CELL CULTURE FROM LYMPHOID ORGAN OF *MACROBRACHIUM ROSENBERGII*

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CERTIFICATE

This is to certify that the dissertation entitled "**Cell Culture from lymphoid organ of *Macrobrachium rosenbergii***" is a record of bonafide research work done by **Miss. Rajitha, M** (1998-2000 batch) of M.F.Sc (Fishery Resource Management) programme under our guidance and it has not previously formed the basis for any publication or for the award of any degree, diploma or other similar titles.

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I hereby declare that the dissertation entitled **"Cell culture from lymphoid organ of *M.rosenbergii*"** is a record of my own research work done under the guidance of **Dr. K. Pani Prasad**, Fish Pathology Laboratory, Central Institute of Fisheries Education, Mumbai during the year 1998-2000 and that the data has not been formed the basis for any other publication or dissertation in the past.

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

ABBREVIATIONS USED

Hr	-	Hour
Min	-	Minutes
g	-	Gram
mg	-	Milli gram
µg	-	Micro gram
ml.	-	Milli litre
µl	-	Micro litre
mmol/kg	-	Molal and molar solution per kilogram
pH	-	*Puissance de hydrogen* (Hydrogen ion concentration)
ppm	-	Parts per million
rpm	-	Revolutions per minute
HCl	-	Hydrochloric acid
KCl	-	Potassium chloride
NaCl	-	Sodium chloride
$MgSO_4$	-	Magnesium sulfate
$MgCl_2$	-	Magnesium chloride
PBS		Phosphate buffer saline
L-15	-	Leibovitz'000s-15 medium
EGF	-	Epidermal growth factor
EMEM	-	Eagle's minimum essential medium
M199	-	Medium-199
FBS	-	Foetal bovine serum
FCS	-	Foetal calf serum

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सारांश

सघन जलकृषि प्रणाली को अपनाने से रोगों की संख्या में वृद्धि हुई है । जिनमें मुख्यत: वायरल बिमारी है । वायरस की पहचान तथा लक्षणों के लिए एक मुख्य उपाय वायरस की वृद्धि है जिसे या तो जीवित झींगा में या फिर संवर्धित कोशिकाओं में किया जाता है । इसको ध्यान में रखते हुए यह अध्ययन मीठे पानी के महाझींगा *माइक्रोब्रेकियम रोजनबर्गाई* के लिम्फोइड अंग से कोशिका संवर्धन तैयार करने के लिए किया गया । यह तैयार संवर्धित कोशिकाओं को भिन्न प्रकार के अध्ययन के लिए इस्तेमाल किया जा सकता है जैसे कि कुछ खास प्रकार का चुनाव, उसकी पहचान, वायरस के लक्षण, अज्ञात रोग तथा विभिन्न दूषित कारकों के स्तर जो कि झींगा को प्रभावित करते है ।

यह अध्ययन माइक्रोब्रेकियम रोजनबर्गाई के लिम्फोइड अंग से कोशिका संवर्धन तकनीकी तैयार करने के लिए किया गया । आधारीय माध्यम लिबोविट्ज - एल 15 को आवश्यक कारक ओसमोलिलिटी, संमुअक तथा उचित तापमान पर कोशिका के संवर्धन प्रणाली विकसित करने के लिए परखा गया । लिबोविट्ज - एल 15 को 15% सुअर गर्भाशय रक्त द्रव (फीटल बोवाइन सीरम), 8% झींगा अर्क, 20 माइक्रोलीटर, बाहयत्वचा विकास कारक, 10 माइक्रोलीटर मानव पुनर्निवेन अन्त:ल्यूकिन, अन्तिम ओसमोलिलिटी, 450 मिलीमोल/कि.ग्रा. तथा जीवप्रतिरोधक के साथ प्राथमिक कोशिका वृद्धि में अच्छे परिणाम प्राप्त किए गए ।

कोशिकाओं वृद्धि का विकास 4 दिन तक बढ़ते क्रम में, चौथे व पांचवे दिन में स्थिर तथा छठवें दिन घटते क्रम में देखा गया । कोशिकाएं सात दिन तक जीवित रहीं । कोशिका वृद्धि के लिए अत्याधिक उचित तापमान 28^{0}C तथा पी.एच. 7.8 पाया गया ।

माध्यम एम 199 को 15% सुअर गर्भाशय रक्तद्रव, 8% झींगा अर्क, 20 माइक्रो ली. बाहयत्वचा विकास कारक, 10 माइक्रो ली. मानव पुनर्निवेन अन्त:ल्यूकिन, 6 मिली. ली. नमक घोल एवं जीव प्रतिरोधक के साथ, कोशिका वृद्धि में एल 15 माध्यम की तुलना में कम उपयुक्त पाया गया । कोशिकाओं 4 दिन से ज्यादा जीवित नहीं रही । ई.एम.ई.एम को 15% सुअर गर्भाशय रक्तद्रव, 8% झींगा अर्क, 20 माइक्रो ली. बाहयत्वचा विकास कारक, 10 माइक्रो ली. मानव पुनर्निवेन अन्त:ल्यूकिन, 6 मिली. ली. नमक घोल एवं जीव प्रतिरोधक के साथ अन्य दोनों माध्यमों की तुलना में कोशिका वृद्धि के लिए कम उपयुक्त पाया गया ।

ABSTRACT

The adoption of intensive aquaculture system has brought about an increase in the number of disease outbreaks especially viral diseases. One of the important ways for identification and characterization of the viruses is multiplying the virus either in living shrimp/prawn or in cell cultures. Keeping this in view the present study was undertaken to develop cell culture from the lymphoid organ of commercially important fresh water prawn, *M.rosenbergii*. Cell culture thus developed can be used for a variety of applications, in particular isolation, identification, characterization of virus, diseases of unknown etiology and also to test the level of various pollutants affecting prawns.

The experiment was carried out to develop primary cultures from the lymphoid organ of *M.rosenbergii*. The basic media Leibovitz's-15 was tested for supplements, osmolality, pH and temperature to establish the culturing system of cells. The L-15 medium supplemented with 15% FBS, 8% prawn extract, epidermal growth factor (20µl /100 ml), human recombinant interleukin (10µl /100ml), with final osmolality 450mmol/kg and antibioties has given good results in the development of primary cultures. The cells were proliferated in an ascending order upto 4 days, 4th and 5th day constnat and descending order from 6th day, survived

upto 7 days and 75% confluence was observed. The optimum maintenance temperature was found at 28^{0}C, and pH 7.8.

M199 medium supplemented with 15% FBS, 8% prawn extract, 20µl EGF, 10µl human recombinant interleukin, 6 ml salt solution and antibiotics showed less growth as compared to L-15 medium. 35% confluence was observed and cells did not survive more than 4 days. EMEM supplemented with 15% FBS, 8% prawn extract, 20µl EGF, 10µl human recombinant interleukin, 6 ml salt solution and antibiotics showed very less cell growth as compared to other two media.

INTRODUCTION

1. INTRODUCTION

1.1 GENERAL ASPECTS

Crustacea constitute a class, which include animal species of biological interest for fundamental research and/or high commercial value. Giant fresh water prawn *Macrobrachium rosenbergii* belongs to the class Crustacea. In many parts of world the giant fresh water prawn is the most coveted species of prawn for culture especially in fresh water areas. There has been renewed interest for the past few years for culture of these species in the country. This is because of its suitability for aquaculture on account of it's best growth rate in fresh and low saline water, omnivorus feeding habit, hardy nature, and compatability for polyculture and high prices in domestic and international market. Giant prawn is widely cultured in Asian countries such as Thailand, Taiwan, Malaysia and Vietnam, India, Indonesia, Hawaii and South Carolina states of USA.

The ever-increasing demand of prawn in the international market has tremendous interests among aquafarmers and industrialists to venture into aquafarming in India. By this, there has been a shift from the traditional extensive farming system to modified extensive, semi intensive and intensive culture systems. However, the adoption of intensive aquaculture system in an unplanned and unscientific manner has brought about an increase in the stress on prawns and resulted in many diseases.

It is widely recognized that infectious diseases are major limiting factors in culture practices. Although many aspects of crustacean virus

diseases have been investigated in host animals, suitable *in vitro* cell lines/tissue culture systems are necessary for the isolation, purification, and characterization of the infective agents, the development of diagnostic methods and production of material for immunological and vaccination studies.

