Development of Lymphoid Cell Culture System from *Penaeus monodon* and Molecular approaches for its Transformation

Thesis submitted to the

Cochin University of Science and Technology

In partial fulfillment of the requirements

for the award of the degree of

DOCTOR OF PHILOSOPHY

in

ENVIRONMENTAL BIOTECHNOLOGY

Under the Faculty of Environmental Studies

By

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KOCHI 682016, KERALA, INDIA

September 2012

Certificate

This is to certify that the research work presented in this thesis entitled ***"Development of lymphoid cell culture system from Penaeus monodon and molecular approaches for its transformation"*** *is based on the original work done by Mr. Jayesh P under my guidance, at the National Centre for Aquatic Animal Health, School of Environmental Studies, Cochin University of Science and Technology, Cochin-682 016, in partial fulfillment of the requirements for the award of the degree of* ***Doctor of Philosophy*** *and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.*

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Declaration

I hereby do declare that the work presented in this thesis entitled ***"Development of lymphoid cell culture system from Penaeus monodon and molecular approaches for its transformation"*** *is based on the original work done by me under the guidance of Dr. I.S. Bright Singh, Professor, School of Environmental Studies, and Coordinator, National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin-682 016, and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.*

Cochin 682 016
September 2012

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Acknowledgements

It is a pleasant task to express my sincere thanks to all those who contributed and supported for the successful completion of this thesis.

I express my deep and sincere gratitude to my supervising guide Prof. (Dr.) I.S. Bright Singh for conceptualization and implementation of this very tough research topic, in addition to his peerless guidance, motivation and patience all the way through my doctoral research. I was extraordinarily fortunate in having the opportunity to work on this frustrating and the most challenging research area on shrimp cell line development under his guidance. I thankfully acknowledge the quality of my mentor to transform the worst and frustrating mind to a mind blooming with confidence and creativeness through his academic and emotional guidance over the years for the accomplishment of this mission. Now, I strongly believe that "everything happens for a reason", and his encouragement, moral support, caring, and the endless freedom I enjoyed, changed my easygoing mind to a warrior, to fight with the hurdles at the toughest 'scientific battle' to achieve goals for the benefit of the society. Dear Sir, your valuable contributions in every turning point in my academic life will always be treasured and I look forward to an exciting acquaintance in the future.

I thankfully acknowledge Dr. Mohankumar, Dean, Faculty of Environmental Studies, for all the support provided in the smooth conduct of my research.

I sincerely place on record my thanks to Prof. Ammini Joseph, Director, School of Environmental Studies for facilitating my research under the Faculty of Environmental Studies and for all the support rendered since my post graduation.

My warm thanks are due to Prof. (Dr.) Mohandas for his kind support, motivation and critical comments during this study. He was approachable at any time and was open to valuable discussion on any matter and I will cherish his fatherly affection forever.

Words fail me to express my thankfulness to my dear teacher, Dr. Rosamma Philip for the affection she showered, generous care and the 'thought provoking talk over a cup of tea'.

She was always there when I really needed a 'Crazy discussion', and her ideas and concepts have remarkably influenced me. I warmly thank for her excellent guidance for in silico gene analysis.

I owe my deep gratitude for a life time to all my teachers of the past and present, Dr. V.V.N Kishore (TERI University), Prof. Yesodharan, Dr. Suguna Yesodharan, Dr. S. Rajathy, Mr. Anand, Dr. Harindranathan Nair, Dr. V. Sivanandan Achary, Dr. R. Indira Nair, Dr. Johny Joseph, Dr. Ramakrishnan Palat, Mr. Radakrishnan and Mr. Balakrishnan for the way they molded my life to what I am today.

I gratefully acknowledge Dr. Manuel Serrano, Howard Hughes Medical Institute, Cold Spring Harbor, New York, for providing me the oncogene for the study through addgene, USA; Dr. Arun K. Dhar, Viracine Therapeutics Corporation, USA, for IHHNV promoter; Dr. R.B Narayanan, Anna University for baculovirus expression system and Dr. Swetha Vikash, Dr. Vikash for GFP vectors.

For the successful completion of any research the financial support is most valuable and I gratefully acknowledge the financial support from the Department of Biotechnology, Government of India, New Delhi, through the project BT/PR 8050/AAQ/03/289/2006 "Identification and removal of blocks on in vitro transformation and establishment of permanent cell lines from Penaeus monodon". I acknowledge the financial support in the form of Junior and Senior Research Fellowships.

The road to my Ph. D started at the National Centre for Aquatic Animal Health (NCAAH) and I am fortunate to have been a part of NCAAH family. I deeply appreciate the state-of-the-art facilities and the research ambiance at this Centre, where my research life of an "aquaphobic turned to an aquanaut".

I thank the Cochin University of Science and Technology for providing me the fellowship (UJRF), excellent library, high speed internet connection and valuable online access to journals and database and for all academic and administrative support provided for the smooth conduct of the study.

Thanks to the administrative staff of the School of Environmental Studies, for their timely help and support, especially, the support rendered by Mrs. Girija, Assist. Registrar, CUSAT (former Section Officer of the school).

Sincere gratitude to my friends overseas Mr. Dileep, Mr. Jiby K Kuryan and Mr. Linoj Kumar (Canada), Mrs. Laiby Paul (Belgium), and Mr. Rajesh (USA), for their support in providing me scientific journals and ebooks.

I express my thanks to Ms. Jiya (NIO, Cochin) Mr. Saji, (STIC – CUSAT) and Scientists at CIFT, Cochin, for the great help in the analysis of samples.

I thank the management and staff M/s Abad hatchery, Cochin and Mr. Sudhakaran, Kodungallore for supplying me the animals for experimentation.

This thesis has been kept on track and been seen through to completion with the support and encouragement from my well wishers, friends and colleagues.

I am much indebted to my friend, Ms. Priyaja, whose care, understanding, untiring support and motivation helped me a lot especially during my hard times with the 'issues' at work. I would have not been in this realm, if I couldn't get such a great friend during my times of hardship. I owe to her for being an understanding friend adjusting to my irritating and bursting character. She was always beside me during the happy and hard moments to push me and motivate me for the successful completion of my research work. She made my voyage through molecular biology smooth and possible.

Words are short to express my thankfulness to my dear chechi, Dr .Valsamma Joseph for the affection she showered, generous care and the calm support in my times of 'confusion'. She was always there when I really needed and her 'philosophical thoughts' and concepts have remarkably influenced me.

I sincerely thank Dr. Sajeevan for his suggestions, encouragement and warm friendship.

Thanks to Dr. Seena Jose for sharing my peaks of convulsion in shrimp cell culture. Her calm and peaceful face and optimistic nature 'down regulated" my frustration and anger.

From the 'untold sufferings' in shrimp cell line development, she prepared me to face the disappointment which had happened during the hard time of cell line research.

Thanks to Mrs. Archana K Nair, project colleague, for the support rendered for me at the beginning of this work.

My friend, Ms. Vrinda deserves special mention for being a good friend and for her love and moral support. She supported me a lot in solving the toughest problems in 'TRAP assay for identifying telomerase activity.

I treasure the friendship and loving inspiration and 'dewy-eyed innocence' of Ms. Ann Rose Bright. Her 'childly charm' turned my trying times to times of life with unforgettable 'mouth-watering and spicy' moments. I extend my thanks to her for the critical comments on designing the cover page of my thesis.

I am indebted my 'sporty' junior Mr. Christo, for being a little brother to me and for his ceaseless love, care and support.

I extend my huge, warm thanks to Dr. Rejish for his brotherly affection, concern and love. Thanks to my 'scientist senior' Dr. Anas Abdulaziz, for his constructive criticism that prepared me to face the 'scientific issues'. Heartfelt thanks to Dr. Somnath Pai, for the healthy debate on thought provoking topics during my 'scientific hours' at lab. I appreciate and thank my roommate and senior colleague Dr. Sreedharan for his caring friendship and help especially at the beginning of my research life. My sincere thanks to Mr. Haseeb for his affection, friendship and for all the support rendered to my entire doctoral life. My thanks are due to Dr. Sudheer for the valuable suggestions.

I am thankful to the 'big shots', our past and present post doctoral fellows Dr. Jasmine, Dr. Swapna P Antony, Dr. Sabu, for their suggestions and encouragement. My sincere and loving thanks to all my dear doctoral colleagues Mr. Sunish, Dr. Gigi Paulose, Mr. Ranjit Kanjur, Mr. Deepesh, Ms. Rose Mary Jose, Dr. Divya Jose, Mrs. Surekha, Mr. Prem Gopinath, Mrs. Riya, Mrs. Ammu, Mrs. Deepa and Mrs. Preena, for being with me and their

support. A special thanks to Mrs. Sreelakshmi, for all the 'support and special care' at the beginning of my doctoral life that helped me to learn lessons on 'human management'.

I wish to extend my warmest thanks to the new members of NCAAH family, Dr. Gopalakrishnan, Dr. Divya, Mrs. Remya, Ms. Asha, Ms. Jisha, Ms. Sanyo, for their support and friendship.

I would also like to thank M.Tech students Mr. Mathews Varkey, Mr. Linu Balan, Mr. Arka Saha, Mr. Mantosh Kumar, Mr. Sourav Parkashi, for their friendship.

I specially thank my M. Sc Classmates Mr. Rajesh, Mr. Haneesh Panicker Mr. Surendran, Ms. Nisha Yesodharan, Mrs. Divya, Mrs. Anu, Ms. Sathya, Ms. Poornima, Mrs. Vidhubala, and my PG seniors Mr. Saiju Sasidharan, Mr. Ganesh, Mr. Basheer, for being with me and for their friendship and suggestions. Cheers to my juniors Mr. Maneesh, Mr. Jiby, Mr. Dileep, Mr. Tijo, Mr. Rakesh, Mr. Sanoop…, for their love, care and untiring support.

Special thanks to my Delhi friend Mr. Biju Narayan for his love and encouragement. Thanks are extended to Ms. Neena Jose, for her friendship.

I extend my thanks to all NCAAH staff, Mr. Soman, Mrs. Surya, Mr. Jaison, Mr. Biju, Mr. Anish, Kusumam chechi, and Mrs. Parisa for their support given during my research. Many thanks to Ms. Blessy Jose for the endless support she offered during my hard time of research.

I sincerely thank all the research scholars of School of Environmental Studies for their help and friendship. Thanks to Mr. Arun Augustine, Mr. Abesh Reghuvaran, Mrs. Kala Jacob, Mrs. Sareen, for their support and help.

The credit to the style of this thesis goes to Mr. Syam, Indu Photos and I thank him for putting excellent professional touch.

I fondly cherish the love, care and support of my hostel mate's and friends, Mr. Prajith K.K, Mr. Phiros sha, Mr. Krishnamohan, Mr.Gireesh, Mr. Vijay, Mr. Vipin, Mr. Shaiju, Mr. Anil kumar, Mr. Anit, Mr. Harisankar, Mr. Haris, Mr. Sarin, Mr. Solly Soloman, Dr. Prabhakaran, Mr. Akhilesh, Mr. Akhil, Mr. Raghul……and it goes on…. Special thanks to

my degree friends Mr. Arun T.K, Mr. Bineesh, Mr. Prasanth, Mr. Shiju, Mr. Shefi Shukur, Mr. Arun K, Mr. Shijil, and Mr. Haroon…, for their friendship.

And most of all, I would like to share this moment of happiness with my loving, supportive, encouraging, family where the most basic source of my life energy resides. The never-ending support of my Pappa (Chandrasekharan), Amma (Sobhana) and sisters (Priya and Maya) has been unconditional all these years and without their encouragement, prayers and understanding it would have been impossible for me to finish this work. I very fondly acknowledge my brother-in-laws Mr. Satheesh and Mr. Rajesh, and my little ones Sayooj (Appu), Sanjay (Tuttu), Karthik (Nandu), and Hrishikesh (Sachu) for their love and support. I sincerely thank all my relatives for their love and encouragement…...

This thesis is dedicated to all my teachers and friends……

Jayesh P.

