, Y.S. 2008. Infection with Hematodium sp in mud crabs *Scylla serrata* cultured in low salinity water in southern China. *Dis Aquat Org*, 82: 145-150.

Liang, T., Ji, H., Du, J., Ou, J., Li, W., Wu, T., Meng, Q., Gu, W., Wang, W. 2012. Primary culture of hemocytes from *Eriocheir sinensis* and their immune effects to the novel crustacean pathogen *Spiroplasma eriocheiris*. *Mol Biol Rep*, 39: 9747-54

Lightner, D.V. 1993. Diseases of penaeid shrimp. In: McVey J.P (ed). CRC Handbook of Mariculture 2nd Edn. Crustacean Aquaculture, CRC Press, Boca Raton, FL Volume 1. pp: 393–486.

Lightner, D.V. 1996. A handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid shrimp. World Aquaculture Society, Baton Rouge, Louisiana USA. pp: 304.

Lightner, D.V. 1998. Disease of cultured penaeid shrimp, CRC Hand Book of Mariculture. Boca, Raton. Flurida: CRC Press, Inc Volume 1. pp: 289-321.

Lightner, D.V., Redman, R.M. 1998. Shrimp diseases and current diagnostic methods. *Aquaculture*, 164: 201-220.

Lightner, D.V., Redman, R.M., Bell, T.A. 1983a. Observations on the geographic distribution, pathogenesis and morphology of the baculovirus from *Penaeus monodon* Fabricius. *Aquaculture,* 32: 209-233.

Lightner, D.V., Redman, R.M., Bell, T.A. 1983b. Infectious hypodermal and hemopoietic necrosis, a newly recognized virus disease of penaeid shrimp. *J Invert Pathol,* 42: 62-70.

Limuswan, Ch. 1999. Shrimp culture in Thailand toward year 2000. In: Tonguthai, K., Chinabut, S., Somsiri, T., Chanratchakul, P., Kanchanakan, S (eds). The AAHRI Newsletter. Department of fisheries, Kasertsart University, Bangkok. pp: 5- 6.

Lincoln, C.K., Gabridge, M.G. 1998. Cell culture contamination: Sources, consequences, prevention and elimination. *Method Cell Biol*, 57:49-65.

Liping, W., Yumei, D., Shuji, Z. 1990. Studies on a cell culture from the hepatopancreas of the oriental shrimp, *Penaeus orientalis* Kishinouge. *Asian Fish Sci*, 3: 299-307.

Liu. W., Qian, D., Yan, X.J. 2011. Studies on pathogenicity and prevalence of white spot syndrome virus in mud crab, *Scylla serrata* (Forskal), in Zhejiang province, China. *J Fish Dis,* 34: 131–138.

Lo, CF., Ho, C.H., Peng, S.E., Chen, C.H., Hsu, H.C., Chiu, Y.L., Chang, C.F., Liu, K.F., Su, M.S., Wang, C.H., Kou, G.H. 1996. White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. *Dis Aquat Org,* 27: 215 -225.

Loh, P.C., Lu, Y., Brock, J.A. 1990. Growth of the penaeid shrimp virus infectious hypodermal and hematopoietic necrosis virus in a fish cell line. *J Virol Methods*, 28: 273-80.

Loomis, F.W., Lenhoff, H.M. 1956. Growth and sexual differentiation of hydra in mass culture. *J Exp Zool*, 132: 555-568.

Lowry, H.O., Rosenbrough, N.L., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J Biol Chem*, 193: 265-275.

Lu, C.C., Tang, K.F.J., Kou, G.H., Chen, S.N. 1993. Development of a *Penaeus monodon*-type baculovirus (MBV) DNA probe by polymerase chain reaction and sequence analysis. *J Fish Dis*, 16: 551-559.

Lu, Y., Tapay, L.M., Loh, P.C., Brock, J.A., Gose, R. 1995. Development of a quantal assay in primary shrimp cell culture for yellow head baculovirus (YBV) of penaeid shrimp. *J Virol Methods,* 52: 231–236.

Luedeman, R.A., Lightner, D.V. 1992. Development of an *in vitro* primary cell culture from the penaeid shrimp *Penaeus stylirostris* and *Penaeus vannamei*. *Aquaculture*, 101: 205–211.

Machii, A., Wada, K.T. 1989. Some marine invertebrates tissue culture. In: Mitsuhashi, J (ed). Invertebrate Cell System Applications, CRC. Boca Raton, Volume II. pp: 225-223.

Machii, A., Wada, K.T., Awaji, M., Nakamura, H.K., Townsley, S.J. 1988. Some characters of cells of the midgut gland and chytrids from primary cultures of the prawn *Penaeus japonicus*. In: Kuroda, Y., Kurstak, E., Maramorosch, K (eds). Japan Scientific Soceities Press, Springer Verlag. pp: 11-14.