1.2 LYMPHOID ORGAN

The lymphoid organ is located in the left and right side of the anterodorsal surface of the hepatopancreas. From the histological structure and manner in which the out-going and in-coming vessels are connected it can be easily imagined that this organ belongs to vascular system in addition, the interstitial tissues surrounding it become lymphoid tissues. For this reason, the organ can regard as a Lymphoid (Oka, 1969).

1.3 CELL CULTURE.

Growing of cells *in vitro* condition with all required materials and conditions provided in laboratory condition are called as cell culture. culture of animal cells is usually divided into 3 classes. Primary cells, cell strains, cell lines. The ultimate source of cells for cell culture is the intact animal. The cells may be obtained from various organs and tissues of embryonic, infant or adults origin (human, mice, fishes or shrimps).

The primary culture defined as the culture, which is obtained directly from the animal's organs and tissues. Primary cell culture retains some of the characteristics of the tissue from which they have been derived. With some exceptions, cell culture can be classified in two general morphological types. Fibroblast like cells are thin and elongated and

epithelial cells are polygonal in shape. Most of the primary cell cultures have a finite life span of five to ten divisions in vitro.

1.4 APPLICATIONS OF CELL CULTURE

Cells cultured in an artificial environment provide a convenient tool for studying the living system at the cellular level. The cells can be monitored without having to sacrifice any experimental animals for the purpose. These provide homogenous populations of cells of virtually identical genetic make up grown under controlled and constant environmental condition. Following are some of the common applications of cell cultures.

1.4.1 Virology

The main application of cell culture in aquaculture so far has been in the isolation, identification, and study of viruses that provoke epizootic outbreaks causing economic loss. Most cell lines are derived from commercially important species and a large number of cell lines from different species is required due to host cell specificity of virus (Cheng *et al* 1993).

Viruses need living cells to multiply because they are obligate intracellular parasites. The cell monolayers inoculated with the test virus and the characteristic appearance of cytopathic effect (CPE) indicates the presence of pathogen.

Primary lymphoid cell cultures from penaeid shrimp were used in the development of a quantal assay protocol ($TCID_{15}$) for the filtration of

two highly pathogenic shrimp viruses, the yellow-head virus (YHV) and non-occluded Chinese baculovirus (CBV) developed by Philip *et al.* (1996).

Monolayer cultures were established from ovary, heart, lymphoid and peripheral hemocytes of penaeid shrimps including *P. monodon*, *P. japonicus* and *P. pencillatus*. By electron microscopy, virions of white spot disease virus (WSDV) and yellow head virus (YHV) were observed in the nuclei and cytoplasm of cultured cells (Chen *et al.*, 1999).

Cell cultures from embryonic tissues of commercial important species of crustaceans, fish can be used to indicate vertical transmission of viral diseases. Oka organ cell lines from penaeid shrimp *P. stylirostris* were used for the studies on simian virus-40 (Tapay *et al.*, 1994 and 1995).

1.4.2 Toxicology

Established cell lines are used as bio-indicators in cytotoxicity assays of aquatic pollutants (Alguacil *et al*, 1991) and direct acting and metabolism mediated toxicants have been evaluated for their cytotoxicities (Babich *et al*, 1991). The requirement for species specific pollution studies caused for establishing cell lines from individual species (Lyons *et al.*, 1996).

1.4.3 Cell Biology

Several aspects of cell biology like cell physiology, differentiation and cytogenetics have been studied using primary and continuous cell

lines. Transport characteristics of hepatopancreatic and antennal gland epitheloid tissue were studied from primary cell cultures by Cattey, 1990.

Hence the present study was undertaken with the following objectives.

1. To develop primary cell culture from lymphoid organ of *M.rosenbergii*
2. To compare different media for initiating primary cultures
3. To evaluate supplements and additives on growth and attachment cells

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

The risk of diseases in aquaculture has prevented the rapid growth of this industry and current emphasis is on early detection of disease, its diagnosis, and prevention through management and immunotherapy. Disease due to viral etiology, have resulted in huge losses to the aquaculture industry and in this context cell culture becomes important owing to the host specificity of viruses, for confirmative disease diagnosis and investigation, it is imperative to have cell culture developed from the concerned crustacean species. Lack of such established cell culture has limited our knowledge regarding prawn viruses. Prawn cell cultures and prawn viruses also form very good educational models in teaching virology, as there is very little danger from handling prawn pathogens.

2.1 CELL CULTURE

Viruses cannot replicate in synthetic media and require living cells as host. Until 1950's the only tools available to grow and study viruses were animals and embryonating the eggs. To solve this problem, the cell culture technique was developed, it requires less space, is less expensive and is more convenient than the use of animals.

Tissue culture has become a wide spread technique as cultured cells are being used in a spectrum of disciplines ranging from biochemistry to molecular and cell biology. Tissue culture techniques/procedures are simple but require extreme care to avoid contamination (Paul, 1975; Freshney, 1987; Adams, 1990 and Baserga, 1990).

The advantages of cultivating cells and tissues in completely defined nutrient medium were reported more than 80 years ago. Realization of this objectives, particularly for fastidious cell types, require development of more complex nutritional formulations and discovery of the various growth and attachment factors required by cells in culture (Ham and Mckeehan, 1979; Waymouth, 1978).

Most of the cultured cells grow in highly specialized microenvironment which may be mimicked *in vitro* by diluting or eliminating serum and replacing it with mixture of metabolic precursors, macromolecules and biophysical elements (Bottenstein *et al.* 1979; Ham 1982; Jayme and Blackman, 1985; Freshney, 1987).

2.1.1 Crustacean Cell Cultures

The availability of cellular tools are important to industries which use intensive aquaculture methods which have increased risk of disease problems and crustaceans cell culture has gained attention as a potent model for the development of diagnostic reagents and probes for use in the shrimp, Cray fish and lobster industries (Toullec, 1999).

Chen *et al.*, (1986) obtained observable cell growth from heart and gonads of grass prawn *P. monodon* by using Leibovitz's -15 medium (L-15) supplemented with 10% of foetal calf serum (FCS), 30% muscle extract, 0.006 g/ml sodium chloride (NaCl) and 10% lobster haemolymph. The culture could be maintained for approximately two months at 28-31^{0}C with weekly change of medium.

Itami *et al.*, (1989) obtained primary cell cultures of the lymphoid organ, epitheloid cells and fibroblastic cells in various synthetic media and the cells survived for 42 days in L-15 (2X), 40 days in Eagles MEM and 54 days in M199 media.

Monolayers of ovarian epitheloid cells from *P. stylirostris* and *P. vannamei* were developed by Luedman and Lightner (1992) *with* 80% confluence in a two-day period at an osmolality of approximately 700-750 mmol/Kg, temperature range of 25-28^{0}C and incubation in normal atmosphere. They used lobster haemolymph, shrimp muscle extract, foetal bovine serum and hybridoma quality foetal bovine serum.

Hsu *et al.*, (1995) tried to establish a subculture system for cells from the Oka organ (lymphoid tissue) of the grass prawn, *P. monodon* and found that Leibovitz's 15 media supplemented with 10% foetal bovine serum, 5g/L NaCl at pH 7.63 – 8.1 supports the growth effectively as a basic culture medium. The basic culture media was tested for osmolality, serum concentration, serum sources and pH.

Toullec *et al.*, (1996) cultured tissues and dissociated cells from various organs of the shrimp *P. vannamei* and embryos of *P. indicus* in different media and observed cell out growths with ovaries, epidermal regeneration buds and embryos with a survival for several months in M 199 based medium for ovaries and embryos, and Grace's medium for regeneration buds. In every case, a 10% foetal bovine serum supplement was used.

Philip *et al.*, (1997) developed the primary lymphoid cell cultures from penaeid shrimp for the development of a quantal assay protocol for the titration of two highly pathogenic shrimp viruses, the yellow head virus (YHV) and non occluded Chinese baculovirus (CBV). This report represented the first convenient quantitative assay protocol using cell cultures derived from the penaeid shrimp to titer shrimp viruses.