Appendix

Contents

Chapter 1

General Introduction

Animal cell culture, the concept of growth and maintenance of cells *in vitro* in a nutrient medium, started way back in 1907, when Harrison (Harrison, 1907) "Father of animal tissue culture", succeeded in growing nerve tissue of frog in lymph clots. Three years after, Burrows (Burrows, 1910) cultured chick-embryo tissue, and in 1943 Earle *et al*. (1943) succeeded in developing primary cell culture from mouse fibroblast. All these success stories led to the development of the first continuous human cell line (HeLa) in 1952 (Gey *et al*., 1952). These events generated a new wave of interest in cell and tissue culture research, and a new field of investigation was opened with an explosive expansion in biological sciences, during the second half of 20th century (Freshny, 2000). Today, human cell cultures, for that matter vertebrate cell lines, have emerged as one of the most powerful tools to address many fundamental questions in biology and medicine (Claydon, 2009). In contrast, development of *in vitro* models for invertebrates (especially marine invertebrates), which contribute to 95% of the animal kingdom (Ruppert and Barnes, 1994), is far less advanced due to elusive biological reasons. In this context, this chapter summarizes the advancements in cell culture development from crustaceans with the focus on the perception and orientation in shrimp cell culture, towards the attainment of stable cell lines.

1.1. Crustacean cell culture

Crustacea constitute a class of animal species of biological interest for fundamental research and/or of high commercial value (Toullec, 1999). According to Johnson *et al*. (2008) a decapod crustacean, shrimp, represents one of the most economically important aquaculture species with a value of over 10 billion US $ annually. Unfortunately, in the mid 1990s the shrimp industry was struck with the white spot virus (WSV) leading to an economic loss of over 3 million US $ on annual basis (Lundin, 1995), and over 40% of world shrimp culture was affected (Lundin, 1995; Lotz, 1997). Even though enormous literature has been generated on WSV, understanding of the viral morphogenesis and development of an appropriate therapy and prophylaxis could not be accomplished due to the absence of a permanent cell line from shrimp or from a susceptible crustacean. Besides, such cell lines shall serve as excellent tools in toxicology as well. In addition, it can certainly be argued that there is a real need for development of *in vitro* techniques for aquatic invertebrate cell culture to ease pressures on wild stock to optimize growth condition (Mothersill and Austin, 2000). The published report on cell culture by Quiot *et al*. (1968) is considered as the first active long-term cell culture from a crustacean (Mothersill and Austin, 2000).

Despite the absolute requirement of a permanent *in vitro* model from this group of animals (Spann and Lester, 1997), till date, no authentic and reproducible cell culture system, from any aquatic invertebrate has been reported. Further, while comparing with mammalian cell culture techniques, culture methods available for invertebrates are under developed, even for the maintenance of primary cell cultures *in vitro* (Mothersill and Austin, 2000). This is mainly because specific culture media for crustacean cells have not been developed (Toullec, 1999), for the formulation of which it is imperative to consider biochemistry of various species and the requirement of each cell/tissue type *in vitro*.

1.2. Cell culture from shrimp: an economically important crustacean

Development of continuous shrimp cell lines has ever been a challenging task, for a long period of over 25 years (Jayesh *et al.*, 2012). However, it still remains unattained presenting researchers more questions than answers (Chen *et al.*, 1986; Owens and Smith, 1999). The *in vitro* cell culture system helps to analyze the inter-related environmental and pathogenic factors that interact with genetic and physiological traits of the cultured animals and to acquire knowledge for health protection and disease management in aquaculture (Villena, 2003). Moreover, primary cell cultures obtained from various organs / tissues represent the first step towards the establishment of cell lines and they provide useful information concerning the most suitable cell culture conditions involved in the survival and proliferative capacity of various tissues (Toullec, 1999). However, as on today, no permanent cell line could be made available from marine invertebrates in general (Rinkevich, 2011) and shrimp in particular (Jayesh *et al.*, 2012). The major fall out of the situation is the impediment which it imposes on the isolation of crustacean viruses (Claydon, 2009; Jose, 2009; Claydon *et al.*, 2010b). The fact is that the requirement of continuous cell lines is so high to investigate the radiating viral threats to shrimp aquaculture (Flegel, 2006; Walker and Winton, 2010; Zwart *et al.*, 2010).

In the realm of cell line development, despite the current advancements in decoding the nutritional requirements of cells *in vitro*, molecular approaches at genomic level for transformation and immortalization of shrimp cells remain unknown and un-attempted. This might be due to the lack of information on the molecular mechanisms that inhibit neoplastic transformations in shrimp. Besides, tumours have only rarely been observed in the decapod crustaceans (Vogt, 2008). Therefore, a thread bear analysis on the very successful history of insect and mammalian cell line development might open up new vistas for focused research towards establishment of shrimp cell lines. Moreover, uncovering the underlying molecular and regulatory mechanisms of the absence of neoplasia and carcinoma in

shrimps might provide new leads for the development of anti-ageing and anti-cancer interventions in humans as well (Vogt, 2011).

1.2.1. The history of shrimp cell culture

The earliest attempts on shrimp cell culture development appeared as published document in 1986 by Chen and colleagues from National Taiwan University, Taiwan (Chen *et al*., 1986). They had chosen *Penaeus monodon* as the species of choice from which several cell culture systems could be generated using various tissues and organs. Three years after the first publication in shrimp cell culture, in 1989, researchers (Chen *et al*., 1989) published an attempt of shrimp cell culture from *P. penicillatus* and on the same year first report on the susceptibility of primary lymphoid cell culture to monodon-type baculovirus was published (Chen and Kou, 1989). This is considered as the first report on *in vitro* cultivation of penaeid virus in shrimp cell culture. Even though only limited success could be obtained, several researchers commenced attempting to develop cell cultures from various tissues and organs of different species of penaeids (Ke *et al*., 1990; Rosenthal and Diamant, 1990; Luedeman and Lightner, 1992; Nadala *et al*., 1993; Ghosh *et al*., 1995; Hsu *et al*., 1995; Tong and Miao, 1996; Sano, 1998; Itami *et al*., 1999; Kasorchandra *et al*., 1999; West *et al*., 1999; Kumar *et al*., 2001; Fan and Wang, 2002; Chun-Lei *et al*., 2003; Maeda *et al*., 2003), and this included test of their susceptibility to shrimp viruses as well (Lu *et al*., 1995a; Maeda *et al*., 2004; Jiang *et al*., 2005). In 2000, report on the ultra structure of white spot syndrome virus (WSSV) grown in primary lymphoid cell culture was published (Wang *et al*., 2000), however, its morphogenesis could not be fully elucidated for want of certified shrimp cell lines. To date the morphology and ultrastructure of WSSV have not been fully understood, however, several characteristics of this virus have emerged in recent years (Sa´nchez-Paz, 2010). In addition to the effort on spontaneous transformation and immortalization by continuous maintenance and repeated passage of the cells *in vitro* and the 'organized neglect' (Grace, 1982) in

the process of cell culture development, in the year 1995 researchers attempted to induce transformation in shrimp cells by transfection with oncogene (Tapay *et al*., 1995). Accordingly, in 2000 first transgenic expression in shrimp cells could be accomplished (Shike *et al*., 2000a) followed by the development of vesicular somatitis virus – glycoprotein (VSV-G) pseudotyped retroviral vectors (Hu *et al*., 2008) and their successful integration in shrimp primary cell culture genome (Hu *et al*., 2010). However, this also did not lead to immortalization of cell cultures. The lack of success in spontaneous and induced cell line development subsequently paved the way for the attempts on developing fusion cell line (Claydon, 2009; Claydon *et al*., 2010b) that too, with little success. More recently, researchers succeeded in viral gene expression (Jose *et al*., 2010), determinations of cytotoxicity and genotoxicity (Jose, 2009; Jose *et al*., 2011), viral multiplication (George *et al*., 2011), and immune gene expression (Jose *et al*., 2012) employing primary cell culture systems developed from different species of penaeids.

1.2.2. Animal species used in shrimp cell culture trials - a major concern

Since the first attempt on shrimp cell line development, performed in 1986 by Chen *et al*. (1986), *P. monodon* remained the best sought-after candidate species among all penaeids in the development of cell cultures; may be due to its availability in all South East Asian Countries and its popularity as the most widely cultured species. Of the 50 selected publications, 17 reported (34%) *P. monodon* (Chen *et al*., 1989; Hsu *et al*., 1995; Chen and Wang, 1999; Fraser and Hall, 1999; Kasornchandra *et al*., 1999; West *et al*., 1999; Wang *et al*., 2000; Manohar *et al*., 2001; Roper *et al*., 2001; Uma *et al*., 2002; Assavalapsakul *et al*., 2003, 2005, 2006; Catap and Nudo, 2008; Claydon *et al*., 2010b; Jose *et al*., 2010, 2011), as the species of choice, eight researchers (16%) used *P. japonicus* (Machii *et al*., 1988; Sano, 1998; Chen and Wang, 1999; Itami *et al*., 1999; Lang *et al*., 2002a, 2004; Maeda *et al*., 2003, 2004), seven (17%) selected *P. chinensis* (Tong and Miao, 1996; Huang *et al*., 1999; Fan and Wang, 2002; Chun-Lei *et al*., 2003; Jiang

et al., 2005; Hu *et al*., 2008, 2010), and *P. vannamei* (Ellender *et al*., 1992; Luedeman and Lightner, 1992; Nadala *et al*., 1993; Lu *et al*., 1995b; Toullec *et al*., 1996; George and Dhar, 2010; George *et al*., 2011). Moreover, six authors (16%) selected *P. stylirostris* (Luedeman and Lightner, 1992; Nadala *et al*., 1993; Tapay *et al*., 1995; Lu *et al*., 1995a; Shike *et al*., 2000a; Shimizu *et al*., 2001), as the donor animal of tissues and organs. Besides, in two publications (4%) *P. indicus* (Toullec *et al*., 1996; Kumar *et al*., 2001) and *P. aztecus* (Ellender *et al*., 1992; Najafabadi *et al*., 1992) were the species used. There is only one report (2%) of using *P. penicillatus* (Chen and Wang, 1999), for extracting tissues and organs for cell culture development (Fig. 1). This indicated that the species selection was based on availability and personal choice and not on the basis of any advantage which one might obtain by selecting a species.

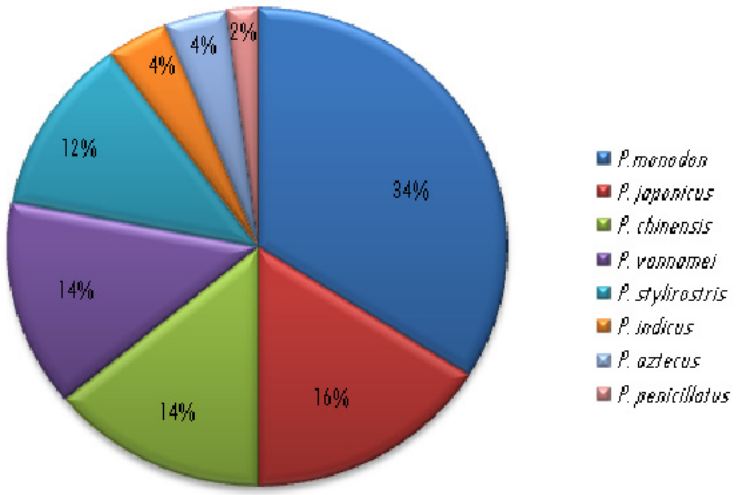


Fig.1. Graphical representation of trends in selection of penaeid species used for cell culture development (% of the 50 selected publications)

1.2.3. *Penaeus monodon* an economically important penaeid shrimp

More than 360 species of finfish and shellfishes are being cultured worldwide and around 25 of them are of high value and traded globally (FAO, 2010). A successful harvest has always been very much encouraging, and this has

spurred the expansion of aquaculture production in terms of area and geographical range. Shrimp continues to be the largest single commodity in terms of value, accounting for 15 percent of the total fishery products traded internationally (FAO, 2006), and *P. monodon* (Fig. 2) is the highly preferred penaeid species (Pechmanee, 1997), in South East Asian countries including India (Sudheer, 2009). Meanwhile, intensive aquaculture practices globally, since 1990, paved the way for the spread of shrimp viral diseases resulting in severe damage to the industry (Bachère, 2000; Valderrama and Engle, 2004). However, according to 'FAO Status of World Fisheries and Aquaculture, 2010,' in the year 2008, the capture fisheries and aquaculture production of decapods was 10,230 tonnes, corresponding to 41 billion US $ (FAO, 2010; Vogt, 2011). This trend in production is unlikely to perpetuate, because there are more than 20 (Bonami, 2008) among the 1100 recognized invertebrate viruses (Adams, 1991) now known to occur in shrimps which include nine that pose serious threat to their culture (Flegel, 2006; Claydon *et al.*, 2010a; Walker and Winton, 2010; Lightner, 2011). Altogether, considering the emerging viral threat on this economically important food commodity, development of a permanent shrimp cell line to bring out effective strategies to combat the viruses is pivotal.