Maeda, M., Mizuki, E., Itami, T., Ohba, M. 2003. Ovarian primary tissue culture of the kuruma shrimp *Marsupenaeus japonicus*. *In Vitro Cell Devel Biol Anim*, 39: 208-212.

Maeda, M., Saitoh, H., Mizuki, E., Itami, T., Ohba, M. 2004. Replication of white spot syndrome virus in ovarian primary cultures from the kuruma shrimp, *Marsupenaeus japonicus. J Virol Methods*, 116: 89 - 94.

Magbanua, F. O., Natividad, K.T., Migo, V.P., Alfafara, C.G., Pena, F.O., Miranda, R.O., Albaladejo, J.D., Nadala, E.C.Jr., Loh, P.C., Tapay, L.M. 2000. White spot syndrome virus (WSSV) in cultured *Penaeus monodon* in the Philippines. *Dis Aquat Org*, 42: 77-82.

Martin, R., Tardent, P. 1963. Kultur von hydroiden-zellen *in vitro*. *Rev Suiss Zool*, 70: 312-316.

McGarrity, G.J. 1976. Spread and control of mycoplasmal infection of cell cultures. *In Vitro*, 12: 643-647.

McGarrity, G.J., Sarama, J., Vanamman, V. 1985. Cell culture techniques (Excellent overview of mycoplasma contamination). *ASM News*, 51: 170-183.

MC-Limans, W.F., Davis, E.V., Rake, G.W. 1957. The analytical methods for biological assay. *Immunology*, 79: 428.

Mintz, G.R., DeFrancesco, S., Lennarz, W. J. 1981. Spicule formation by cultured embryonic cells from the *Sea urchin*. *J Biol Chem*, 25: 13105-13111.

Mitsuhashi, J. 2002. Invertebrate Tissue Culture Methods (Springer Lab Manuals). Hicom, Japan Binding Tokyo-Shoeki, Japan. pp: 47-363.

Monnier-Dadkhah, Z., Bride, M., Colard, C. 1991. Realisation d'un bioessai a l'aide de cultures cellularies gonadiques d'escargot: influence d' extradites de ganglions nerveux sur l'activitie synthetique des cellules cibles. *Cell Mol Biol*, 37: 687-703.

Morgan, S.J., Darling, D.C. 1993. Animal Cell Culture. In: Graham, J.M., Billington, D (eds). BIOS Scientific Publishers Ltd, Oxford UK. pp: 51-65.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*, 65: 55–63.

Mothersill, C., Austin, B. 2000. Aquatic invertebrate cell culture, Chichester, UK: Springer Praxis. pp: 409.

Moulton, J.E. 1978. Preparation of primary cultures of tick cells. *Am J Vet Res*, 39: 1558-1564.

Mulford, A.L., Austin, B. 1998. Development of primary cell cultures from *Nephrops norvegicu*s. *Methods Cell Sci*, 19: 269 - 275.

Mulford, A.L., Lyng, F., Mothersill, C., Austin, B. 2001. Development and characterization of primary cell cultures from the hematopoietic tissues of the Dublin Bay prawn, *Nephrops norvegicus*. *Methods Cell Sci*, 22: 265 - 275.

Muller, W.E.G., Wiens, M., Batel, R., Steffen, R., Borojevic, R., Custodio, M.R. 1999. Establishment of a primary cell culture from a sponge: primmorphs from *Suberites domuncula*. *Mar Ecol Prog Ser*, 178: 205 - 219.

Mullis, K.B. 1990. The unusual origin of the polymerase chain reaction. *Sci Am*, 262: 56-65.

Nadala, E.C. Jr., Tapay,. L.M., Cao, S., Loh, P.C. 1997. Detection of yellow-head virus and Chinese baculovirus in penaeid shrimp by Western blot technique. *J Virol Methods*, 69: 39 - 44.

Nadala, E.C., Loh, P.C., Lu, Y.1993a. Primary culture of lymphoid, nerve and ovary cells from *Penaeus stylirostris* and *Penaeus vannamei*. *In Vitro Cell Devel Biol*, 29: 620 - 622.

Naganuma, T., Akutsu, T., Ishida, T., Kato, C., Horikoshi, K. 1996. *In situ* RT-PCR from the β tubulin mRNA in abalone cells in primary cultures. *J Mar Biotechnol*, 4: 75-81.

Naganuma, T., Degnan, B.M., Horikoshi, K., Morse, D.E. 1994. Myogenesis in primary cell cultures from larva of the abalone, *Haliotis rufescens*. *Mol Mar Biol Biotechnol*, 3: 131-140.

Nakano, H., Koube, H., Umezawa, S., Momoyama, K., Hiraoka, M., Inouye, K., Oseko, N. 1994. Mass mortalities of cultured kuruma shrimp, *Penaeus japonicus*, in Japan in 1993: epizootiological survey and infection trials. *Fish Pathol*, 29: 135–139.