Chen and Wang (1999) established monolayer cultures from ovary, heart, lymphoid tissue and peripheral haemocytes of penaeid shrimps including *P. monodon, P. japonicus* and *P. penicillatus* and observed that the most favorable conditions for the culture of penaeid shrimp cells *in vitro* was in CMRL and L-15 tissue culture media when used within osmolarity ranges of 620 – 760 mmol/Kg, at a temperature of 25^0C for tissue of *P. japonicus* and 28^0C for tissue of *P. monodon* and *P. penicillatus*. Lymphoid tissue of Oka organ was found superior for the formation of confluent cell monolayers and was subculture up to three times. Muscle extract was found to enhance the survival of cultured cells of penaeid cells *in vitro*

Leigh and Jan (1999) attempted to produce continuous cell lines from penaeid tissue and the osmolarity of *P. monodon* haemolymph was measured at 687 mmol/Kg. The best media for cell growth and maintenance was observed to be double strength L-15 supplemented with 10% foetal bovine serum and 10% prawn muscle extract was made by homogenizing 30g of prawn muscle in 50/50 ratio of distilled water/autoclaved seawater which was clarified at 2k, 14k, 14k X g for 30 min each.

Kasornchamdra *et al.*, (1999) developed primary shrimp cell cultures from lymphoid organ and ovaries of black tiger shrimp, *P. monodon* by using double strength Leibovitz's L-15 medium supplemented with 15% foetal bovine serum, 1% glucose, 5 g/l sodium chloride and 15% shrimp meat extract, and L-15 media was found to give 80% confluent monolayer cells of *P. monodon* because it has strong buffering capacity and it's chemical composition.

2.1.2 *Macrobrachium rosenbergii*

Frerichs (1996) obtained good yields of dissociated, uncontaminated, viable cell suspensions from embryonic tissue of *M. rosenbergii* by physical disruption of harvested eggs in the presence of buffered iodophore disinfectant and malachite green. Primary cultures in the form of proliferating foci of cells were readily initiated using a wide range of mammalian and insect cell culture media with osmolality adjusted to 450 mmol/kg water and addition of 20% heat-inactivated foetal bovine serum, and the passage of primary cultures resulted in the loss of adherence of cell to the culture vessel surface, cessation of cell multiplication and consequent failure to establish a cell line.

2.2 MEDIA

In *in vitro* culture, the media provide nutrients for maintaining homeostasis to adapt and respond to the microenvironment and for growth, division and differentiation of the growing cell. They also serves as reservoirs of metabolites (Trumbore, 1966). A general similarity between the nutritional requirement of different cells was observed by Paul (1965).

Epitheloid and fibroblast cells were observed in the primary culture of lymphoid organ of *P. japonicus* by using different media like PRMI 14, L-15 (2X), Eagle's MEM and M 199, while 18% calf serum, 27% muscle extract of kurma shrimp, 10% blood homogenate of *Charybdis japonicus* and 6 gm of sodium chloride were added to the media. (Itami *et al.*, 1989). Best growth of gonad cells of *P. monodon* occurred in a culture system containing double strength (2X) L-15 medium supplemented with 10% foetal calf serum (FCS), 30% muscle extract, 0.006 gm/ml NaCl and 10% lobster or grass prawn haemolymph (Chen *et al.*, 1986).

Philip *et al.*, (1997) used 2X L-15 medium supplemented with 20% foetal bovine serum and 8% shrimp head extract, 6% salt solution, 20 ug/ml. epidermal growth factor, and 10 units/ml. human recombinant interlukin-2. Toullec, 1999 modified commercially prepared media (M 199, EMEM and L-15) and the use of double strength M 199 and L-15 gave similar effects in closely related species and some times in same species of shrimps.

Table 1. Different Components of Media

Components	Leibovitz-15	EMEM	M 199
Inorganic salts(mg/l)			
NaCl	8000.00	6800.00	6800.00
KCl	400.00	400.00	400.00
$CaCl_2$	140.00	200.00	
$CaCl_2.2H_2O$			
$MgCl_2.6H_2O$	200.00		
$MgSO_4.7H_2O$		200.00	200.00
$NaH_2PO_4.2H_2O$		150.00	158.30
$Na_2HPO_4.7H_2O$	359.00		
$NaHCO_3$		2200.00	2200.00
L-amino acids(mg/l)			
Alanine	225.00		25.00
Arginine	500.00		
Asparagine	250.00		
Aspartic acid			30.00
Cysteine	120.00	24.02	
Cystine			20.00
Glutamic acid		292.30	66.82
Glutamine	300.00		100.00
Glycine	200.00		50.00
Histidine	250.00	52.50	
Isoleucine	125.00	52.50	20.00
Leucine	125.00		60.00
Lycine	75.00	14.90	
Methionine	75.00	33.02	15.00
Phenylalanine	125.00		25.00
Proline			40.00
Serine	200.00	47.64	25.00
Threonine	300.00	10.20	30.00
Tryptophan	20.00	36.22	10.00
Tyrosine	300.00	46.90	40.00
Valine	100.00		25.00

Vitamins/Cofactors(mg/l)			
Choline.Cl	1.00	1.00	0.50
Folicacid	1.00	1.00	0.01
Inositol	2.00	2.00	0.05
Nicotinamide	1.00	1.00	0.025
Pantotheniate.Ca	1.00	1.00	0.01
Pyridoxine.HCl	1.00		0.025
Riboflavine.PO_4.2Na	0.10		
Thaimine.PO_4	1.00		
Other components(mg/l)			
Phenol red	10.00	10.00	10.00
D-glucose		1000.00	1000.00
D-galactose	900.00		
Pyruvate sodium	550.00	110.00	
CO_2 (gas phase)%	Atmosphere	5.00	5.00

2.2.1 Supplements

2.2.1.1 Serum

Different percentages of foetal bovine serum (FBS) have been used for cell culture of different shrimps as supplement at concentrations of 10 to 20% in the media with an optimum of 10% (Toullec, 1999). The author observed improved secretory capacity in Y-organ cells cultures from the crab, *Carcinus maenas* by the addition of FBS. Luedman and Lightner (1992) used 10% FBS for development of an *in vitro* primary cell culture

system from the penaeid shrimp, *P. stylirostris* and *P. vannamei*. Philip *et al.*, (1997) used 20% FBS as supplement in 2X L-15 medium. Chen *et al* (1986) cultured the tissue of grass prawn, *P.monodon* by using 10% of FCS.

FBS is notable for it's lack of toxicity, good attachment, pH maintenance and growth stimulating properties. Hormones, micronutrients, growth and cell addition factors, which are known to exist in serum, stimulate cell spreading in culture (Hashimoto *et al.*, 1997). Undefined growth inhibitory factors may be present in high concentrations FBS and are avoided (Loo *et al.*, 1987).

2.2.1.2 Antibiotics

Antibiotics have been universally used in combination to minimize risk of contamination in shrimp cell cultures. Antibiotics are necessary for long term cell survival because bacteria and fungi are commonly found as a part of the haemolymph and cell mixture. Streptomycin seemed to be the most affective antibiotic against the bacterial strains. In addition, Penicillin and amphotericin B was added to eliminate any possible airborne bacterial and fungal contamination (Toullec, 1999). Philip *et al.*, (1997) used 100 I.U/100 µg/ml. penicillin/ streptomycin to develop cell lines from shrimp lymphoid organ. Leigh and Jan (1999) used penicillin 10000 IU/ml, streptomycin 10000 ng/ml, fungizone 25 ng/ml. and found that 15 out of the 101 primary cultures established form *P. monodon* succumbed to bacterial contamination. Hsu *et al.*, (1995) used penicillin (10000 units/ml), streptomycin (10000 µg/ml), amphotericin B (500 ng/ml) and gentamycin

(1 μg/ml.) in the holding media. Fraser and Hall (1999) used penicillin (10000 IU/ml), streptomycin (10000 μg/ml) and amphotericin B (10 μg/ml) in all cell culture media and washing buffers.

2.2.1.3 Salt solution

Osmolality of all media solutions diluents throughout all experiments were increased using a sterile salt solution. Osmolality was increased to approximate as closely as possible the osmolality of shrimp tissue (Toullec, 1999). Hsu *et al.* (1995) observed that cell cultures with 5-g /l NaCl (472 mmol kg^{-1} osmolality) had the best attachment percentage and appearance.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 LOCATION

The experiment was performed in the Fish Pathology Laboratory, Central Institute of Fisheries Education, Mumbai.

3.2 COLLECTION OF SAMPLES

Macrobrachium rosenbergii live samples were collected from Bhivandi creek, weighing approximately 30-40g, and maintained in the laboratory. They were provided with continuous aeration and artificial pelleted feed.