Fig.2. Adult *Penaeus monodon*

1.2.4. Most commonly used medium for shrimp cell culture

Despite the necessity of an exclusive medium for shrimp cell culture several researchers, over decades, have been modifying commercially available media to suit the requirements of shrimp cells *in vitro* (Roper *et al*., 2001; Claydon, 2009; Jose, 2009). Among the commercial media used, Leibovitz's – 15 (L -15) has been the most popular one for shrimp cell culture. Of the 50 selected publications 32 (64%), were based on L-15 as the basal medium (Chen *et al*., 1986, 1988, 1989; Fuerst *et al*., 1991; Ellender *et al*., 1992; Najafabadi *et al*., 1992; Nadala *et al*., 1993; Lu *et al*., 1995; Tapay *et al*., 1995; Tong and Miao, 1996; Toullec *et al*., 1996; Mulford and Austin, 1998; Chen and Wang, 1999; Shike *et al*., 2000a; Wang *et al*., 2000; Kumar *et al*., 2001; Manohar *et al*., 2001; Roper *et al*., 2001; Shimizu *et al.,* 2001; Uma *et al*., 2002; Chun-Lei *et al*., 2003; Jiang *et al*., 2005; Assavalapsakul *et al*., 2003, 2005, 2006; Catap and Nudo, 2008; Hu *et al*., 2008; Claydon *et al*., 2010b; Jose *et al*., 2010, 2011, 2012), six (12%) selected Grace's Insect Medium (Luedeman and Lightner, 1992; Nadala *et al*., 1993; Toullec *et al*., 1996; Wang *et al*., 2000; George and Dhar, 2010; George *et al*., 2011), five (10%) M199 (Ghosh *et al*., 1995; Toullec *et al*., 1996; Itami *et al*., 1999; Shimizu *et al*., 2001; Lang *et al*., 2002b), and three (6%) MPS (Tong and Miao, 1996; Fan and Wang, 2002; Hu *et al*., 2010). A couple of other media such as Pj-2 (Machii *et al*., 1988), NCTC 135 (Wang *et al*., 2000), MM Insect medium and TC 100 medium (Nadala *et al*., 1993) were also tested for the development of cell lines from shrimp (Fig. 3). However, it was rather inappropriate to point out any medium mentioned above as the most effective one as it has been a personal choice. This highlights the importance of a new medium exclusively for shrimp cell culture.

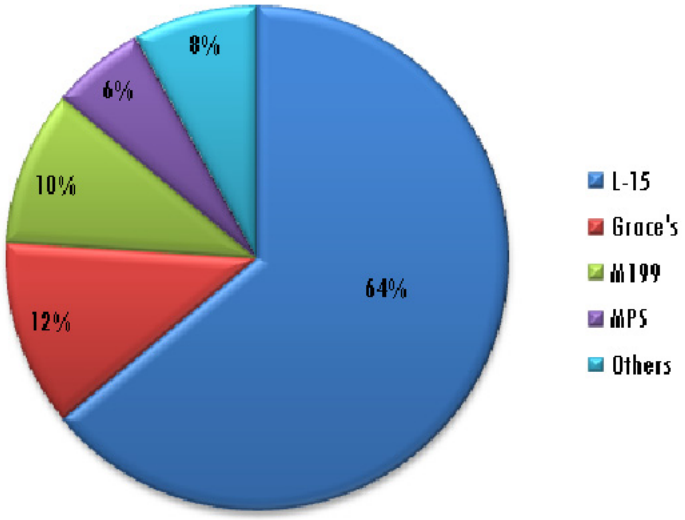


Fig.3. Graphical representation of trends in selection of growth media used for shrimp cell culture (% of the 50 selected publications)

1.2.5. Organic and inorganic supplements added to improve growth of shrimp cells *in vitro*

Considering the inadequacy of the available growth media several attempts have been made to improvise the composition by adding supplements in isolation as well as in multiples. Several investigators selected crustacean body fluids and extracts for improving the basal medium. Among them shrimp extract was the popular one with varying concentrations such as 4% (Lu *et al*., 1995a), 8% (Nadala *et al*., 1993; Tapay *et al*., 1995), 10% (Chen *et al*., 1989; Toullec *et al*., 1996; George and Dhar, 2010; George *et al*., 2011), 27% (Kumar *et al*., 2001) and 30% (Chen *et al*., 1986). Haemolymph of lobsters at 10% (Chen *et al*., 1986) was also used. Moreover, ovary extracts (Chen and Wang, 1999), chitosan and nerve nodule extracts (Fan and Wang, 2002) were also incorporated in the medium as growth promoting factors. Fetal bovine serum (FBS)/ fetal calf serum (FCS) as the supplements with a concentration 10% (Luedeman and Lightner, 1992; Lang *et al*., 2002a; Maeda *et al*., 2003, 2004; George and Dhar, 2010; George *et al*., 2011), 15% (Assavalapsakul *et al*., 2003,2005,2006), 18% (Chen *et al*., 1986) and 20%

(Machii *et al*., 1988; Nadala *et al*., 1993; Lu *et al*., 1995; Tapay *et al*., 1995; Shike *et al*., 2000a; Wang *et al*., 2000; Fan and Wang, 2002; Jiang *et al*., 2005; Hu *et al*., 2010; Jose *et al*., 2011, 2010) were added as the source of minerals, proteins, lipids, hormones (Freshney, 2000) and as the growth-promoting substances (Mitsuhasi, 2002). Considering the importance of inorganic salts for the maintenance of ionic balance and osmotic pressure (Mitsuhasi, 1989), researchers have used KCl, $MgSO_4$, $MgCl_2$, and $CaCl_2$ at concentrations ranging from 0.9 to 3 g l^{-1} to supplement the required quantity in the growth medium (Luedeman and Lightner, 1992; Itami *et al*., 1999; Lang *et al*., 2002a) Moreover, to adjust osmolality, NaCl at a concentration ranging from 6 to 12 g l^{-1} (Chen *et al*., 1986; Luedeman and Lightner, 1992; Fan and Wang, 2002; Jiang *et al*., 2005) has also been added besides the balanced salt solutions (Tapay *et al*., 1995; Jiang *et al*., 2005).

Addition of vitamins (Jose *et al*., 2010, 2011), proline (Luedeman and Lightner, 1992; Toullec *et al*., 1996; Maeda *et al*., 2003, 2004) and glutamine (Ghosh *et al*., 1995; Toullec *et al*., 1996) has been proven to be the choice of supplements in the growth media. In addition, lactalbumin hydrolyzate at a concentration of 0.1-1 g l^{-1} (Machii *et al*., 1988; Itami *et al*., 1999; Lang *et al*., 2002b; Maeda *et al*., 2003, 2004; Assavalapsakul *et al*., 2003, 2005, 2006), tryptose phosphate broth at 2.95 mg ml^{-1} (Jose *et al*., 2010, 2011) and TC Yeastolate at 1 g l^{-1} (Maeda *et al*., 2003, 2004) have also been used as the source of peptides, amino acids and carbohydrates. As the additional energy source 0.3- 2g l^{-1} glucose (Machii *et al*., 1988; Maeda *et al*., 2003, 2004; Jiang *et al*., 2005; Jose *et al*., 2010, 2011) and 0.55 g l^{-1} sodium pyruvate (Fan and Wang, 2002) have also been supplemented. Buffering agents such as HEPES (Ghosh *et al*., 1995; Toullec *et al*., 1996; Lang *et al*., 2002a) and $NaHCO_3$ have been incorporated by many researchers (Luedeman and Lightner, 1992; Ghosh *et al*., 1995; Fan and Wang, 2002; Lang *et al*., 2002b). Growth factors such as epidermal growth factor (EGF)

at a concentration 20-30 ng ml^{-1} (Nadala *et al*., 1993; Lu *et al*., 1995a; Tapay *et al*., 1995) and 10 U ml^{-1} of human recombinant interleukin-2 (Tapay *et al*., 1995) have been used to improve the proliferation of cells *in vitro*. All these modifications have led to improvisation of growth media with enhancement in growth and multiplication of primary cell cultures, but have never lead to establishment of any cell line.

1.2.6. Tissues and organs used for shrimp cell culture development

Ovary and the lymphoid tissue were the most commonly used donor tissues for cell culture development. Of the 90 selected experiments with 15 different tissues, 20 were conducted with lymphoid tissue (Chen *et al*., 1986, 1989; Najafabadi *et al*., 1992; Nadala *et al*., 1993; Hsu *et al*., 1995; Lu *et al*., 1995a, 1995b; Tapay *et al*., 1995; Tong and Miao, 1996; Chen and Wang, 1999; Itami *et al*., 1999; West *et al*., 1999; Wang *et al*., 2000; Lang *et al*., 2002a, 2004; Assavalapsakul *et al*., 2003, 2005; Catap and Nudo, 2008; Hu *et al*., 2008; Jose *et al*., 2012) and 18 with ovary (Chen *et al*., 1986, 1988, 1989; Luedeman and Lightner, 1992; Nadala *et al*., 1993; Tong and Miao, 1996; Mulford and Austin, 1998; Chen and Wang, 1999; Itami *et al*., 1999; Toullec,1999; West *et al*., 1999; Shike *et al*., 2000; Shimizu *et al*., 2001; Lang *et al*., 2002a; Maeda *et al*., 2003, 2004; Hu *et al*., 2010; George and Dhar, 2010) . Ten experiments were with haemocytes (Ellender *et al*., 1992; Ghosh *et al*., 1995; Chen and Wang, 1999; Itami *et al*., 1999; Jiang *et al*., 2005; Claydon *et al*., 2010b; George and Dhar, 2010; Jose *et al*., 2010, 2011; George *et al*., 2011), four with eyestalk (Tong and Miao, 1996; Mulford and Austin, 1998; Kumar *et al*., 2001; George and Dhar, 2010). Besides, testis (Mulford and Austin,1998; Toullec, 1999), heart (Chen *et al*., 1986; Mulford and Austin, 1998; Tong and Miao, 1996; Chen and Wang, 1999; Lang *et al*., 2002a), hepatopancreas (Chen *et al*., 1986, Machii *et al*., 1988; Najafabadi *et al*., 1992; Ghosh *et al*., 1995; Mulford and Austin, 1998; Toullec, 1999; Wang *et al*., 2000; Manohar *et al*., 2001; Lang *et al*., 2002a), gill (Chen *et*

al., 1986; Mulford and Austin, 1998), nerve (Chen *et al*., 1986; Tong and Miao, 1996; Mulford and Austin, 1998; Toullec, 1999; Lang *et al*., 2002a; Chun- Lei *et al*., 2003), muscle (Chen *et al*., 1986; Lang *et al*., 2002; George and Dhar, 2010), hematopoeitic tissue (Chen *et al*., 1988; West *et al*., 1999; Mulford *et al*., 2001), embryonic tissue (Tong and Miao, 1996; Toullec *et al*., 1996; Fan and Wang, 2002); epidermis (Toullec *et al*., 1996; Toullec, 1999) gut (Chen *et al*., 1986; Mulford and Austin, 1998) and Y organ (Toullec, 1999) were also widely used for cell culture development (Fig. 4). Among the tissues used, the most advancement was obtained from lymphoid and ovarian tissue only.