Nandala, E.C., Loh, P.C., Lu, Y. 1993b. Primary culture of lymphoid, nerve and ovary cells from *Penaeus stylirostris* and *Penaeus orientalis* Kishinouye. *Asian Fish Sci*, 3: 299-307.

Nunan, L.M., Lightner, D.V. 1997. Development of a non-radioactive gene probe by PCR for detection of white spot syndrome virus (WSSV). *J Virol Methods*, 63: 193–201.

Otta, K., Shubha, G., Joseph, B., Chakraborty, A., Karunasagar, I., Karunasagar, I. 1999. Polymerase chain reaction (PCR) detection of white spot syndrome virus (WSSV) in cultured and wild crustaceans in India. *Dis Aquat Org,* 38: 67-70.

Owens, L., Smith, J. 1999. Early attempts at production of prawn cell lines. *Methods Cell Sci,* 21: 207 - 211.

Pantoja, C.R., Lightner, D.V. 2001. Detection of hepatopancreatic parvovirus (HPV) of penaeid shrimp by in situ hybridization at the electron microscope level. *Dis Aquat Org,* 44: 87-96.

Parenrengi, A., Zafran, Boer, D.R., Rusdi, I. 1993. Identifikasi dan patogenisitas beberapa bakteri Vibrio pada larva kepiting bakau (*Scylla serrata*). (Identification and pathogenicity of various vibrios on the mangrove crab, *Scylla serrata*, larvae). *Journal Penelitian Budidaya Pantai (Res Coastal Aquacul),* 9: 125 - 129.

Paul, B. 2000. Population genetic structure of the marine penaeid prawn - Penaeus indicus. Ph.D Thesis. Cochin University of Science and Technology, Kochi. pp.87.

Peponnet, F., Quiol, J.M. 1971. Cell cultures of crustacea arachnida and merostomacea. In: Vago, C (ed). Invertebrate Tissue Culture. Volume I. pp: 341-359.

Phromjai, J., Boonsaeng, V., Withyachumnarnkul, B., Flegel, T.W. 2002. Detection of hepatopancreatic parvovirus in Thai shrimp *Penaeus monodon* by in situ hybridization, dot blot hybridization and PCR amplification. *Dis Aquat Org,* 51: 227 - 232.

Plummer, T.D. 1979. An introduction to practical biochemistry. In: McGraw second (ed) Hill Book company (UK) Limited. Maidenhead. Berkshire, England. pp: 145-241

Pomponi , S. A., Willoughby, R., Kaighn, M. E., Wright, A. E. 1997. Development of techniques for *in vitro* production of bioactive natural products from marine sponges.

In: Maramorosch, K., Mitsuhashi, J (eds). Invertebrate cell culture: Novel Directions and Biotechnology applications. Science Publishers, USA. pp: 231 - 237.

Poncet, J., Serpentini, A., Thiébot, B., Villers, C., Bocquet, J., Boucaud-Camou, E., Lebel, J. 2000. *In vitro* synthesis of proteoglycans and collagen in primary cultures of mantle cells from the nacreous mollusk, *Haliotis tuberculata*: a new model for study of molluscan extracellular matrix. *Mar Biotechnol,* 4: 387-398.

Pretlow, T.G., Pretlow, T.P. 1989. Cell Separation by gradient centrifugation methods. *Methods Enzymol,* 171: 462 - 482.

Pudney, M., Varma, M.G., Leake, C.J. 1973. Culture of embryonic cells from the tick *Boophilus microplus* (Ixodidae). *J Med Entomol,* 10: 493 - 496.

Quinitio, E.T., Parado-Estepa, F. D., Millamena, O.M., Rodriguez, E., Borlongan, E. 2001. Seed production of mud crab, *Scylla serrata* juviniles. *Asian Fisheries Sci*, 14: 161-174.

Quiot, J. M., Vey, A. 1978. Culture *in vitro* de cellules embryonnaires d'ecrevisses. *Freshwater Crayfish*. 4: 391-398.

Rahman, M.H. 1981. The culture of embryonic cells from the tick *Hyalomma rufipes* (Koch, 1844). *Ann Trop Med Parasitol*, 75: 573-574.

Ramaswamy, P., Brennan, G.P., Jayakumar R. 1995. A record and prevalence of monodon baculovirus from postlarval *Penaeus monodon* in Madras, India. *Aquaculture,* 130: 129-135.

Rannou, M. 1968. Formation de spicules dans des cultures cellulaires de cnidaire (Gorgone). *Vie Milieu Ser,* 19 : 53-57.

Reed, L.J., Muench, H. 1938. A simple method of estimating fifty percent end points. *Am J Hygiene*, 27: 493-497.