3.3 CULTURE FLASKS

Vessels in which the cells are seeded and cultivated plays a profound role in the *in vitro* growth of cells. It not only achieves physical contaminenet of cells, but also influences the chemical environment by absorption or release of proteins or ions. Hence, the material and quality of the culture vessel is important for successful proliferation of cells.

3.3.1 Preparation of Glassware

Glassware made commercially is often toxic to a number of different types of cells. This toxicity problem in most cases can be solved by proper cleaning procedures. The glassware used in the experiments was of neutral glass of Borosil make. They were soaked overnight in a mild acid solution (HCl). Later they were rinsed thoroughly with tap water, several times, then with demineralised water seven times and finally with triple distilled water 6-7 times. The distilled glass water rinse is an

important step of the cleaning cycle, since piped water may contain pyrogens or dissolved metal ions that may interfere with the cell growth. After drying, they were properly wrapped in aluminium foil, then paper and sterilized in hot air oven at 160^0 C for one and half-hour.

3.3.2 Preparation of Plasticware

All the plasticware used were nontoxic to cell cultures having good optical quality and were recommended suitable for "tissue culture" by manufacturers (Tarsons, India). A large quantity of disposable sterile plasticware was used for the cell culture like tissue culture flask, petridishes, multi well plates, syringes, filters etc. which are available from commercial suppliers.

3.4 MEDIA

Cells require nutrition to maintain homeostasis to adopt and respond to the microenvironment and for growth, division and differentiation. In *in vitro* culture, the cell culture media provide nutrients for the growing cell. These media have all the required elements, ions, vitamins, amino acids, etc.

3.4.1 Leibovitz 15(L-15 medium)

L-15 medium (Sigma, USA) which has galactose as the energy source. L-15 media was prepared by following procedure. A stirbar was placed in a 600-ml beaker or Erlenmeyer flask, and filled with 400-450 ml of triple distilled water and placed on stir plate without heat. A full bottle of dehydrated L-15 media was added to the triple distilled water while stirring (this is the amount of media originally intended for 1 lit of water).

The media was allowed to dissolve for an hour or so. Triple distilled water was added to the 500ml level and continued stirring for about 30 min. The media was sterilized by passing through 0.22 μm Millipore filter. The medium was checked for sterility by culturing 0.5 ml of the filtered medium in brain heart infusion agar for 24- 48 hours and the pH was adjusted to 7.6. The medium was stored at 4^0 C as 2x L-15 medium.

3.4.2 Eagle's Minimum Essential Medium (E-MEM) & M-199

Dehydrated powder of Eagle's MEM and M-199 was obtained from Sigma Aldrich, USA. These two media were prepared according to the same procedure followed for L-15 media. The pH was adjusted to 7.6. The media was sterilized by passing through 0.22 μm Millipore filter. The medium was checked for sterility, and stored at 4^0C.

3.5 SALT SOLUTI0N

The salt solution was prepared by the following method. To 400 ml of triple distilled water the following salts were added in a beaker or Erlenmeyer flask. Care was taken to dissolve each salt before adding next. Once dissolved triple distilled water was added to 500 ml., sterilized by autoclaving at 121^0C, 15 lbs. pressure for 20min and stored at 4^0 C, until further use.

S.no	Salt	Quantity (gm)
1.	Sodium chloride	51.2
2.	Potassium chloride	0.9
3.	Magnesium chloride	5.9
4.	Magnesium sulphate	5.4
5.	Calcium chloride	2.55

3.6 PREPARATION OF PRAWN EXTRACT

20 gms of head soft tissue from the thoracic region of the prawn was removed with the help of sterile forceps and scissors. An approximate 10 % mixture was prepared using 2 x L-15 media (20-g prawn in 200ml. of total volume). The mixture was homogenized at 15000 rpm for 2 min or until smooth. The homogenate was centrifuged at 3000 rpm for 30 min at 4^0C. The supernatant was collected and centrifuged at 10,000 rpm for 1 hr at 4^0C. The supernatant was collected and centrifuged at 29,000 rpm for 3 hrs at 4^0C. The sediment was discarded and the supernatant was used as the prawn extract. The supernatant was filtered through 0.2-μm filter, sterility test performed and stored at -20°C, for further use.

3.7 ANTIBIOTIC INCUBATION MEDIA (AIM)

AIM solution was prepared by adding the following components.

S.no	Ingredient	Quantity (ml)
1.	Amphotericin (@250µg/ml)	1
2.	Gentamycin (@50 mg/ml.)	0.5
3.	Penicillin/Streptomycin (@10,000IU/10mg/ml)	1
4.	Salt solution	6
5.	2 x L-15 medium	91.5

The solution was stored at 4^0C, until further use.

3.8 GROWTH MEDIUM

The growth media was prepared by the addition of the following ingredients:

S. No	Ingredients	Quantity
1.	Foetal Bovine Serum	20 ml
2.	Prawn Extract	8 ml
3.	Epidermal growth Factor (@100 µg/ml.)	20 µl
4.	Human recombinant interlukin-2 (@ 10 µg/ml.)	10 µl
5.	Penicillin/Streptomycin (@ 10,000 IU/10µg /ml.)	1 ml
6.	Salt Solution	6 ml
7.	2 x L-15 Media	65 ml

The media was filtered through 0.2-µm filter, sterility test was performed and sterile media was stored at 4^0C, until further use.

3.9 PRAWN LYMPHOID ORGAN

The giant freshwater prawn (*M.rosenbergii*, approximately 30-40 g each) was immersed in the 1% of bleaching powder for 10 min. The prawn was taken out and the surface was sterilized with 500-ppm iodine solution. Under sterile conditions the exoskeleton (of head) was slowly removed

taking care not to puncture the haepatopancreas. The haepatopancreas was removed and the lymphoid organ lies just beneath it. The lymphoid organ was removed carefully with the help of sterile scissors and forceps and transferred to sterile petridish with AIM solution. The lymphoid organ was cut into small pieces using sterile scissors and incubated with 5-10 ml of AIM solution for 45 min with periodical shaking. The process of incubation in AIM solution was repeated twice.

3.10 PRIMARY CELL CULTURE

After incubation of lymphoid organ in AIM solution, the AIM solution was replaced with growth media and incubated for 45 min. Then the tissue fragments were transferred to culture flasks and the medium is removed. The tissue pieces were not disturbed for 30 to 45 min, to allow the pieces to stick to the bottom of the flask. Then 10 ml of the growth media was added to each flask and incubated at 26-28^{0}C and observed everyday for multiplication of the cells under inverted light microscope. The surface area of the flask which was spread by growing cells in comparison to the available area for growth was calculated in percentage.

3.10.1 Effect of Different Media on Cell Confluence

Different media like L-15, EMEM, M199 were tested for their ability to support growth of the cells.

3.10.2 Effect of Different pH on Cell Confluence

L-15 media at different at pH of 7.5, 7.8, 8.0, 8.2 was tested on the confluence and growth of the cells of lymphoid organ of *M.rosenbergii*.

3.10.3 Effect of Different Osmolality of L-15 Media on Cell Confluence

The effect of osmolality on confluence of the cells of the lymphoid organ of *M.rosenbergii* was tested different levels of osmolality at 350, 450, 550 and 650 mmol/kg of L-15 media. Sodium chloride added to the media at the rate of 0.85, 4.25, 8.861 and 12.376g/l to obtain above osmolality respectively.

3.10.4 Effect of Temperature on Cell Confluence

The confluence was observed at different temperatures *viz* 24°C, 28^0C, 32^0C, and 37^0C in the L-15 growth media with 20% FBS,20 µl EGF, p^H 7.8 and osmolality of 450 mmol/kg.

3.10.5 Effect of the Quantity of FBS in Media on Cell Confluence

FBS at 5, 10,15,20 and 25% in the growth media was tested on the growth of the cells..

3.10.6 Effect of Quantity of Epidermal Growth Factor (EGF) in Media on Cell Confluence

The EGF was added at different levels of 5,10,15,20,25,30 µl per 100 ml of growth media (L-15) and was tested for cell confluence.

RESULTS

4. RESULTS

Commercially available three basal media were selected for development of cell culture from *M. rosenbergii* lymphoid organ because of differences in their carbohydrate and aminoacid concentration. The comparison of cell proliferation and attachment with different media has been tested and assessed the effect of osmolality, pH of the media, quantity of FBS in the media, quantity of EGF, and incubation temperature on the growth of cells.