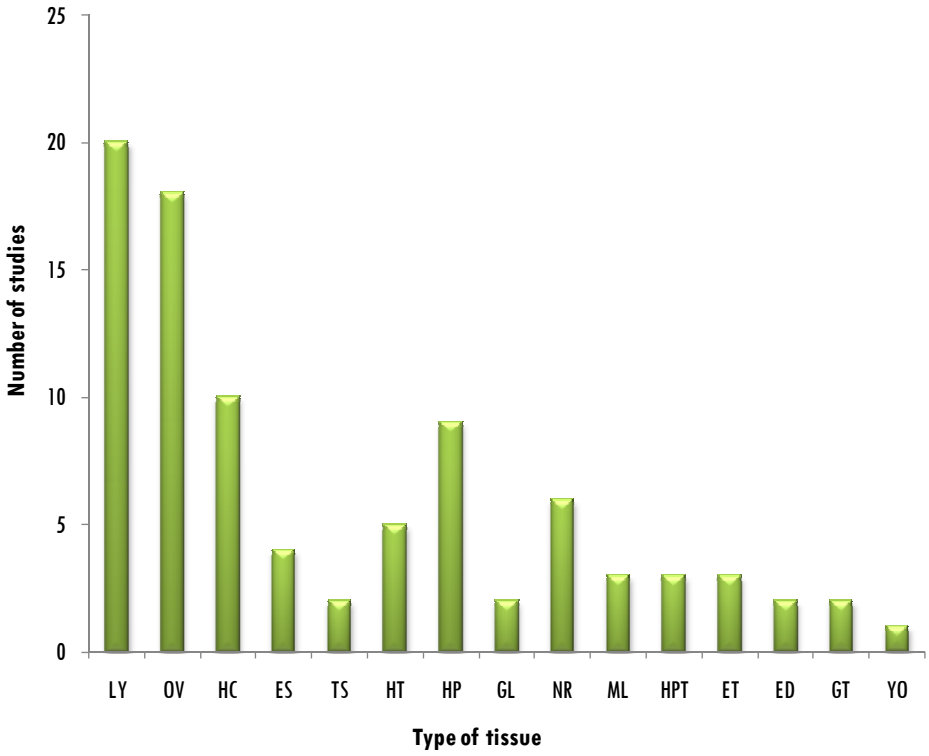


Fig.4. Trends in various tissues used for shrimp cell culture development. LY-Lymphoid, OV-Ovary, HC- Haemocytes, ES- Eye stalk, TS - Testis, HT- Heart, HP-Hepatopancreas, GL-Gill, NR-Nerve, ML-Muscle, HPT- Haematopoeitic tissue, ET-Embryonic, ED-Epidermis, GT-Gut, YO-Y organ (results from 90 experiments)

1.2.7. Longevity and sub-culturing of the shrimp cell culture

The ultimate objective of every shrimp cell culture development programme was the establishment of corresponding cell lines. However, this objective has not been achieved so far. Even though not able to be sub cultured, various researchers could maintain cell cultures for different duration. Accordingly, researchers could maintain ovarian cell culture for 66 days (George and Dhar, 2010), 45 days (Maeda *et al*., 2003), 20 days (Chen and Wang, 1999), 10 days (Luedeman and Lightner, 1992) and to several months (Tong and Miao, 1996; Toullec *et al*., 1996) along with single passage (Mulford and Austin, 1998), and 3 passages (Chen *et al*., 1986; Chen and Wang, 1999). Lymphoid cell cultures were reported to be passaged 2 times (Chen *et al*., 1989) 3 times (Chen and Wang, 1999), and maintained for 54 days (Itami *et al*., 1999), 20 days (Chen and Wang, 1999), and for a period greater than 3 weeks (Nadala *et al*., 1993) to 3 months (Tong and Miao, 1996). However, Hsu *et al*. (1995) claimed to have attained more than 90 passages for a lymphoid organ cell culture which was later reported as Thraustochytrid contamination by Rinkevich (1999). At the same time Tapay *et al*. (1995) reported to have attained even 44 passages of lymphoid cell culture. With eye stalk cell culture, several workers reported to have maintained them for 12 days (George and Dhar, 2010), 3 months and attained 4 passages (Kumar *et al*., 2001). Besides, haemocyte cultures were maintained for 48 days (George and Dhar, 2010), 20 days (Jiang *et al*., 2005), 10 days (Itami *et al*., 1999), 8 days (Jose *et al*., 2010, 2011), and 4 days (Chen and Wang, 1999). Embryonic cell cultures could be maintained for several months (Toullec *et al*., 1996) and attained 10 passages (Fan and Wang, 2002). Moreover, researchers could maintain nerve cells for 15 days (Chun-Lei *et al*., 2003) and up to 3 months (Nadala *et al*., 1993), heart tissue for 4 days (Chen and Wang, 1999) and hepatopancreas for 30 days (George and Dhar, 2010). The striking observation was that there existed no consistency in the number of days which a cell culture could be maintained by different workers.

1.2.8. Virus susceptibility tests in various shrimp cell culture system.

Lymphoid organ cell culture system from penaeid shrimp has been claimed as the best option for *in vitro* growth of several pathogenic viruses. Many researchers claimed the *in vitro* growth of monodon-type baculovirus in lymphoid cell culture from *Penaeus monodon* (Chen and Kou, 1989; Catap and Nudo, 2008). Susceptibility of Yellow head virus in lymphoid cell culture from *P. monodon* (Chen and Wang, 1999; Assavalapsakul *et al*., 2003, 2006; Tirasophon *et al*., 2005), *P. japonicus* and *P. penicillatus* (Chen and Wang, 1999), and from *P. vannamei* (Lu *et al*., 1995a, 1995b) have been reported. Moreover, Lu *et al*. (1995b) suggested the *in vitro* growth of yellow head virus in cell culture from nine different tissues and organs including gill, hepatopancreas, head soft tissue, abdominal muscle, eyestalk, lymphoid organ, heart, nerve cord and midgut. Susceptibility of white spot syndrome virus (WSSV) in lymphoid cell culture from *P. monodon* (Wang *et al*., 2000; Jose *et al*., 2012) from *P. monodon, P. japonicus* and *P. penicillatus* (Chen and Wang, 1999), ovarian cell culture from *P. japonicus* (Maeda *et al*., 2003), hepatopancreatic cell culture from *P. monodon* (Uma *et al*., 2002) haemocytes from *P. chinensis* (Jiang *et al*., 2005) have also been reported. Recently, Jose *et al*. (2010) conducted a detailed investigation on the viral titration and viral gene expression in *P. monodon* haemocyte culture. Still more recently, George *et al*. (2011) investigated the multiplication of Taura Syndrome Virus (TSV) in haemocytes from *P. vannamei*. In spite of the successful attempts by several researchers to grow a few shrimp viruses in cell culture systems from penaeids, strangely enough there has not been any attempt by other laboratories either to validate the methodology or to uses them as the protocol for shrimp virus cultivation. However, with the available techniques it is possible to generate and maintain primary cell cultures from shrimp and use them for virus titration and viral gene expression.

1.3. Lymphoid organ cell culture- a promising *in vitro* system

Lymphoid organ was first described in *P. orientalis* by Oka (Oka, 1969) and found exclusively in penaeid prawns, and do not possess in other crustaceans such as crabs, lobsters and crayfish (Rusaini and Owens, 2010). Lymphoid organ consisted with two distinct lobes located dorso-anterior to the ventral hepatopancreas (Bell and Lightner, 1988) in the cephalothoracic region of the shrimp (Fig. 5a & 5b). Each lobe is composed of two parts: lymphoid tubules and interstitial spaces, permeated with haemal sinuses filled with large numbers of haemocytes (Duangsuwan *et al*., 2008). Histology (Fig. 6a & 6b) and three dimensional organization of lymphoid organ were also well studied (Duangsuwan *et al*., 2008).

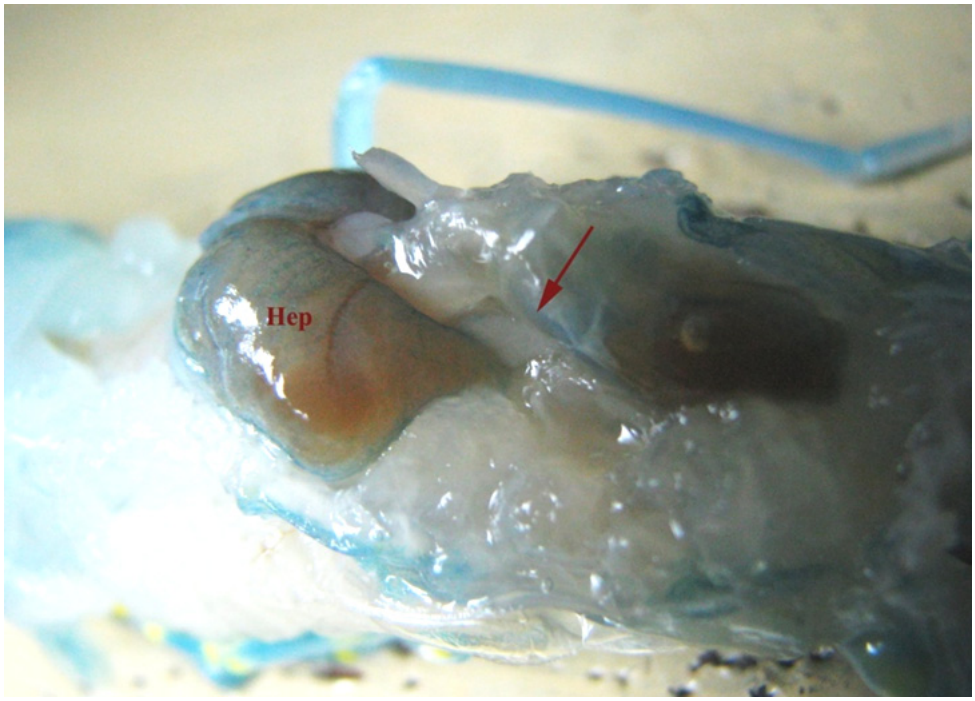


Fig.5a. Cephalothoracic region of the *P. monodon* showing lymphoid organ (arrow) and Hep-hepatopancreas (Jose, 2009).

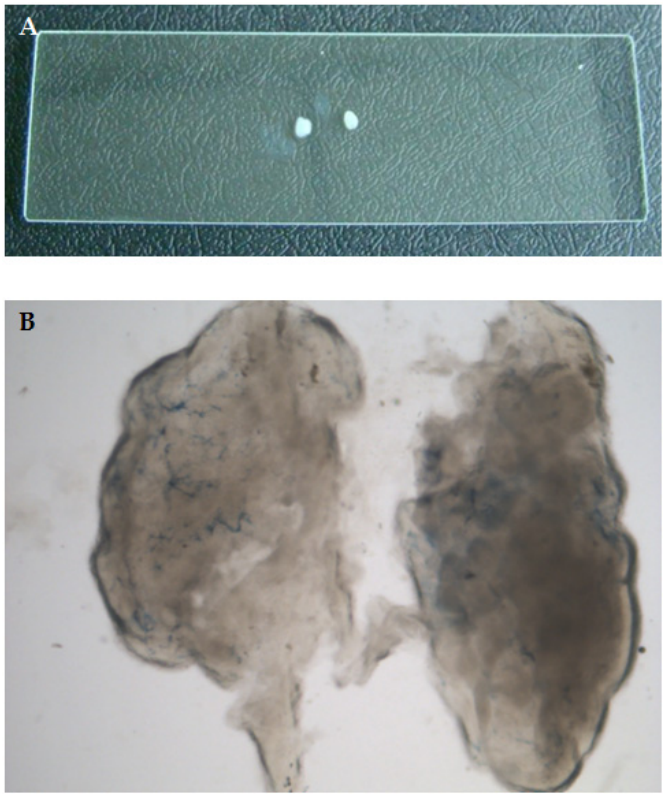


Fig.5b. Lymphoid organ removed from *P. monodon*. A: Two lobes of lymphoid organ on standard glass slide; B: lymphoid organ under light microscope (4x magnification)

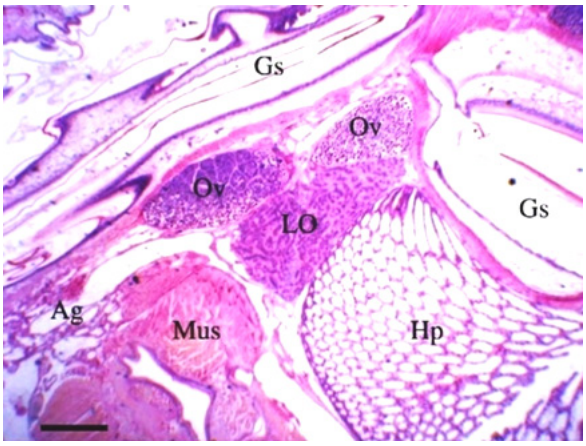


Fig. 6a. Overall longitudinal view of the lymphoid organ and surrounding tissue of *P. monodon* female, H & E stain, scale bar = 200 µm. Ag: antennal gland; Gs: gastric sieve; Hp: hepatopancreas; Mus: muscle; Ov: ovary and LO: Lymphoid organ (Rusaini, 2006).