Rinckevich, B., Blisko, R., IIan, M. 1998. Further steps in the initiation of cell cultures from embryos and adult sponge colonies. *In Vitro Cell Dev Biol*, 34: 753-756.

Rinkevich, B. 2005. Marine invertebrate cell cultures: new millennium trends. *Mar Biotechnol*, 7: 429-39.

Roper, K.G., Owens, L., West, L. 2001. The media used in primary cell culture of prawn tissue : A review and comparative study. *Asian Fisheries Sci*, 14: 61-75.

Rosenthal. J., Diamant, A. 1990. *In vitro* primary cell culture from *Penaeus semisulcatus*. In: Perkins, F.O., Cheng, T.C (eds). Pathology Marine Science. San Diego: Academic Press. pp: 7-13.

Saalbach, A., Aust, G., Haustein, U.F., Herrmann, K., Anderegg, U. 1997. The fibroblast-specific MAb AS02: a novel tool for detection and elimination of human fibroblasts. *Cell Tissue Res*, 290: 593 - 599.

Sager, R. 1992. Tumor suppressor genes in the cell cycle. *Curr Opin Cell Biol*, 4: 155-160.

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., Arnheim N. 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science*, 230: 1350 - 54.

Sánchez-Paz, A. 2010. White spot syndrome virus: an overview on an emergent concern. *Vet Res*, 41: 43.

Sano, T., Nishimura, T., Oguma, K., Momoyama, K., Takeno, N. 1981. Baculovirus infection of cultured kuruma shrimp, *Penaeus japonicus* in Japan. *Fish Pathol*, 15: 185-191.

Sara, M., Liaci, L., Melone, N. 1966a. Bispecific cell aggregation in sponges. *Nature,* 210: 1167-1168.

Sara, M., Liaci, L., Melone, N. 1966b. Mixed cell aggregation between sponges and the anthozoon *Anemonia sulcata*. *Nature,* 210: 1168-1169.

Sasaki, M., Honda, T., Yamada, H., Wake, N., Barrett, J.C., Oshimura, M. 1994. Evidence for multiple pathways to cell senescence. *Cancer Res*, 5423: 6090 – 6093.

Sashikumar, A., Desai, P.V. 2008. Development of primary cell culture from *Scylla serrata*: Primary cell cultures from *Scylla serrata*. *Cytotechnology*, 56: 161-169.

Saver, M.A,, Wilkens, J.L., Syed, A.N. 1999. In situ and *in vitro* identification and characterization of cardiac ganglion neurons in the crab, *Carcinus maenas. J Neurophysiol*, 81: 2964-2976.

Schmitt, K., Daubener, W., Bitter-Suermann, D., Hadding, U. 1998. A safe and efficient method for elimination of cell culture mycoplasmas using ciprofloxacin. *J Immunol Methods*, 109: 17-25.

Shashikumar, A., Desai, P.V. 2011. Development of cell line from the testicular tissues of crab *Scylla serrata. Cytotechnology,* 63: 473-480.

Shashikumar, A., Desai, P.V. 2012. Susceptibility of testicular cell cultures of crab, *Scylla serrata* (Forskal) to white spot syndrome virus. *Cytotechnology*, 65: 253-62.

Shen, Y., Lai, Q. 1994. Present status of mangrove crab *Scylla serrata* (Forskal) culture in China. Naga, the ICLARM Quarterly, ISSN 0116-290X. pp: 280-289.

Shi, Z.L. 2000. Etude d'un virus Bacilliforme des de vetter Penaeidae (white spot syndrome virus WSSV): clonage, analysis partiel du genome et outills de diagnostics. PhD thesis, Universite des Science et Techniquesq du Languedoc, Mountpellier.

Shike, H., Shimizu, C., Kimpel, K.S., Burns, J.C. 2000. Expression of foreign genes in primary cultured cells of the blue shrimp *Penaeus stylirostris*. *Mar Biol,* 137: 605–611.

Shimizu, C., Shike, H., Klempel, K.R., Burns, J.C. 2001. Hemolymph analysis and evaluation of newly formulated media for culture of shrimp cells (*Penaeus stylirostris*). *In Vitro Cell Dev Biol Anim*, 37: 322–329.

Simkiss, K., Taylor, M.G. 1994. Calcium Magnesium Phosphate granules: Atomistic simulations explaining cell death. *J Exp Biol,* 190: 131-139.

Snieszko, S.F. 1974. The effect of environmental stress on outbreaks of infectious disease of fishes. *J Fish Biol*, 6: 197-208.

Sousa, N.L., Avila., P.C., Widdicombe, J.H. 2003. Polarized cultures of human airway epithelium from nasal scrapings and bronchial brushings. *In Vitro Cell Dev Biol Anim,* 39: 266–269.