4.1 PHYSICAL PARAMETERES

M.rosenbergii weighing approximately 25 to 30g were used for the experiment (Plate 1).

4.2 EFFECT OF DIFFERENT MEDIA ON CELL CULTURE

Among the three media attachment of lymphoid organ to culture flask is well in L-15 media and good in M-199 but is less in EMEM. After 24 h of incubation cells started to proliferate. When L-15 media was supplemented with 15% FBS, 8% shrimp extract, 20µl EGF, 10µl human recombinant interleukin and antibiotics allowed the growth of cells. The cells grew in an ascending order upto 4 days, 4^{th} and 5^{th} day constant and in descending order from 6^{th} day. The cells deteriorated after 7 days (Table 8 and Fig 7). Two types of cells were observed they are fibroblast cells that are thin and elongated and epitheloid cells which are spindle shaped cells (Plate 2).

. Cell growth in low level was observed by using M199 with supplements like 20% FBS, 8% prawn extract with antibiotics but cells did

not survive more than 4 days but in case of EMEM cell attachment and confluence is very less as comparative to other two media (Table 2 and Fig.1).

4.3 EFFECT OF OSMOLALITY OF L -15 MEDIA

The L- 15 media at an osmolality of 450 mmol/kg was found to be optimum osmolality for lymphoid organ culture of *M.rosenbergii* (Table 3 and Fig.2).

4.4 EFFECT OF pH OF MEDIA ON CELL CONFLUENCE

Among the various pH of media, the media at pH 7.8 supported the confluence of the cells (Table 4 and Fig .3).

4.5 EFFECT OF TEMPERATURE ON CELL CONFLUENCE

The incubation temperature of 28^{0}c was found to be optimum incubation temperature for growth of cells (Table 5 and Fig.4).

4.6 EFFECT OF FBS ON CELL CONFLUENCE

15% FBS in the growth media was found to have higher percentage of confluence of growth cells (Table 6 and Fig.5) .

4.7 EFFECT OF EGF ON CELL CONFLUENCE

The EGF was added at different levels of 5,10,15,20,25,30 µl to the growth media. The media with 20µl gave the best confluence as well as growth (Table 7 and Fig .6).

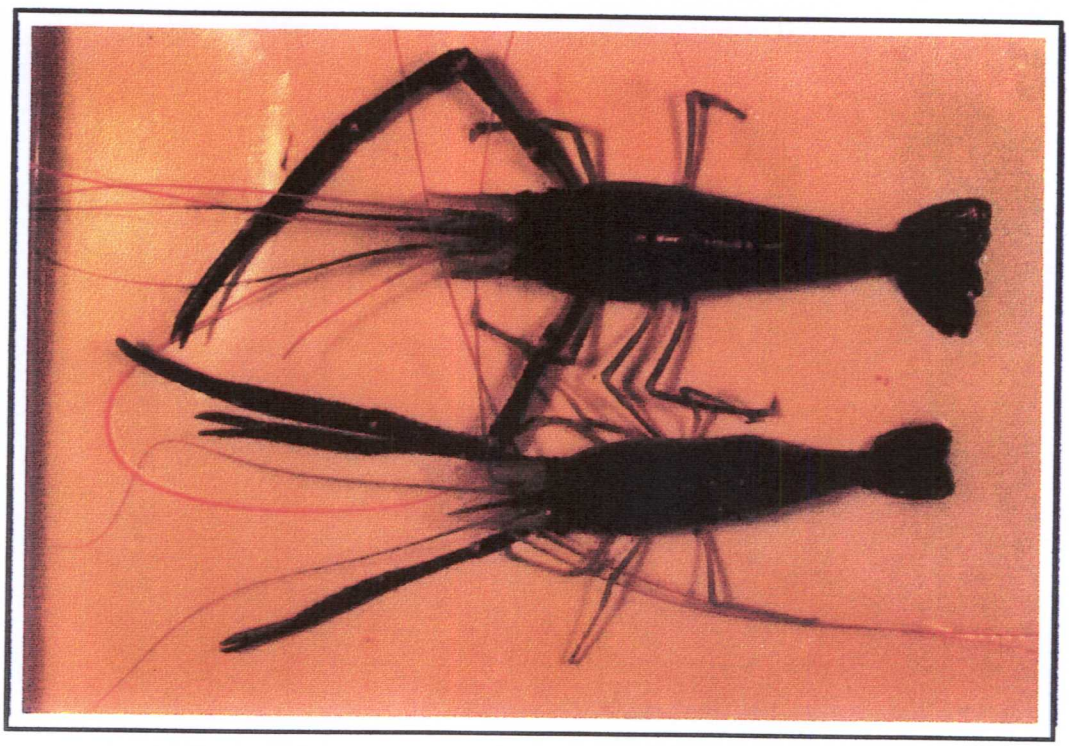

Plate 1 *M. rosenbergii*

Plate 2 **Lymphoid Cell Culture - 48 hours old (10X magnification)**

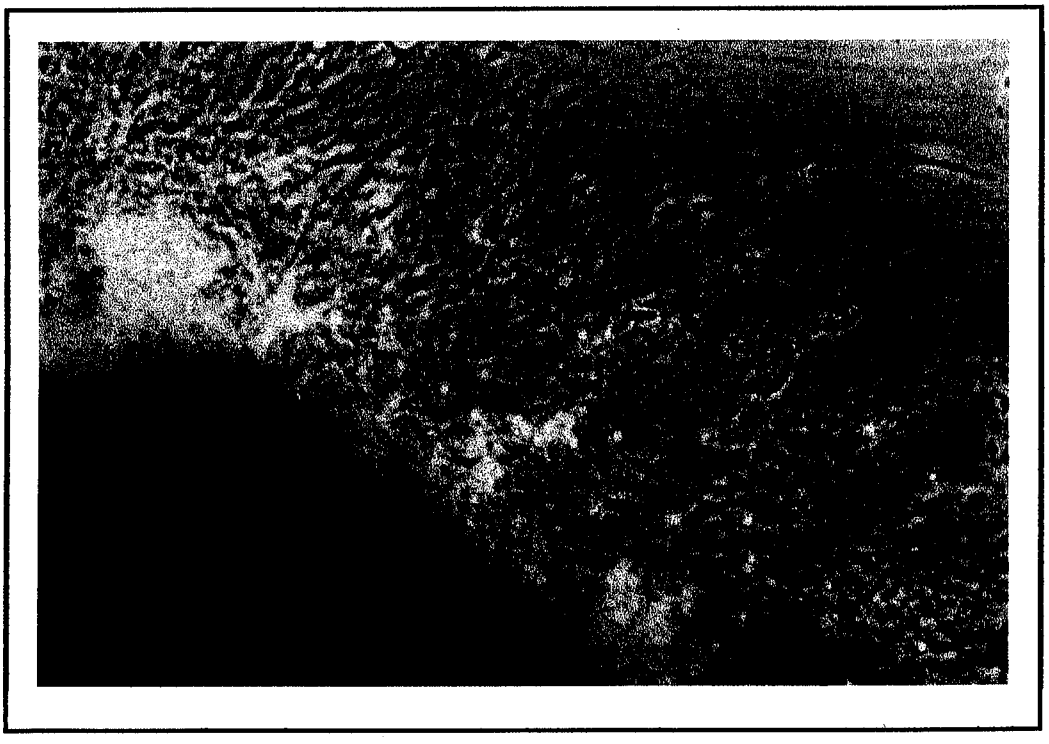

Plate 3 **Lymphoid Cell Culture - 4 days old (10X magnification)**

Table No. 2: Effect of media with pH 7.6, Osmolality (450 mmol/kg) on Cell Confluence