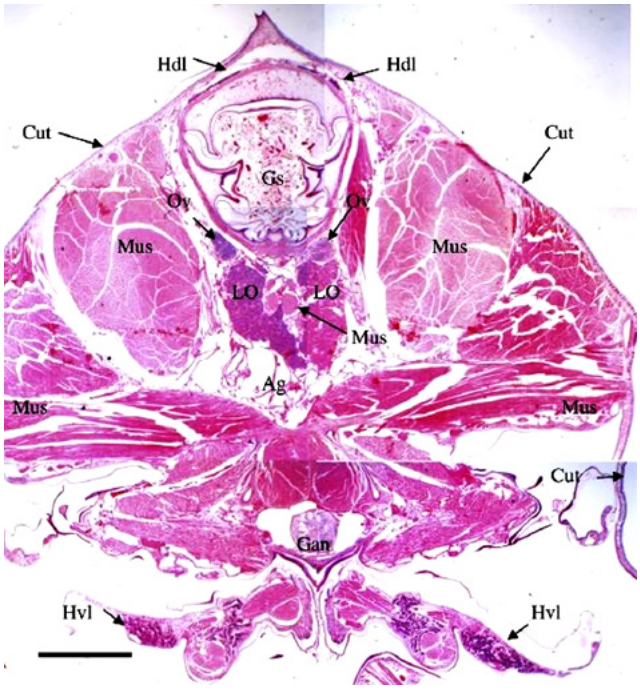


Fig. 6b. Transverse section of the lymphoid organ (LO) and surrounding tissue of *P. monodon*. The LO consists of two lobes located ventro-lateral of the gastric sieve and dorsal of antennal gland. H & E stain. Scale bar =100 µm. Ag: antennal gland; Cut: cuticle; Gan: ganglion; Gs: gastric sieve, Hdl: haematopoietic dorsal lobules; Hvl: haematopoietic ventral lobules; Mus: muscle; LO: lymphoid organ; Ov: ovary (Rusaini, 2006).

Lymphoid cells were found to be susceptible to most of the viruses such as; Lymphoidal parvo like-virus (Owens *et al*., 1991), Monodon-type baculovirus (Chen and Kou, 1989; Catap and Nudo, 2008), Spawner-isolated mortality virus (Fraser and Owens, 1996), White spot syndrome virus (Chen and Wang, 1999; Wang *et al*., 2000; Rodríguez *et al*., 2003; Jose *et al*., 2012), Yellow head virus (Chantanachookin *et al*., 1993; Lu *et al*., 1995a, 1995b; Chen and Wang, 1999; Assavalapsakul *et al*., 2003, 2006; Tirasophon *et al*., 2005), Lymphoid organ virus (Spann *et al*., 1995) , Taura syndrome virus (Hasson *et al*., 1999), Infectious myonecrosis virus (Tang *et al*., 2005) , Mourilyan virus (Rajendran *et al*., 2006), Laem-Singh virus (Sritunyalucksana *et al*., 2006), Rhabdovirus of penaeid shrimp (Nadala *et al*., 1992), and Lymphoid organ vacuolization virus (Bonami *et al*.,

1992). In addition, viral proteins in infected lymphoid cells were also successfully detected by immunefluorescence using specific antibodies (Wang *et al*., 2000; Jose *et al*., 2012). Jose *et al.* (2012) used lymphoid cell culture system from *P. monodon* for studying WSSV mediated viral and immune gene expression. The same system was also used for studying the BrdU incorporation and for determining the metabolic activity using MTT assay. Lang *et al*. (2002a, 2002b) confirmed and recorded the mitotic division in lymphoid cells *in vitro*. Shike *et al*. (2000a) confirmed that lymphoid cell culture system could be used for foreign gene expression. Altogether, as the lymphoid organ probably was a prime target and site for replication of most systemic viruses (Rusaini and Owens, 2010) and confirmed to be useful for cellular and molecular studies, the development of an immortal cell line as a 'model *in vitro* system' from lymphoid organ will provide more acceptance than any other cell type from *P. monodon*.

1.4. Importance of 'specific' medium for shrimp cell culture - a stepping stone for cell line development

Several hindrances stand on the way of the development of shrimp cell lines. One among them is the unsettling fact of an appropriate shrimp cell culture medium, that the media used for shrimp cell culture development have been mostly the modified commercially available preparations, despite the fact that the media composition happens to be the most important factor which determines the success of any cell line development (Mitsuhashi, 2001). To date, a medium exclusively for *in vitro* growth of shrimp cell cultures has not been designed, and the fact that an appropriate medium is required to establish shrimp cell lines in tune with the quantum change which the Grace's insect cell culture medium (Grace, 1958, 1962, 1982, Grace and Brzostowski, 1966) has brought about; ever since the publication of Grace's insect cell culture medium, over 500 insect cell lines could be established (Lynn, 2001; Smagghe *et al*., 2009). Likewise, to formulate an exclusive shrimp cell culture medium, in-depth analysis of the biochemistry of

body fluids (Najafabadi *et al*., 1992; Shimizu *et al*., 2001) is the prime requirement. Moreover, to tide over the difficulties in developing a complete medium for shrimp cell culture, attention must be directed towards satisfying the nutritional requirements of each cell type.

1.5. Molecular approaches for *in vitro* transformation of shrimp cells and its immortalization

Given the tremendous advancements in human and veterinary virology thanks to the availability of a variety of cell lines, any radical change in crustacean virology would be possible only if appropriate cell lines for *in vitro* cultivation of intracellular pathogenic agents (Claydon and Owens, 2008) could be made available. Considering the past experience in this realm more focus should be on the molecular approaches to immortalize shrimp cells by disrupting cell cycle regulator genes and the telomere maintenance. Usually somatic cells do not spontaneously immortalize in culture, but instead enter replicative senescence after a finite number of population doublings (Hayflick and Moorhead, 1961; Hayflick, 1965). In contrast to mammals and most insects, decapod crustaceans can enlarge their organs in the adult life period and regenerate lost appendages, organs with indeterminate growth (Vogt, 2011). The high regeneration capabilities of the crustacean cells (including shrimp) do not show neoplastic transformation and thus it prevents spontaneous immortalization. Neoplastic transformation can be achieved by transfection with active oncogenes (Ratner *et al*., 1985), the technique which has not yet been fully applied to crustacean and aquatic invertebrate cells (Claydon and Owens, 2008). Moreover, unveiling the molecular and regulatory mechanisms that prevent neoplastic transformation in shrimp cells (decapod crustaceans) might provide new leads for the development of anti-ageing and anti-cancer interventions in humans (Vogt, 2011).

To date, oncogenic mammalian virus gene, simian virus 40 large T (SV40-T) antigen (Tapay *et al*., 1995; Hu *et al*., 2008, 2010) has only been used for

transformation of primary shrimp cell culture. The first transformation attempt in lymphoid organ primary cell culture of *P. stylirostris* was made in 1995 (Tapay *et al*., 1995) with the pSV-3 neo plasmid vector encoding SV40-T antigen gene from Simian virus-40 by lipofection. Although, Tapay *et al*. (1995) claimed three transformed cells (OKTr-1, OKTr-23 and OKTr-25) with enhanced cell proliferation, extended life span, altered growth and morphology, and passaged 44, 18 and 3 times for OKTr-1, OKTr-23 and OKTr-25 respectively and were to stain positively (OKTr-1, OKTr-23) with mouse-anti- SV40-T antigen, further improvement has not been reported, indicating the failure of the stable transformation. Further, retroviral vectors pseudotyped with the envelop glycoprotein of vesicular somatitis virus was proved to be infective to primary cell cultures from *P. stylirostris* (Shike *et al*., 2000a), however, without any direct evidence of integration. Even though, researchers (Hu *et al*., 2008, 2010) proved the use of VSV-G pseudotyped pantropic retroviral vectors by confirming the stable expression of SV40-T gene in post transfected cells, the attempts failed to induce *in vitro* transformation. Moreover, Claydon and Owens (Claydon and Owens, 2008), transfected human papilloma viruses (HPV) E6 and E7 genes into the *C. quadricarinatus* cells by lipofection and the successful transfection was demonstrated by the presence of oncogene mRNA by RT- PCR. While transfection of the oncogenes was successful and transfected cells survived more than 150 days, cell proliferation was stagnant due to the lack of telomere maintenance.

Telomerase activity in cultured cells is a limiting proliferating factor, as inactivation of pRb and p53 pathways (Smeets *et al*., 2011) in combination with activation of a telomere maintenance mechanism is suggested to be necessary for immortalization of somatic cells (Bodnar *et al*., 1998; Vaziri and Bachimol, 1999). Ablation of cell cycle checkpoint genes through mutation or viral oncogene expression is necessary to lead escape from senescence, additional doublings, and entrance into crisis phase, and finally the emergence of immortal clones. In the vast

majority of cases, telomerase is reactivated and telomeres are stabilized (Forsyth *et al*., 2004). Moreover, researchers proved that the introduction of telomerase activity in normal human cells caused an extension of replicative life span (Bodnar *et al*., 1998; Vaziri and Bachimol, 1998; Simons, 1999). In our study, we could not find any telomerase activity in primary lymphoid cell culture using telomeric repeat amplification protocol (TRAP). Even though, this is contradictory to the reported active telomerase activity in cultured lymphoid organ cells for up to 30 days (Lang *et al*., 2004), till date, no additional report has been seen in literature to confirm the telomerase activity in the cultured shrimp cells.

As spontaneous and induced transformation of somatic penaeid cells has not taken place (Claydon *et al*., 2010b) attempts to create hybrid cells by fusing cells from an immortal cell line of insects (*Epithelioma papulosum cyprinid* and *Spodoptera frugiperda*) with haemocytes from *P. monodon* were attempted and accordingly three fusion-cells could be produced (F11, F12 and F13). However, shrimp genes and viral susceptibility could not be observed in the fusion-cells; this happens to be the first attempt to produce hybrid cells from shrimp cells.

1.6. Critical analysis on shrimp cell line development and significance in this study

The 'futile attempts' in shrimp cell line development might be the outcome of the neglect on 'know your animal' (Lynn, 1999) philosophy, as the successful history of insect cell lines started from the in-depth knowledge gained on the insect biochemistry with which an appropriate and exclusive insect cell culture medium could be developed (Wyatt *et al*., 1956; Wyatt, 1956). Despite the modification of commercially available medium based on haemolymph analysis (Ellender *et al*., 1992; Shimizu *et al*., 2001) an exclusive medium for the growth and development of shrimp cells *in vitro* has not been accomplished. Even though Wyatt (Wyatt *et al*., 1956) was not totally successful, her contribution was essential to Grace's ultimate success in the

development of Grace's insect cell culture medium (Grace, 1958, 1962; Grace and Brzostowski, 1966) which resulted in the development of over 500 insect cell lines (Lynn, 1999, 2001). Such a scientific temper should be imbibed in the shrimp cell culture research for successful development of a continuous cell line. Moreover, lack of third party validation and confirmation of results achieved by researchers in sister institutions has also hampered the progress of research in shrimp cell culture development with a diminishing output. The transformation studies include identification of a putative promoter system to construct transformation and transduction vectors specific to shrimp. For the safe and stable transduction and immortalization, recombinant baculovirus with specific promoter for transcription initiation in shrimp cells are to be addressed. Development of hybrid cell line might also will pave way for the development of penaeid virus susceptible fusion cell lines. Moreover transgenic expression of oncogene and telomerase reverse transcriptase might also lead to a successful outcome of a valid shrimp cell line. With these concepts, the present study was undertaken with the following objectives:

1. A novel medium for the development of *in vitro* cell culture system from *Penaeus monodon*
2. Screening and optimization of growth factors and their potential impacts on lymphoid cell culture: Cellular activity and viral susceptibility
3. Differential expression of telomerase in various tissues and primary lymphoid cell culture, and identification and partial sequencing of telomerase reverse transcriptase (*TERT*) gene in *Penaeus monodon*
4. Construction and evaluation of the versatile recombinant baculoviral vector systems with hybrid promoters designed for the expression of foreign gene in shrimp cells
5. Transfection and transduction mediated oncogene expression in lymphoid cell cultures from *Penaeus monodon* for its *in vitro* transformation

Chapter 2

A novel medium for the development of *in vitro* cell culture system from *Penaeus monodon*