Stafford, J.L., Belosevic, M. 2003. Transferrin and the innate immune response of fish: identification of a novel mechanism of macrophage activation. *Dev Comp Immunol*, 27: 539-54.

Stephens, E.B., Hetrick, F.M. 1979. Cultivation of granular amoebocytes from the American oyster. *Tissue Culture Assoc Manual*, 5: 991-912.

Takahashi, M., Ota, S., Shimada, T., Hamada, E., Kawabe, T., Okudaira, T., Matsumura, M., Kaneko, N., Terano, A., Nakamura, T., et al. 1995. Hepatocyte growth factor is the most potent endogenous stimulant of rabbit gastric epithelial cell proliferation and migration in primary culture. *J Clin Invest,* 95: 1994-2003.

Takahashi, Y., Itami, T., Kondo, M., Maeda, M., Fujii, R., Tomonaga, S., Supamattaya, K., Boonyaratpalin, S. 1994. Electron microscopic evidence of bacilliform virus infection in kuruma shrimp (*Penaeus japonicus*). *Fish Pathol,* 29: 121-125.

Tamse, C.T., Collin, C., Kuzirian, A.M., Smith, P.J.S. 1995. Culture of *Hermissenda crassicornis* (mollusca) neurons. *In Vitro Cell Dev Biol Anim,* 31: 653-656.

Tangkrock–Olan, N., Ketpadung, R. 2010. A comparative study on the blood osmolality of the mud crab (*Scylla serrata*) and the blue swimming crab (*Portunus pelagicus*) exposed to different salinities: A case study for the topic "Osmotic Regulation" in high school biology. *Asian J Biol Education,* 4: 8-14

Tapay, L.M., Lu, Y., Brock, J.A., Nadala, E.C.Jr., Loh, P.C. 1995. Transformation of primary culture of shrimp (*Penaeus stylirostris*) lymphoid (Oka) organ with simian virus- 40 (T) antigen. *Proc Soc Exp Biol Med*, 209: 73-78.

Tapay, L.M., Nadala, E.C.B., Loh, P.C. 1999. A polymerase chain reaction protocol for the detection of various geographical isolates of white spot virus. *J Virol Methods,* 82: 39–43.

Tong, S.L., Miao, H.Z. 1996. Attempts to initiate cell cultures from *Penaeus chinensis* tissues. *Aquaculture,* 147: 151–157.

Toullec, J.Y. 1999. Crustacean primary cell culture: A technical approach. *Methods in Cell Sci,* 21: 193–198.

Toullec, J.Y., Crozat, Y., Patrois, J., Porcheron, P. 1996. Development of primary cell cultures from the penaeid shrimps *Penaeus vannamei* and *Penaeus indicu*s. *J Crustacean Biol,* 16: 643–649.

Tripp, M.R., Bisignani, L.A., Kenny, M.T. 1966. Establishment of cell line from the pacific oyster. *In Vitro Cell Dev Biol*, 29: 901-903.

Uma, A., Prabhakar, T.G., Koteeswaran, A., Ravikumar, G. 2002. Establishment of primary cell culture from hepatopancreas of *Penaeus monodon* for the study of white spot syndrome virus (WSSV). *Asian Fish Sci*, 15: 365-370.

Uma, A., Ramanathan, N., Saravanabava, K. 2007. A Study by PCR on the prevalence of white spot syndrome virus, *Monodon Baculovirus* and hepatopancreatic parvovirus infection in *Penaeus monodon* post larvae in Tamil Nadu, southeast coast of India. *Asian Fish Sci*, 20: 227-239.

Varma, M.G., Pudney, M., Leake, C.J. 1975. The establishment of three cell lines from the tick *Rhipicephalus appendiculatus* (Acari, Ixodidae) and their infection with some arboviruses. *J Med Entomol*, 11: 698-706.

Villena, A.J. 2003. Applications and needs of fish and shellfish cell culture for disease control in aquaculture. *Rev Fish Biol Fisheries*, 13: 111-140.

Walton, A., Smith, V.J. 1999. Primary culture of the hyaline haemocytes from marine decapods. *Fish Shellfish Immunol*, 9:181–194.

Wang, C.H., Lo, C.F., Leu, J.H., Chou, C.M., Yeh, P.Y., Chou, H.Y., Tung, M.C., Chang, C.F., Su, M.S., Kou, G.H. 1995. Purification and genomic analysis of baculovirus

associated with white spot syndrome (WSBV) of *Penaeus monodon*. *Dis Aquat Org*, 23: 239-242.

Wang, C.H., Yang, H.N., Tang, C.Y., Lu, C.H., Kou, G.H., Lo, C.F. 2000. Ultrastructure of white spot syndrome virus development in primary lymphoid organ cell cultures. *Dis Aquat Org*, 41: 91–104.