Media	% of confluence
L-15	70
Medium 199	35
EMEM	20

Table No. 3: Effect of Osmolality (mmol/kg) of Media (L-15) on Cell Confluence

Osmolality (mmol/kg)	% of confluence
350	60
450	75
550	60
650	40

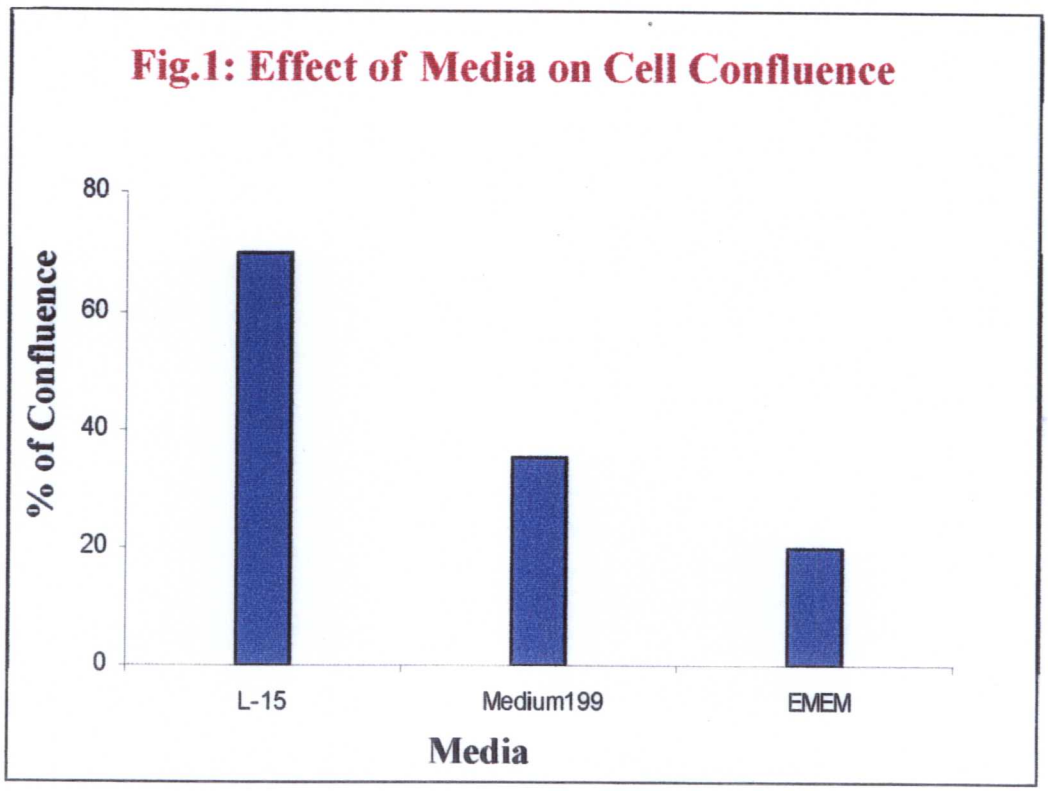
Fig.1: Effect of Media on Cell Confluence
% of Confluence
80
60
40
20
0
L-15
Medium199
EMEM
Media

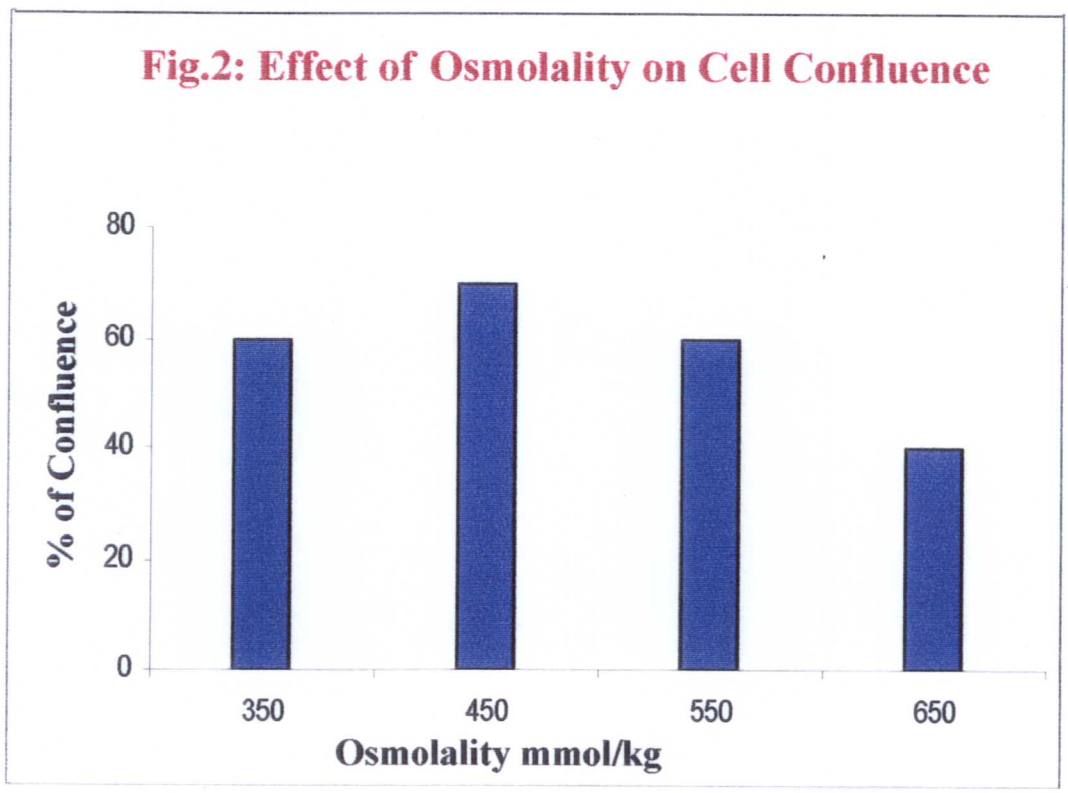
Fig.2: Effect of Osmolality on Cell Confluence
% of Confluence
80
60
40
20
0
350
450
550
650
Osmolality mmol/kg

Table No. 4: Effect of pH of the media (L-15) on Cell Confluence

pH	% of confluence
7.5	35
7.8	75
8.0	70
8.2	40

Table no. 5: Effect of Temperature on Cell Confluence

Temperature (^{0}c)	% of confluence
24	50
28	78
32	60
37	55

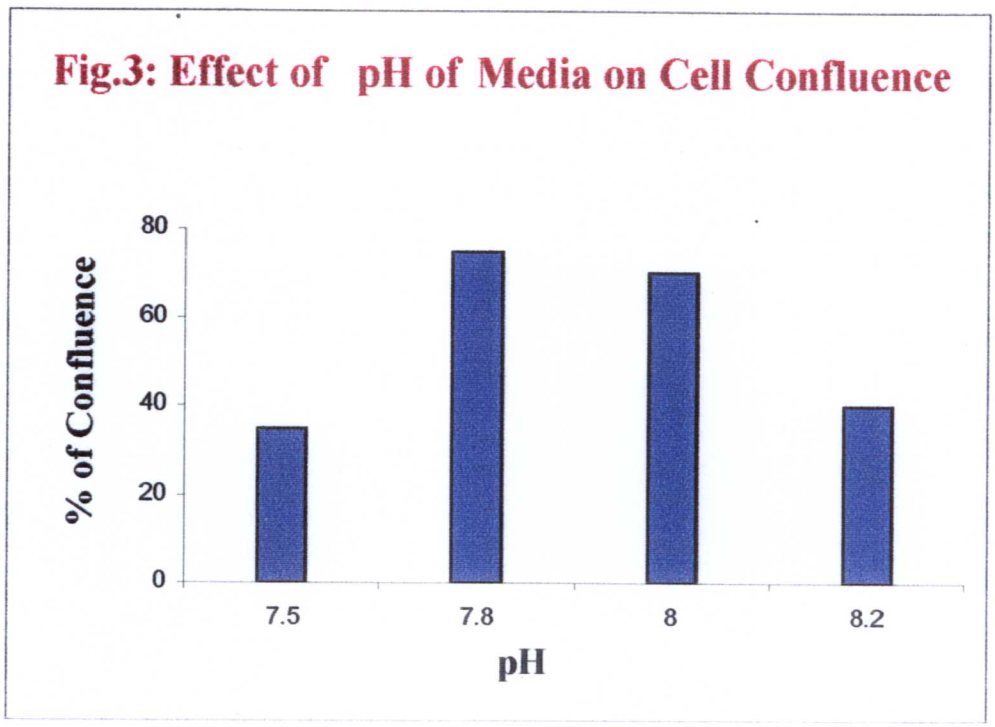
Fig.3: Effect of pH of Media on Cell Confluence
% of Confluence
80
60
40
20
0
7.5
7.8
8
8.2
pH