2.1. Introduction

Attempts on the development of continuous cell lines from shrimps have a long and arduous history amidst the absolute requirement of certified cell lines to address the viral diseases which spelled havoc in shrimp industry. Lack of a cell line has been hampering the progress of research in shrimp viruses especially in the study of viral morphogenesis and in the development of prophylactic and therapeutic measures. The most prominent reason for the non attainment of cell lines from shrimps and for that matter from crustaceans altogether might be the lack of an appropriate medium like Grace's insect cell culture medium which paved the way for the development of over 500 insect's cell lines (Lynn, 2001). Considering this necessity, several commercially available media have been modified over decades to suit the requirements of cell cultures (Jose, 2009). Among them Leibovitz's – 15 (L -15) has been the most popular one subjected for modifications (Chen *et al*., 1986,1988, 1989; Nadala *et al*., 1993; Lu *et al*., 1995; Tapay *et al*., 1995a; Tong and Miao, 1996; Toullec *et al*., 1996; Mulford and Austin, 1998; Chen and Wang, 1999; Shike *et al*., 2000; Wang *et al*., 2000; Shimizu *et al.,* 2001; Kumar *et al*., 2001; Chun-Lei *et al*., 2003; Maeda *et al*., 2003, 2004; Jiang *et al*., 2005; Assavalapsakul *et al*., 2006; Jose *et al*., 2010,

2011, 2012). They have been modified by supplementing with growth factors in isolation as well as in multiples (Chen *et al*., 1986; Chen *et al*., 1988; Machii *et al*., 1988; Chen *et al*., 1989; Luedeman and Lightner, 1992; Nadala *et al*., 1993; Ghosh *et al*., 1995; Lu *et al*., 1995a; Tapay *et al*., 1995; Toullec *et al*., 1996; Mulford and Austin, 1998; Chen and Wang, 1999; Itami *et al*., 1999; Shike *et al*., 2000; Wang *et al*., 2000; Kumar *et al*., 2001; Mulford *et al*., 2001; Lang *et al*., 2002a; Fan and Wang, 2002; Maeda *et al*., 2003, 2004; Jiang *et al*., 2005; George and Dhar, 2010; Jose *et al*., 2010, 2011, 2012). Meanwhile, Shimizu *et al*. (2001) reported a modified L-15 medium based on the haemolymph composition with some success in prolonging the longevity of primary cell culture from shrimp. More recently, Jose *et al*. (2010) modified L-15 (2x) by supplementing it with 2% glucose, MEM vitamins (1x), tryptose phosphate broth (2.95 mg ml^{-1}), 20% FBS, and 0.2 mM N-phenylthiourea. Jose *et al*. (2012) selected Grace's Insect Medium (Sigma Aldrich) supplemented with magnesium chloride hexahydrate (2 g l^{-1}), 10% aqueous sodium bicarbonate (3.5 ml l^{-1}), L-proline (20 mg l^{-1}), sucrose (200 mg l^{-1}), trehalose (200 mg l^{-1}), foetal bovine serum (10%), fish muscle extract (4%), shrimp muscle extract (4%), shrimp haemolymph (8%), chloramphenicol (0.06 µg l^{-1}), streptomycin (100 µg ml^{-1}), and penicillin (100 IU ml^{-1}). Mulford *et al*. (2001) revealed that 2x Leibovitz's medium supplemented with 10% (v/v) heat inactivated FBS, 5% (v/v) muscle extract, 0.06 g l^{-1} of L-proline, 1 g l^{-1} glucose prepared in 25‰ seawater was effective for cell migration, survival and longevity. Though the haemolymph composition of *P. aztecus* and *P. stylirostris* had been reported earlier (Najafabadi *et al*., 1992; Shimizu *et al*., 2001), a medium exclusively for shrimp cell culture based on it could not be attained so far, other than the modification of the existing media. This might be cited as one of the reasons for the non attainment of immortal cell line from shrimp.

In this context we made an attempt to develop seawater based cell culture medium exclusively for shrimp cell culture and named it as Shrimp Cell Culture

Medium (SCCM). Experiments were carried out using various tissues from *P. monodon* for determining its suitability to develop cell cultures. Primary cell cultures developed by employing this medium from lymphoid and ovarian tissues could be sub cultured twice using shrimp cell dissociation "cocktail" developed in this study.

2.2. Materials and methods

2.2.1. Design of the experiment

The whole experiment was designed to formulate a medium exclusively for shrimp cell culture. The haemolymph components of *P. monodon,* the free amino acids, fatty acids and metal ions were used as background information about the physiological conditions required for *in vitro* growth of cells. Seawater and artificial seawater were screened for suitable base for the medium. Physical observation was carried out to screen the most suitable combinations initially and further confirmations were done based on MTT assay.

2.2.2. Experimental animals

Shrimps required for the experiments were maintained in Recirculating Aquaculture System (RAS) integrated with nitrifying bioreactor (Kumar *et al*., 2009) maintained at 27‰. Post larvae, nested PCR negative to white spot syndrome virus (WSSV), were stocked in the system and reared for three months, maintaining the water quality parameters within a narrow range (pH 6.8-7.8; total ammonia - nitrogen < 0.1 mg l^{-1}; nitrite - nitrogen <1.0 mg l^{-1}; total alkalinity ($CaCO_3$) 75-125 mg l^{-1}; total hardness 5000-6000 mg l^{-1}) and fed pelleted feed (Higashimaru). Shrimps weighing 15-20 g were used as the donor animals for various tissues, besides nauplii directly collected from a seed production centre.

2.2.3. Analysis of haemolymph

2.2.3.1. Collection of haemolymph

For free amino acid and fatty acid analysis, haemolymph was withdrawn aseptically from rostral sinus using capillary tubes containing 100 µl of 10% sodium citrate (Jose *et al*., 2011) and the total volume of each sample was measured to calculate the dilution factor (Shimizu *et al*., 2001). Pooled haemolymph from 20 animals weighing 20-30 g were centrifuged at 1000xg for 10 min to remove haemocytes, lyophilized the plasma and stored at -20 ºC. Haemolymph was collected without anticoagulant also for metal ion analysis. Osmolality of the haemolymph was measured immediately after collection using Fiske 1-10 Osmometer (Fiske Associates, USA).

2.2.3.2. Analysis of free amino acids

Aliquot of 160 µg pooled lyophilized haemolymph was collected in a test tube and added 10 ml 6 N HCl. The test tube was filled with nitrogen, sealed and kept at 121 ºC for 24 h. The hydrolysed sample was filtered and flash evaporated repeatedly adding distilled water until the traces of chlorine were removed. The residue obtained was made up to 10 ml with 0.05 M HCl.

Samples were filtered through a polyvinylidene fluoride membrane filter (PVDF, Millipore) of 0.45 µm pore size and injected 20 µl to an amino acid analyzer (HPLC-LC 10 AS) equipped with cation exchange column packed with a strongly acidic cation exchange resin, styrene divinyl benzene copolymer, with sulphonic group. The column used was Na type, ISC-O7/S 1504 Na, having a length of 19 cm and diameter 5 mm. The instrument was equipped with Shimadzu FL 6A fluorescent detector and Shimadzu CR 6A Chrompac recorder. A gradient mobile phase was applied with buffer A and buffer B for the effective separation of amino acids. The oven temperature was maintained at 60 ºC. The total run was programmed for 62 min. The amino acid analysis was done with non-switching

flow method and fluorescence detection after post-column derivatization with o-phthaldehyde. In the case of proline and hydroxyproline, imino group was converted to amino group with hypochlorite. Amino acid standard (Sigma chemical Co., St. Louis, USA) was also run to calculate the concentration of the amino acids in the sample. The amount of each amino acid was expressed as µmol ml^{-1} haemolymph (Antoine *et al*., 1999).

2.2.3.3. Analysis of fatty acids

For fatty acid analysis, gas chromatograph with flame ionization detector (GC-FID) was employed (Agilent Technologies, model 6890). The pooled and lyophilized haemolymph was converted into fatty acid methyl esters (FAMEs) by saponification, methylation and extraction (Carvalho and Malcata, 2005) into hexane: methyl *tert*-butyl ether. A 25 m (length) x 0.2 mm ID x 0.33 µm film thickness, cross linked 5% phenylmethyl silicone fused silica capillary column was used to separate the fatty acids. While operation the initial temperature of 170 ºC was increased to 310 ºC at the rate of 40 ºC min^{-1} and held for 1.5 min. Helium was used as the carrier gas at a constant flow rate of 1.3 l min^{-1}. The peaks were analyzed using the software Sherlock (MIDI, Inc., USA) to identify the relative amounts of fatty acids in the sample and were expressed as percentage of the total fatty acids. Identification of the peaks was accomplished by comparison of retention times to those of authentic standards.

2.2.3.4. Analysis of metal ions

As the analysis of major ions in the haemolymph is crucial to formulate cell culture medium (Najafabadi *et al*., 1992), metal ion strength of haemolymph and seawater at 27‰ were analyzed using Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES Thermo Electron IRIS INTREPID II XSP DUO). Before analysis, seawater was sterilized by autoclaving at 15 lbs for 15 min, filtered through Whatman No.1 filter paper to remove precipitates. Lyophilized

haemolymph (100 µg) from different age group of *P. monodon* was diluted with Milli-Q water to the required volume for analysis (Shimizu *et al.*, 2001; Huang *et al.*, 1999). Identification of the elements was accomplished by comparison with authentic standards (Merck, Germany).

2.2.4. Formulation of shrimp cell culture medium (SCCM) base composition

All chemicals used for the preparation, unless specifically stated otherwise, were purchased from Sigma-Aldrich, USA. The shrimp cell culture medium contained (mg l^{-1} in artificial/natural seawater) L-alanine 70, L – arginine 45, L-asparagine 15, L-aspartic acid 10, L-cystine 1, L-cysteine 1, L-histidine 15, L-leucine 20, L-lysine 60, L-isoleucine 10, L-methionine 5, L-phenyl alanine 10, L-proline 100, L-serine 15, L-taurine 100, L-threonine 15, L-tryptophan 15, L-glutamine 150, L-glutamic acid 10, glycine 20, L-tyrosine 80, L-valine 20, choline bitartarate 1.8, D-pantothenic acid (hemicalcium) 1, folic acid 1, myo-Inositol 2, pyridoxal-HCl 1, riboflavin 0.1, thiamine 1, niacinamide 1, glucose 1000, ribose 10, trehalose 10, sodium pyruvate 500, potassium dihydrogen phosphate 2, di-sodium hydrogen phosphate 11.5, cholesterol 0.2, and phenol red 0.01. This composition was considered as the base for SCCM to which additional ingredients such as antibiotic mixture and fetal bovine serum (FBS) were added as described elsewhere, and the efficacy was evaluated through development of cell cultures and their subsequent visual observation under inverted microscope (Leica DMIL) connected with CCD camera (Leica DFC 420C).

2.2.5. Artificial seawater and natural seawater as liquid base

Based on the report of Dall (1981), the isosmotic point of *P. monodon* was identified equivalent to 27‰. Natural seawater at 27‰ having 810±20 mOsm kg^{-1} osmolality was used for the whole experiment. Seawater was sterilized by autoclaving at 15 lbs for 15 min, filtered through Whatman No.1 filter paper to remove precipitates and stored in a 20 l container at room temperature. Artificial

seawater was prepared by dissolving the following ingredients one after the other: NaCl, 2.3926 g; Na_2SO_4, 0.4 g; KCl, 0.0677 g; $NaHCO_3$, 0.0196 g; KBr, 0.0098 mg; H_3BO_3, 0.0026 g and NaF, 0.0003 g in 75 ml MilliQ water with constant stirring. To this solution, 5.327 ml of 1.0 M $MgCl_2.6H_2O$, 1.033 ml of 1.0 M $CaCl_2.2H_2O$ and 0.09 ml of 0.1 M $SrCl_2.6H_2O$ were added and made up to 100 ml with MilliQ water (Kester *et al.*, 1967). The differential effects of artificial and natural seawater were evaluated by visual observation of the extent of attachment and monolayer formation of primary cell cultures from various tissues using an Inverted phase contrast microscope (Leica DMIL) connected with CCD camera (Leica DFC 420C).

2.2.6. Effect of inorganic salts and trace elements

To equalize the inorganic salt and trace element concentration of SCCM to those of haemolymph, additional inorganic salts and trace elements were added to the tune of 0.518 mg l^{-1} barium chloride, 124.2 mg l^{-1} copper chloride, 37.38 mg l^{-1} zinc sulphate, 8.28 mg l^{-1} ferric citrate and 0.26 mg l^{-1} manganese chloride. The effect of this modification was evaluated visually by observing the extent of attachment and monolayer formation of the cells using an Inverted phase contrast microscope (Leica DMIL) connected with CCD camera (Leica DFC 420C).

2.2.7. Effect of organic supplements

Five different mixtures of organic supplements were added to the SCCM and the effect of these supplements were evaluated by the extent of cell attachment and monolayer formation and compared with that of the basal SCCM as control. The following were the various supplements in final concentration added to SCCM.