Watanabe, W., Sudo, K., Asawa, S., Konno, K., Yokota, T., Shigeta, S. 1995. Use of lactate dehydrogenase to evaluate the anti-viral activity against influenza A virus. *J Virol Methods*, 51: 185–192.

Wen, C. M., Kou, G.H., Chen, S. N. 1993. Establishment of cell lines from the pacific oyster. *In Vitro Cell Dev Biol Anim*, 29: 901-903.

Weng, S.P., Guo, Z.X., Sun, J.J., Chan, S.M., He, J.G. 2007. A reovirus disease in cultured mud crab, *Scylla serrata* in southern China. *J Fish Dis*, 30: 133-139.

West, L., Mahony, T., McCarthy, F., Watanabe, J., Hewitt, D., Hansford, S. 1999. Primary cell cultures isolated from *Penaeus monodon* prawns. *Methods Cell Sci*, 21: 219–223.

Williams, S.T., Ozawat, T. 2006. Molecular phylogeny suggests polyphyly of both the turban shells (family Turbinidae) and the superfamily Trochoidea (Mollusca, Vetigastropoda). *Mol Phylogenet Evol*, 39: 33-51.

Wilson, H. V. 1910. Development of sponges from dissociated tissue cells (document no. 750). *Bull Bureau Fish US*, 30: 1-30.

Wilson, H.V. 1907. On some phenomena of coalescence and regeneration in sponges. *J Exp Zool*, 5: 245-258.

Wolff, E. 1962. Culture organotypique de tissue de jeunes limules (*Xiphosura polyphemus*). *Bull Soc Zool Fr,* 87: 120-126.

Wongteerasupaya, C., Vickers, J.E., Sriurairatana, S., Nash, G.L., Akarajomrn, A., Boonsaeng, V., Panyim, S., Tassanakajon, A., Withyachumnarnkul, B., Flegel, T,W. 1995. A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*. *Dis Aquat Org,* 21: 69-77.

Yavin, E., Yavin, Z. 1974. Attachment and culture of dissociated cells from rat embryo cerebral hemispheres on polylysine-coated surface. *J Cell Biol,* 62: 540-546.

Yoon, J.M., Park, S.Y. 2006. Genetic comparison between *Crucian carp* (*Carassius auratus* Linnaeus) and *Crucian carp* (C. cuvieri Temmnick and Schlegel). *J Anim Sci Technol,* 48: 637-650.

Yu-Ying, F. L., Baker, F. D., Andrew, W. 1963. A method for tissue culture of hydra cells. *Proc Soc Exp Biol Med,* 113: 259-262.

Zeng, H., Ye, H., Li, S., Wang, G., Huang, J. 2010. Hepatopancreas cell cultures from mud crab, *Scylla paramamosain*. *In Vitro Cell Dev Biol Anim,* 46: 431-437

Zhang, X., Zhang, W., Xue, L., Zhang, B., Jin, M., Fu, W. 2010. Bioremediation of bacteria pollution using the marine sponge *Hymeniacidon perlevis* in the intensive mariculture water system of turbot *Scophthalmus maximus*. *Biotechnol Bioeng,* 105: 59-69.

Zhao, Q., Zhang, W., Jin, M., Yu, X., Deng, M. 2005. Formulation of a basal medium for primary cell culture of the marine sponge *Hymeniacidon perleve*. *Biotechnol Prog,* 21: 1008-1012.

Zilli, L., Schiavone, R., Scordella, G., Zonno, V., Verri, T., Storelli, C., Vilella, S. 2003. Changes in cell type composition and enzymatic activities in the hepatopancreas of *Marsupenaeus japonicus* during the moulting cycle. *J Comp Physiol B,* 173: 355-363.

Zilli, L., Schiavone, R., Storelli, C., Vilella, S. 2007. Analysis of calcium concentration fluctuations in hepatopancreatic R Cells of *Marsupenaeus japonicus* during the molting cycle. *Biol Bull*, 212: 161-168.

FUTURE WORK

The explant and dissociated cultures of the gills, hepatopancreas, heamolymph, heart, muscles, brain do not survive for long and proliferate effectively and they tend to enter into senescence. Therefore, further investigation is required to explore why the cells show poor anchorage, survivability and proliferation. One has to identify the genes responsible for cell proliferation and senescence.

In the present work a finite cell line is developed while maintaining cell susceptibility to WSSV, further work is required to develop a technique for making infinite cell line which does not loose the normal crab/crustacean behaviour as well as susceptibility to different pathogens. Also the susceptibility of testicular finite cell line to many other crustacean viruses needs to be investigated.