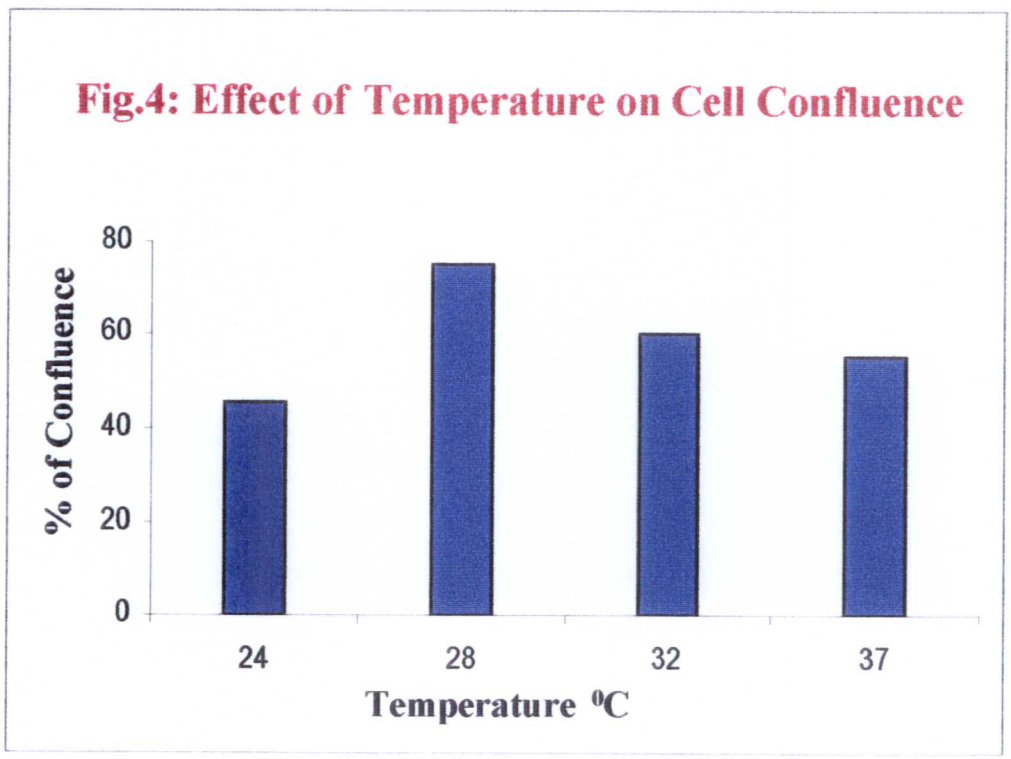
Fig.4: Effect of Temperature on Cell Confluence
% of Confluence
80
60
40
20
0
24
28
32
37
Temperature 0C

Table No. 6: Effect of Foetal Bovine Serum (FBS) on Cell Confluence

Foetal Bovine Serum	% of confluence
5	35
10	40
15	75
20	75
25	75

Table No. 7: Effect of Epidermal Growth Factor on Cell Confluence

Epidermal Growth Factor (EGF)	% of confluence
5µl	65
10 µl	67
15µl	70
20µl	75
25µl	65
30µl	60

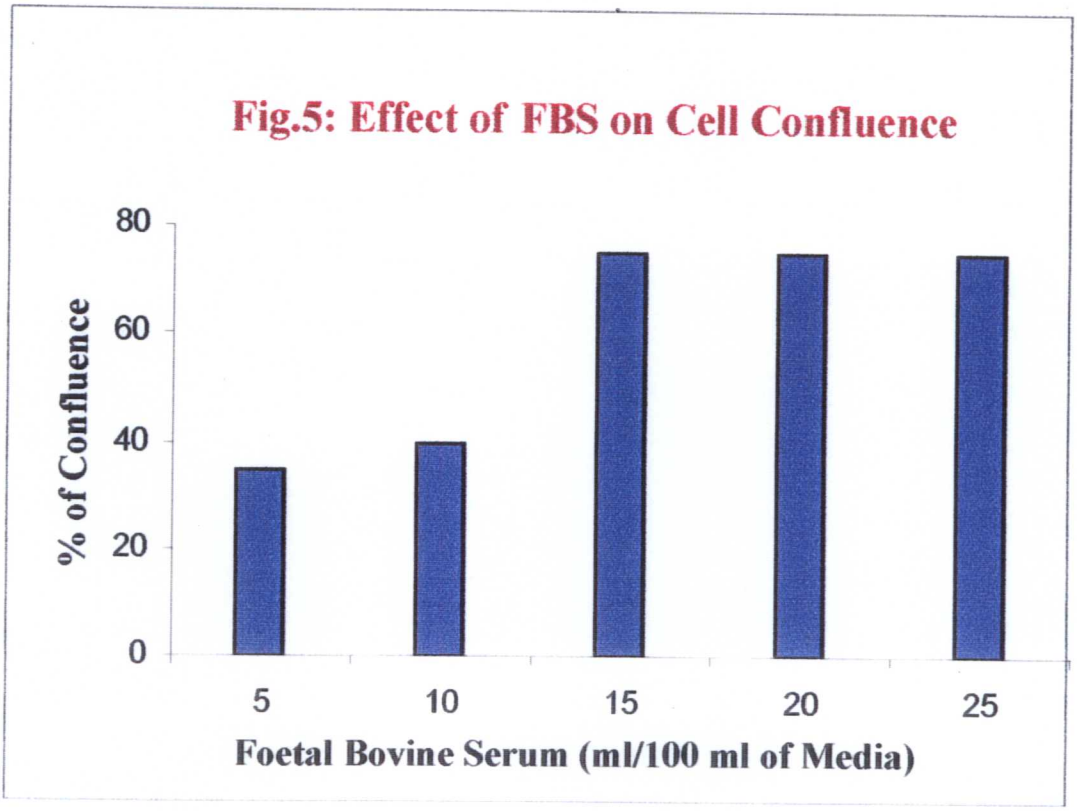
Fig.5: Effect of FBS on Cell Confluence
% of Confluence
80
60
40
20
0
5
10
15
20
25
Foetal Bovine Serum (ml/100 ml of Media)

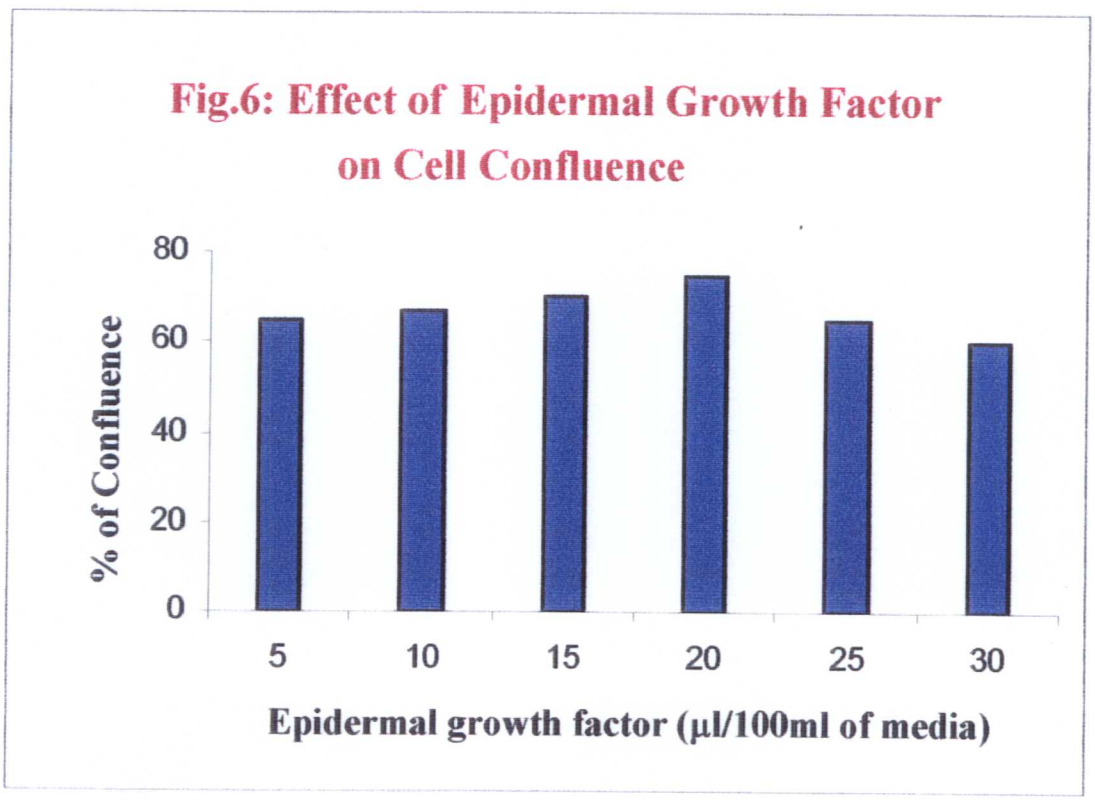
Fig.6: Effect of Epidermal Growth Factor on Cell Confluence
% of Confluence
80
60
40
20
0
5
10
15
20
25
30
Epidermal growth factor (µl/100ml of media)