Supplement A: consisted of a mixture of arachidonic acid 0.02 µg l^{-1}, linoleic acid 0.1 µg l^{-1}, linolenic acid 0.1 µg l^{-1}, myristic acid 0.1 µg l^{-1}, oleic acid

0.1 µg l^{-1}, palmitic acid 0.1 µg l^{-1}, stearic acid 0.1 µg l^{-1}, cholesterol 2.2 µg l^{-1}, tween-80 20.2 µg l^{-1}, and tocopherol acetate 0.7 µg l^{-1}.

Supplement B: consisted of precursors of signal molecules such as, flavin adenine dinucleotide (FAD-Na_2) 0.83 mg l^{-1}, adenosine 5′-triphosphate magnesium salt (ATP) 0.55 mg l^{-1}, nicotinamide adenine dinucleotide phosphate (NADP) 1.14 mg l^{-1}, adenosine 5′-monophosphate (AMP- Na_2) 0.35 mg l^{-1}, Coenzyme A (CoA-Na_2) 0.77 mg l^{-1} .

Supplement C: consisted of precursors of nucleic acid synthesis such as, adenosine 0. 27 mg l^{-1}, guanosine 0.28 mg l^{-1}, cytosine 0.11 mg l^{-1}, thymine 0.13 mg l^{-1}, deoxy ribose 0.13 mg l^{-1}, uracil 0.11 mg l^{-1}, uridine 5′-triphosphate (UTP) 0.55 mg l^{-1}.

Supplement D: consisted of Kreb's cycle intermediates which include ketoglutaric acid 0.15 mg l^{-1}, malic acid 0.13 mg l^{-1} and succinic acid 0.12 mg l^{-1}.

Supplement E: consisted of vitamins viz., ascorbic acid 0.01 mg l^{-1}, biotin 0.05 mg l^{-1}, nicotinamide 0.01 mg l^{-1}, nicotinic acid 0.01 mg l^{-1}, pyridoxin, 0.01 mg l^{-1}, calciferol 0.01 mg l^{-1}, tocopherol 0.01 mg l^{-1}, p-aminobenzoic acid 0.01 mg l^{-1}.

2.2.8. Preparation of shrimp cell culture medium (SCCM)

Different constituents of SCCM were prepared separately and mixed as follows. A 100x concentration amino acid mixture ("amino mix-I") containing L-alanine, L – arginine, L-asparagine, L-cysteine, L-histidine, L-lysine, L-methionine, L-proline, L-Serine, L-taurine, L-threonine, glycine, L-valine was prepared in MilliQ water. The "amino mix-II" was prepared by dissolving 100x concentration of L-aspartic acid, L-cystine, L-leucine, L-isoleucine, L-phenyl alanine, L-Tryptophan, L-glutamic acid, L-tyrosine in 1 N HCl. An aliquot of 100x concentration of "sugar mix" was prepared by dissolving glucose, ribose, trehalose, sodium pyruvate, potassium dihydrogen phosphate and di-sodium hydrogen

phosphate in MilliQ water. "Vitamin mix" was prepared by mixing 100x concentrations of choline bitartarate, D-pantothenic acid (hemicalcium), myo-Inositol, pyridoxal-HCl, thiamine and niacinamide in MilliQ water with 100x concentration riboflavin and folic acid in 1 M NaOH. Cholesterol was prepared (100x) separately in MilliQ water. Phenol red solution was prepared by dissolving 1 mg phenol red (100x) in 1 ml NaOH (1M). All the above mixtures were separately filtered through a polyvinylidene fluoride membrane filter (PVDF, Millipore) of 0.22 µm pore size and stored in amber colored bottle at -20 ºC till use.

The final SCCM (1000 ml) was prepared by mixing 10 ml each of amino mix I, amino mix II, sugar mix, vitamin mix, cholesterol and phenol red solution in 100 ml double distilled water, mixed with 685 ml sterile seawater of 810±20 mOsm kg^{-1} osmolality (27‰). An aliquot of 100 ml fetal bovine serum was added, Osmolality of the medium was adjusted to 720±10 mOsm kg^{-1} by adding NaCl and measuring by means of osmometer (Fiske One-Ten Osmometer, Fiske Associates, USA). pH of the medium was adjusted to 6.8 using 1 N NaOH or 1 N HCl, the medium was made up to 1000 ml with distilled water. Further, the medium was supplemented with antibiotic mixture containing penicillin (100 U ml^{-1}), streptomycin (100 µg ml^{-1}) and chloramphenicol (0.0 6 µg ml^{-1}) and added filter sterilized 150 mg l^{-1} glutamine just before use.

2.2.9. Development of primary cell cultures

Prior to dissection, the juvenile shrimp and nauplii were chilled in ice and surface sterilized by immersion in 800 mg l^{-1} sodium hypochlorate in ice cold seawater for 10 min, followed by thorough washing in sterile seawater. Lymphoid organ, heart, hepatopancreas, nerve code, eye stalk, muscle, testis and ovary were removed aseptically and collected in holding medium (SCCM without FBS) of 720 mOsm kg^{-1} osmolality. The tissues and nauplii were washed three times with PBS and minced in to very small pieces using sterile surgical knife. The clumps of

tissue were separated using cell dissociation sieve (CD-1, Sigma) with a 60 mesh screen (Mulford *et al.*, 2001); the suspension was mixed thoroughly with the medium and seeded on 25 mm^2 culture flask/wells (Greiner Bio-One) and incubated at 25 °C. For haemocyte culture, haemolymph was withdrawn aseptically from rostral sinus using capillary tubes containing 100 µl anticoagulant (tris HCl 0.01 M, sucrose 0.25 M, tri sodium citrate 0.1 M) and diluted to obtain 5 x 10^5 cells ml^{-1} using SCCM supplemented with N-phenylthiourea (0.2 mM), 0.06 mg ml^{-1} chloramphenicol, 100 mg l^{-1} streptomycin and 100 IU ml^{-1} penicillin (Jose *et al.*, 2011) and aliquots of 200 µl were dispensed into the wells of 96 well plates (Greiner Bio-One) and incubated at 25 °C.

2.2.10. MTT reduction assay for measuring cellular metabolism

In spite of the visual observation, mitochondrial dehydrogenase activity was measured as the cell viability and metabolic activity which depended on an intact mitochondrial membrane and the respiratory chain. MTT assay measures the mitochondrial dehyrogenase which reflects the metabolic activity of the cells. Succinate dehydrogenase system which belongs to the mitochondrial respiratory chain reduces MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, Sigma-Aldrich Co.) to insoluble formazan crystals, solubilized in dimethyl sulphoxide (DMSO) yielding a purple-coloured solution (Mosmann, 1983). Accordingly, aliquots of 200 µl cell suspension of ovary, lymphoid organ, heart, hepatopancreas and the haemocytes were seeded on to 96 well plates and after desired incubation period (2, 4, 6 and 8 days), the medium was pipetted out and replaced with 50 µl MTT solution (5 mg ml^{-1}) prepared in PBS (720 mOsm kg^{-1}), and the plate was kept for incubation (in dark) at 25 °C for 5 h (Jose *et al.*, 2012). The control consisted of the medium alone with MTT added. Removed the MTT solution and added 200 µl DMSO, (HiMedia Laboratories, Mumbai) mixed well to

confirm the dissolution of formazan crystals, absorbance measured at 570 nm in a microplate reader (InfiniteM-200 Tecan, Austria).

2.2.11. Comparison of SCCM with other selected media

The efficacy of SCCM on *in vitro* growth and viability of cell types from ovary, lymphoid organ, heart, hepatopancreas and the haemocytes was determined by comparing the same with 2x L-15 with 10% FBS (v/v), modified L-15 (Jose *et al.*, 2010) and Grace's insect medium with 10% FBS (v/v). Metabolic activity (viability) of the cultures *in vitro* was analyzed on the 4th day of culture using MTT assay, and the percentage difference was compared.

2.2.12. Cell dislodgement and passaging

Cell dislodgement was performed for passaging the primary cell cultures developed from explants of ovary, lymphoid organ, heart, hepatopancreas and the haemocytes. Trypsin (0.25%, w/v), 1% collagenase type V, 1% accutase, non enzymatic cell dissociation solution-I (prepared in PBS), non enzymatic cell dissociation solution-II (prepared in HBSS without calcium and magnesium) and shrimp cell dissociation 'cocktail' were tested. The 'cocktail' contained 0.25 g trypsin, 0.02 g EDTA, 0.02 g EGTA, 0.04 g polyvinyl pyroline and 0.05 g glucose dissolved in 100 ml PBS (720 mOsm kg^{-1}). pH was adjusted to 6.8 within a range 6.8-7.2 using 1 N NaOH or 1 N HCl and supplemented with antibiotic mixture containing penicillin (100 U ml^{-1}) streptomycin (100 µg ml^{-1}) and chloramphenicol (0.06 µg ml^{-1}), filtered through 0.22 µm pore size polyethersulfone (PES) (Millex GP, Millipore) using syringe filter.

2.2.13. Statistical analysis

The results in the figures are average values of 3 - 6 replicates ± standard deviation. The effects of treatments were statistically analyzed by single factor and two

factor analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$.

2.3. Results

2.3.1. Analysis of haemolymph

Amino acid components from haemolymph recorded (Fig. 1) were aspartic acid (17.24%), threonine (4.6%), serine (7.53%), glutamic acid (12.2%), proline (1.3%), glycine (7.55%), alanine (6.12%), cystine (0.18%), valine (8.35%), isoleucine (5.17%), leucine (9.56%), tyrosine (2.32%) , phenyl alanine (7.69%), histidine (6.84%) , lysine (1.18%), and arginine (0.85%).

Fatty acid profile exhibited long chain fatty acids (up to 20-carbon atoms) along with polyunsaturated fatty acids (PUFA) (Table 1, Fig. 2). Among the fatty acid components of the haemolymph lipids, 81.63% were contributed by palmitic acid (16:0), linoleic acid (18.2 ω -6), oleic acid (18.1 ω-9) and stearic acid (18:0). Other fatty acids recorded were capric acid (0.07%) lauric acid (0.17%), myristic acid (1.28%), pentadecyclic acid (0.48%), margaric acid (1.95%), linolenic acid (0.16%), nonadecyclic acid (0.53%), arachidic acid (0.46%), eicosenoic acid (0.34%), eicosadienoic acid (0.34%) and arachidonic acid (2.58%).

Of the ten elements analyzed, concentration of copper, zinc, barium, iron and manganese were lower in the seawater (27‰) than in the haemolymph of *Penaeus monodon*. Copper and manganese levels in the seawater were very low (<0.01 mg l^{-1}) compared to those of the haemolymph, 148.6±34.50 mg l^{-1} and 0.33±0.10 mg l^{-1} respectively. However, the concentration of sodium, potassium, calcium, boron and strontium were more or less the same (Table 2). Concentration of zinc in seawater was 0.035±0.02 mg l^{-1} and iron 0.053±0.01 mg l^{-1}, whilst in haemolymph the concentrations were 39.415±2.88 mg l^{-1} and 12.98±6.63 mg l^{-1} respectively. Moreover, the barium level in seawater (0.015±0.01 mg l^{-1}) was

hundred times lower than that of the haemolymph concentration (15.29±14.27 mg l^{-1}). Inorganic salts and trace elements were added to the SCCM as supplements to adjust these differences. Osmolality of haemolymph and 27‰ seawater were found to be 730.5±51.2 and 810±20 mOsm kg^{-1} respectively.

2.3.2. Artificial seawater and natural seawater as liquid base

Based on the visual observation of growth and monolayer formation of various primary cell cultures from different tissues and organs (Table 3), it was clear that the artificial seawater was not promising as the liquid base for SCCM in terms of limited proliferation and less attachment of cells compared to natural seawater in spite of the fact that there was chances of variations in the composition of natural seawater from place to place.

2.3.3. Effect of inorganic salts, trace elements and organic supplements

The inorganic salts and trace elements added to equalize the haemolymph metal ion concentration were found to have negative effect on growth and monolayer formation of various cell types (Table 3). Moreover, the experiments by addition of organic supplements like vitamin mixture, lipid mixture, citric acid cycle intermediates, nitrogenous base and energy precursors over and above incorporated in the basal medium did not bring forth any enhancement in the attachment of cells, their proliferation and confluence.