Cytotechnology
DOI 10.1007/s10616-008-9152-1

REVIEW

Development of primary cell culture from *Scylla serrata*

Primary cell cultures from *Scylla serrata*

Anu Sashikumar · P. V. Desai

Received: 16 November 2006 / Accepted: 23 May 2008
© Springer Science+Business Media B.V. 2008

Abstract This paper reports for the first time, the Primary cell culture of hepatopancreas from edible crab *Scylla serrata* using crab saline, L-15 (Leibovitz), 1 × L-15 + crab saline, 2 × L-15 + crab saline, 3 × L-15 and citrate buffer without any serum. We could isolate and maintain E (Embryonalzellen), F (Fibrenzellen), B (Blasenzellen), R (Restzellen) and G (Granular cells). Upon seeding the hepatopancreatic E, F, B, and R cells showed different survival pattern over time than granular cells. A modified L-15 (3×) medium supported the best survival of hepatopancreatic E, F B, and R cells in in-vitro culture. However granular cells could be maintained for 184 days with L-15 (1×) + crab saline. Fetal bovine serum was not effective additive and hampered cell viability in present study.

Keywords Hepatopancreas · *Scylla serrata* · Primary cultures · E (Embryonalzellen) · F (Fibrenzellen) · B (Blasenzellen) · R (Restzellen) and G (Granular cells)

Introduction

Crustacean cell culture has gained momentum due to viral diseases affecting commercially important species. Hence, cell culture techniques were developed: (a) to assist understanding the mechanism of host pathogenesis interaction Chen et al. 1995, (b) to produce large amount of viral material for their characterization (c) to improve tools for diagnosis and cure of diseases. Attempts have been made to establish several cell culture systems of shrimps (Al-Mohanna and Nott 1987; Chen et al. 1986, 1995; Hsu et al. 1995; Ke et al. 1990; Mulford and Austin 1998; Mulford et al. 2001; Nadala et al. 1993; Toullec et al. 1996; Uma et al. 2002) and other crustaceans (Peponnet and Quiol 1971).

Mud crab fishery has become commercially important in many parts of the world. In India mud crab, *Scylla serrata* has gained economic importance since early eighties with the commencement of live crab export to south East Asian countries. Though crab fishery is growing in India, it is blighted by disease outbreaks resulting from environmental and pathogenic factors. This has resulted in growing a need to develop cell culture techniques for understanding the pathogenicity. Earlier studies on crab were related to cytotoxicology and histology (Al-Mohanna and Nott 1985, 1987). Ballard et al. (1993) attempted integumentary cuticular epithelial cell culture of crab. However, there is hardly any report on development of primary cell cultures of commercially important crab species. Therefore, it was decided to develop a primary cell culture of hepatopancreas an organ responsible for detoxification of xenobiotics of *Scylla serrata*.

A. Sashikumar · P. V. Desai (✉)
Department of zoology, Goa University, Taleigao plateau, Goa 403206, India
e-mail: pv26in@yahoo.co.in

Cytotechnology
DOI 10.1007/s10616-011-9365-6

ORIGINAL RESEARCH

Development of cell line from the testicular tissues of crab *Scylla serrata*

Anumol Shashikumar · P. V. Desai

Received: 5 August 2010 / Accepted: 25 May 2011
© Springer Science+Business Media B.V. 2011

Abstract This is the first report on development of a finite cell line from testicular tissues of crab, *Scylla serrata*. Both the explant and segregated tissues of testes yielded cells that could proliferate and grow. These cells ranged in size from 10 to 38 µm with distinct nuclei of varying shapes. The testicular cells survived and proliferated best in L-15-crab saline medium supplemented with epidermal growth factor (20 ng/mL) and glucose (1 mg/mL). The cell proliferation rate was assessed by Methyl tetrazolium assay in terms of change in optical density which clearly indicated a prominent increase in cell density. The testicular cells were subcultured at an interval of 4–6 days. These subcultured cells remained healthy and proliferated for 5 months with a minimum of ten subsequent passages. The finite cell line was characterized in terms of morphology, growth rate, lactate dehydrogenase release (for detecting health status) and 18S rRNA sequencing. This cell line could be a very useful tool for testing infections and replications of crustacean viruses. The present work provides a technique that could be extended for developing other crustacean cell lines.

A. Shashikumar · P. V. Desai (✉)
Department of Zoology, Goa University, Taleigao Plateau, Goa 403206, India
e-mail: pvdesai@unigoa.ac.in

A. Shashikumar
e-mail: anumolkumar@yahoo.co.in

Published online: 29 July 2011

Keywords Cell culture · Cell line · *Scylla serrata* · Crab · 18S rRNA sequence · MTT

Abbreviations

EGF	Epidermal growth factor
FBS	Fetal bovine serum
HS	Horse serum
MTT	Methyl tetrazolium
LDH	Lactate dehydrogenase
PCN	Product code number

Introduction

Crustacean cell culture has gained interest over the last two decades, especially when disease problems began to affect the commercially valuable species (Chen et al. 1986; Leudeman and Lightner 1992; Mulford and Austin 1998). Cell culture techniques offer opportunity for studying the effects of pathogens in in vitro state and to develop diagnostic reagents and probes. The availability of such cellular tool is especially important to aquaculture industries which experience disease problems that are exaggerated by intensive aquaculture methods (Ellender et al. 1992; Toullec et al. 1996).