Table No.8 Cell growth in the media (L-15) having pH 7.61 Osmolality 450 (mmol/kg), FBS 20%, EGF 20µl and 8% of prawn extract

Days	% of confluence
1	25
2	40
3	55
4	75
5	75
6	60
7	45

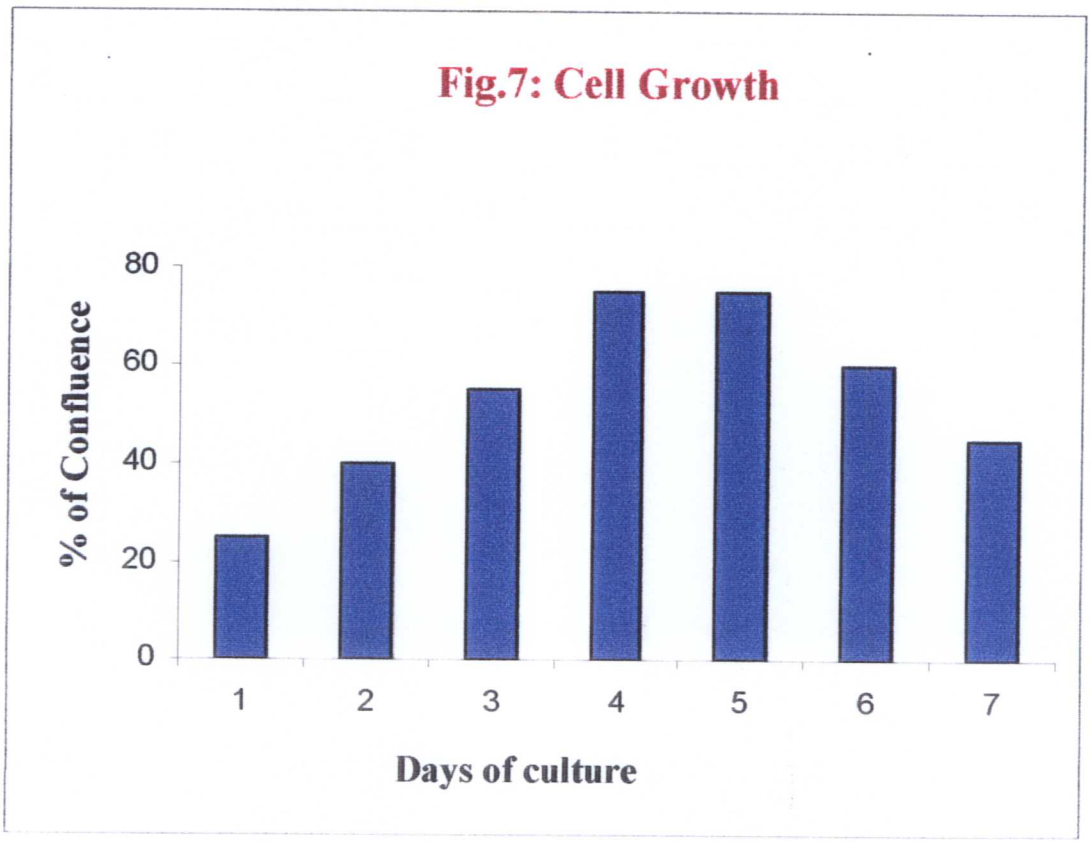
Fig.7: Cell Growth
% of Confluence
80
60
40
20
0
1
2
3
4
5
6
7
Days of culture

DISCUSSION

5. DISCUSSION

Aquaculture, a growing industry in many parts of the world, is geared to meet the increasing demand for aquatic products. Concomitant with the rapid expansion of aquaculture, occurrence of disease became wide spread. Viral diseases are suspected in several outbreaks of diseases in India, which, however are not studied in detail. Cell cultures are of immense importance where diagnosis of unexplained mortalities of prawn is needed. In the present study, conditions for the successful primary lymphoid organ culture of *M.rosenbergii* have been established for that commercially available three basal media, supplements and additives were evaluated for attachment, growth in the culture.

In the present study three commercially important media were tested for their support to attachment and growth of the lymphoid cells of *M. rosenbegii* in vitro. The media L-15, M 199 and EMEM, which are quite different in the number and proportion of their components, did not have similar effect in the same species. L-15 supported the growth better than the other two media. These results are in accordance with the studies of Freshney (1983) in mammalian fibroblasts and in shrimp cell cultures (Leudman and Lightner, 1992 and Chen *et al.*, 1986) (Table 2 and Fig.1)

Media composition was important in the ultimate success of cell culture, other factors also significant. In addition to the components of the media supplements, pH, osmotic pressure and maintenance temperature have a dramatic effect on cell growth. When, Foetal bovine serum is added to media as a supplement at the rate of 15% gave the good results. It was

similar to that of shrimp lymphoid cell culture by Philip (1997). Hsu (1995) obtained good results with 10% FCS in the culture of lymphoid organ of *P. monodon*. Prawn muscle extract containing growth factors had a positive influence on cell growth, at the rate of 8% was used. This is an agreement with Tong and Maio (1996) found 10% muscle extract in conjuction with 20% FBS enhanced cell growth and disagreement with the finding of muscle extract was not necessary for confluent growth (Luedman and Lightner, 1992).

The optimal osmolality for the maintenance of prawn lymphoid cells was found to be at 450mmol/k (Table 3 and Fig. 2). This result was similar to that of embryonic tissue of *M.rosenbergii* cell line cultures (Frerichs, G.N, 1996) and is different that of penaeid shrimp cells (Chen *et al.*, 1999). The optimal maintenance temperature was found at 28^0C, which vary according to the species, it was found 25^0C for tissues of *P. japonicus* and 28^0C for tissues of *P. monodon* (Chen and Wang, 1999). The pH of 7.8 was given good results, is very similar to that of mammalian fibroblasts (Freshney, 1983) and to the primary cell culture to the shrimps (Chen et al., 1986, Luedman and Lightner, 1992), but slightly that found in insect cells (Grace, 1992). EGF at the rate of 20µl per 100ml. of growth medium enhanced the growth rate (Table 7 and Fig.6)

Cell cultures are liable for contamination and is the main obstacle for successful development of a cell culture (Wolf and Quimby, 1969; Sathe etal, 1995). Surface of prawn, is constantly bathed in water and thus harbours plenty of microbes and parasites. If not removed these can get into culture. So, prawn was held in clean water for several days prior to

removal of organ. Further anti microbial chemicals like $KMno_4$, bleaching powder and antibiotics were used which proved to eliminate the externa microbes.

The growth medium supported the multiplication of cells upto 4 days only and further studies are necessary to extend the life of the cells and their ability to multiply.

In the present study primary culture were established from lymphoid organ of *Macrobrachium rosenbergii* could be used as a convenient bioassay tool. In conclusion, methodologies are yet to be developed with regard to the basal media and characterization with regard to karyotyping viral sensitivity, isoenzyme pattern etc is needed for successful development of cell culture.

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* Not Seen in Originals

Appendix

The composition and preparation of media and reagents employed in the study are given as follows.

Brain Heart Infusion (BHI) Agar

Ingredient	Grams/Litre
Calf brain infusion	200.00
Beef extract, infusion form	250.00
Proteose peptone	10.00
Dextrose	2.00
Sodium	5.00
Di-sodium phosphate	2.50
Agar	15.00

To 3.7gms of dehydrated medium 100 ml. of distilled water was added and boiled for few minutes and pH adjusted to 7.4 before sterilization by autoclaving at 121°C, 15 lbs pressure for 20 min.

BUFFER

Phosphate buffer saline (PBS)

NaCl	8.0g
NaH_2PO_4	1.44g
KH_2PO_4	0.2g
KCl	0.2g
Triple Distilled water	1000ml
pH	7.2