Although primary cell cultures from crustacea have been initiated since 1960s, no established cell line of marine crustacean is reported (Claydon and Owens 2008) except a transformed shrimp lymphoid

Cytotechnology
DOI 10.1007/s10616-012-9482-x

ORIGINAL RESEARCH

Susceptibility of testicular cell cultures of crab, *Scylla serrata* (Forskal) to white spot syndrome virus

Anumol Shashikumar · P. V. Desai

Received: 10 January 2012 / Accepted: 24 June 2012
© Springer Science+Business Media B.V. 2012

Abstract Testicular cell culture of crab, *Scylla serrata* (Forskal) was used to study the effects of White spot syndrome virus (WSSV). We are showing the susceptibility of cell culture of crabs to WSSV. The proliferating cell culture of testes were maintained for more than 4 months in a medium prepared from L15 and crab saline supplemented with epidermal growth factor. The cell cultures inoculated with different concentrations of virus showed distinct cytopathic effects such as change in cell appearance, shrinkage and cell lysis. WSSV infection of cultured cells was confirmed by Nested PCR technique. The incorporation of viral DNA in cultured cells was shown by RAPD profile generated using 10-mer primers. The controls that were not exposed to WSSV did not show cytopathic effects. This work shows the usefulness of proliferating testicular cell culture for studying WSSV infection using molecular tools. Thus, this report gains significance as it opens new vistas for diagnostics and drugs for WSSV.

Keywords White spot syndrome virus · *Scylla serrata* (Forskal) · Crab · Cell culture · Nested PCR · Pathogenesis

A. Shashikumar · P. V. Desai (✉)
Department of Zoology, Goa University,
Taleigao Plateau, Goa 403206, India
e-mail: pvdesai@unigoa.ac.in

A. Shashikumar
e-mail: anumolkumar@yahoo.co.in

Published online: 25 July 2012

Abbreviations

VS	Viral suspension
WSSV	White spot syndrome virus

Introduction

White spot syndrome is one of the major infectious viral diseases that cause large scale mortality of commercially cultivated crustacean species which include shrimp, prawn, crab and other arthropods (Wang et al. 1998; Chang et al. 1998; Chen et al. 2000). Numerous studies on morphology, histopathology and gene sequence of White Spot Syndrome Virus (WSSV) are reported but the mechanism of WSSV infection is not understood (Jiang et al. 2006). Although new information is available on WSSV, relatively less is known about host parasite interactions (Jiravanichpaisal et al. 2006). To understand and ultimately to control the viral diseases in crustacea, a specific and sensitive diagnostic tool to investigate this pathogen needs to be developed (Claydon and Owens 2008).

Tissue culture is an important tool employed for the study of pathogenic infections, especially for pathogens such as viruses that replicate intracellularly (Jiravanichpaisal et al. 2006). Claydon and Owens (2008) reported that due to the presence of dominant senescence genes, crustacean primary cell culture has limited proliferations. A few primary cultures of

Diversity and Physiological Processes ; 38 - 45, 2012
Editors; P V Desai & R Roy
© Goa University
ISBN; 978-81-908791-3-2

Characterization of finite cells line from testiculatr cells of crab *Scylla serrata*

Shashikumar Anumol and Desai P V

Key words: *Scylla serrata, Cell line, Testicular cell, Characterization.*

Crustacean fisheries and shrimp aquaculture production are undergoing a significant global expansion. However, the development of aquaculture is affected by the diseases of diverse etiologies that hamper the cultures of several commercially valuable species, resulting into economic loses [1-3]. Prevention and control of diseases are thus an absolute priority for aquaculture industries. The lack of standardized system for isolation, identification and purification of a disease causing viruses, is a major obstacle in controlling crustacean diseases [4]. Primary cell culture is the first step towards developing a cell line which could be used for prognosis and diagnosis of several pathogens that affect the crustacean.

Before the crustacean cell line is tested for virus infectivity, its characterization is essential to identify the purity and genetic stability as well as to check the potential risk of advent of contaminants like bacteria, fungi and mycoplasma in the cell culture. Although primary cell cultures from crustacea have been reported since 1960s, no established cell line of marine crustacean is reported thus far [5] other than that of transformed lymphoid cell line of shrimp [1] and hybridized cell line of *Penaeus monodon* [4]. However, recently we have reported the development of a finite cell line from the testes of a crab, *Scylla serrata* [6]. In the present work we report a few characteristics of the afore mentioned cell line.

Live *Scylla Serrata* (wt ~ 110 ± 0.60 gm) obtained from a local supplier were maintained in aerated seawater (salinity 29 ‰) and