2.3.4. Preparation of shrimp cell culture medium (SCCM)

The base composition of SCCM was finalized by conducting a series of experiments by trial and error method (data not shown) by incorporating different permutation combination of amino acid mix I and II, sugar mix, vitamin mix, cholesterol, FBS and pH. Phenol red, antibiotic mix and glutamine were in fixed quantity. Potassium dihydrogen phosphate and di-Sodium hydrogen phosphate were added as the buffering agents incorporated in the sugar mix. Cholesterol, 0.2

mg l^{-1}, was added as the lipid component and phenol red as the pH indicator. The most appropriate composition was finally selected based on the extent of attachment of the explants and proliferation of cells by visual observation (Table 3). The pH, temperature of incubation and osmolarity of the medium were 6.8-7.2, 25 ºC and 720±10 mOsm kg^{-1} respectively. Natural seawater as the liquid base at 27‰ gave better performance. Concentration of 10-15% FBS (v/v) was found to be most effective.

2.3. 5. Development of primary cell cultures

Among the combinations studied, SCCM supplemented with 10% FBS prepared in 27‰ seawater was the most effective one for attachment of cells, their proliferation and confluence for all cell types tested (Table 3). Among the cell types tested, lymphoid and ovarian cells were the most promising ones having the cell longevity exceeding 50 days, 85±9 days for lymphoid cells and 63±6 days for ovarian cells followed by heart cells (29±1 days) and hepatopancreas (25±5 days). Meanwhile, the longevity of other cell types were, testis 21±3 days, haemocytes 10±3 days, eyestalk 9±2 days, muscle 7±1 days, nerve cord and cells from nauplii 6±1 days (Fig. 3). Moreover, metabolic activity of lymphoid cells in terms of MTT assay showed a significant increase (p <0.05) from day 2 to day 8 compared to the other cell types which were to a large extent static (Fig. 4).

Morphology of lymphoid cells in culture was spherical or elliptical initially, and the cells were found getting attached within 3 h, and after 24 h 90% of the cells were anchored, judged by visual observation. They were epitheloid with large nucleus and granulated cytoplasm exhibiting mitotic division (Fig. 6). Subsequently, 42% increase in cell metabolic activity was observed by MTT assay within 48 h (Fig. 4). Moreover, additional increase of 27% and 16% were observed within 72 and 96 h respectively. A rapid monolayer formation and cell proliferation were characteristic of the lymphoid cells compared to all other cell types.

Haemocytes in culture appeared similar shape of lymphoid cells, however within 6 h transformed to spindle shaped cells anchor dependent. (Fig. 7). Even though, haemocytes were viable for 10 days (judged through MTT assay) direct mitotic division could not be observed under microscope. In ovarian culture, fibroblastic as well as round cells were found proliferating (Fig. 9). Meanwhile, cells from hepatopancreas (Fig. 10) required longer duration to attach to and proliferate (> 24 h) than the heart cells which showed better multiplication within 24 h of seeding (Fig. 8). Cell culture developed from testis was very small compared to all other cell types (Fig. 11). Despite the attachment to the substratum, viability and longevity, cell migration and multiplication were not satisfactory with respect to the cells originated from muscle, eyestalk, nerve cord and nauplii.

2.3.6. Comparison of SCCM with other selected media

Lymphoid and ovarian cells ameliorate in SCCM compared to the same in other selected media. An increase of 107% growth (in terms of MTT assay (p <0.05) was observed in lymphoid cells in SCCM in comparison with the same in 2x L-15, and 59% and 82% growth in comparison with modified L-15 (Jose *et al*. 2010) and Grace's insect medium respectively. Ovarian cells showed an increase of 45%, 37% and 36% (p <0.05) in SCCM, 2x L-15, modified L-15 (Jose *et al*., 2010) and Grace's medium respectively (Fig. 5a & 5b). However, increase in growth and multiplication of cells from heart, hepatopancreas and haemolymph in SCCM was less than 20% compared to the same in the other media.

2.3.7. Cell dislodgement and passaging

The results of cell dislodgement and passaging of primary cell cultures from ovary, lymphoid organ, heart, hepatopancreas and haemocytes using different dissociation methods and their efficacy in cell reattachment and growth are summarized in Table 4. Of the six dissociation agents tested shrimp cell dissociation 'cocktail' showed better survival (40%) of lymphoid cells after two passages. Heart

cells and ovarian cells showed a survival rate of up to 30% where as to hepatopancreas and haemocytes it was toxic with a survival rate 20% and less than 10% respectively. Trypsin (0.25%) and accutase (1%) were found toxic to cells with a survival rate of less than 10%. The non-enzymatic solution I and II were proven to be not suitable for shrimp cells altogether. However, in 1% collagenase V, all cells types showed comparatively better survival (>10%) except haemocytes, which was less than 10%.

2.4. Discussion

On surveying the literature it has been observed that shrimp cell line development has not yet attained considerable success mainly because of the absence of an appropriate growth medium. Even though medium composition was very important among the factors which affect the proliferation of cells from primary cultures (Mitsuhashi, 2001), what has been done so far was to modify and use the available media which otherwise had been designed for mammalian cell culture systems. Among the commercially available cell culture media used Leibovitz's -15 (L-15) has been the most popular one (Chen *et al*., 1986,1988, 1989; Nadala *et al*., 1993; Lu *et al*., 1995a; Tapay *et al*., 1995; Tong and Miao, 1996; Toullec *et al*., 1996; Mulford and Austin, 1998; Chen and Wang, 1999; Shike *et al*., 2000; Wang *et al*., 2000; Kumar *et al*., 2001; Shimizu *et al*., 2001; Chun-Lei *et al*., 2003; Maeda *et al*., 2003, 2004; Jiang *et al*., 2005; Jose *et al*., 2010, 2011, 2012). Other media such as M199 (Ghosh *et al*., 1995; Toullec *et al*., 1996; Itami *et al*., 1999; Shimizu *et al*., 2001; Lang *et al*., 2002a), MPS (Tong and Miao, 1996; Fan and Wang, 2002; Hu *et al*., 2010), and Grace's insect medium (Luedeman and Lightner, 1992; Nadala *et al*., 1993; Toullec *et al*., 1996; Wang *et al*., 2000; George and Dhar, 2010; George *et al*., 2011; Jose *et al*., 2012) were also tested alone as well as with additives for growth of shrimp cells *in vitro*.

Seawater based shrimp cell culture medium (SCCM) was formulated in 27‰ natural seawater as the base since the isosmotic point of salinity for *P. monodon* had indirectly been calculated (Dall, 1981) to be 27‰ by comparing with its coexist *P. merguiensis*. Earlier to this, Mulford *et al*. (2001) had prepared L-15 medium in 25‰ seawater and found effective for cell migration, survival, and cell longevity and claimed rapid migration of cells from explants of hematopoietic tissue of the lobster *Nephrops norvegicus*. Meanwhile, in our study the experiment with artificial seawater was not found to be as effective for growth and proliferation of cells as those in natural seawater.

The great success achieved in the development of insect cell line by Grace (Grace, 1962) was due to the contributions by Wyatt *et al*. (1956) in insect biochemistry. May be motivated by them, Najafabadi *et al.* (1992) and Shimizu *et al*. (2001) attempted to investigate the biochemistry of haemolymph from *P. aztecus* and *P. stylirostris* and modified commercially available L-15 to suit the requirement of shrimp cell culture *in vitro*. Following these lines we undertook determination of the composition of haemolymph of *P. monodon* and quantified 15 fatty acids, 16 amino acids and 10 metal ions, which were hitherto not recorded in this species. Taking queues from this information we formulated a novel shrimp cell culture medium having natural seawater as the base. This was due to the fact that the haemolymph metal ions such as sodium (6784.3±785.8 mg l^{-1}), potassium (524.5±157.9 mg l^{-1}) and calcium (488.8±107.9 mg l^{-1}) were within the range of 27‰ seawater where they were 8075.5±260.9 mg l^{-1}, 512.935±73.2 mg l^{-1} and 443.7±63.1 mg l^{-1} respectively. These values supported the results of Najafabadi *et al*. (1992) who observed 6188.00±795.6 mg l^{-1} sodium, 281.5±62.6 mg l^{-1} potassium and 410±7 mg l^{-1} calcium in the haemolymph of *P. aztecus*. In *P. stylirostris* (Shimizu *et al*., 2001) the values were 8411.1± 549.7 mg l^{-1}, 328.44±50 mg l^{-1}, and 8439.2±44.3 mg l^{-1} respectively (Table 2). Concentration of iron, zinc and strontium in the haemolymph of *P. monodon* were 12.9±6.6 mg l^{-1}, 39.42±2.9

mg l^{-1} and 7.3±2 mg l^{-1}, respectively whilst in seawater it was 0.053±0.01 mg l^{-1}, 0.035±0.02 mg l^{-1} and 7.05±0.44 mg l^{-1}. However, Shimizu *et al*. (2001) estimated the concentrations of these elements in *P. stylirostris* haemolymph as 0.1±0.0 mg l^{-1}, 11.4±1.9 mg l^{-1} and 6.3±0.6 mg l^{-1}.

In our study, the addition of salts such as barium chloride, copper chloride, zinc sulphate, ferric citrate and manganese chloride and the organic supplements such as vitamin mixture, citric acid cycle intermediates, nitrogenous base and energy precursors to 27‰ natural seawater did not make any observable changes in the growth and multiplication of primary cell cultures over and above what has been observed in SCCM. This may be due to the availability of sufficient organic and inorganic elements from the supplemented fetal bovine serum (Freshny, 2000) and the elemental complexity in the natural seawater (Bruland *et al*., 1991).

Amino acid and fatty acid constituents in the haemolymph provided a basic knowledge about the concentration to be used in the new medium. Accordingly various concentrations of the amino acid mixture were tried to find out the most appropriate one which provided maximum cell attachment, multiplicity and survival. Despite the requirement of 0.02% cholesterol for better performance of the shrimp cell culture in SCCM, the addition of lipid mixture by and large did not make any observable changes. In similar lines Kasornchandra *et al*. (1992) and George and Dhar (2010) had recommended 0.01% cholesterol for better performance of shrimp cell culture in media.

Even though the maintenance of proper pH is essential for the successful growth of cells (Nadala *et al*., 1993) no direct measurements of haemolymph pH of *P. monodon* or any penaeid has been published. However, Huang *et al*. (1999) calculated in culture flasks, the optimum pH for hepatopancreatocytes of *Penaeus chinesis* as 6.5 with a suggested range of 6.0 to 7.2. Meanwhile, most researchers selected a pH within the range of 7 to 7.2 (Tong and Miao, 1996; Fan and Wang,

2002; Jiang *et al*., 2005) and 6.8 to 7.2 (Chen and Wang, 1999: Kumar *et al*., 2001). In the present study pH of SCCM was selected as 6.8 within a range of 6.8-7.2 where the medium remained clear without any precipitation supporting better growth of the cell cultures.

Osmolality of the medium was fixed at 720±10 mOsm kg^{-1} in accordance with the haemolymph osmolality, which was found to be 730.5±51.2 mOsm kg^{-1} and the cells *in vitro* were in isosmotic state at this osmolality as judged by visual observation. This was supported by the findings of Kasornchandra *et al*. (1992) and Fraser and Hall (1999), who used 730 ± 10 and 720 ± 20 mOsm kg^{-1} respectively for maintaining cells *in vitro* from *P. monodon*.

In SCCM, we could maintain ovarian cell culture for 63±6 days, lymphoid cells for 85±9 days during which they showed better proliferation among all the cell types tested, and also as evidenced through MTT assay with significant increase in cell metabolic activity (p <0.05). They exhibited better survival (40%) after two passages. Further, cell cultures could be maintained from hepatopancreas for 25±5 days, heart for 29±1 days, haemocytes for 10±3 days, testis for 21±3 days, eyestalk for 9±2 days, nerve cord, and cells from nauplii for 6±1 days and muscle for 7±1 days in SCCM. Several authors claimed to have maintained ovarian cell culture for 45 to 66 days (Maeda *et al*., 2003; George and Dhar, 2010), to several months (Tong and Miao, 1996; Toullec *et al*., 1996). Lymphoid cell culture was reported to be maintained for 54 days (Itami *et al*., 1999) to 3 months (Tong and Miao, 1996), hepatopancreas for 30 days (George and Dhar, 2010), heart tissue for 4 days (Chen and Wang, 1999) and haemocytes for 48 days (George and Dhar, 2010). Even though, Toullec *et al*. (1996) maintained embryonic culture for several months, Chun-Lei *et al*. (2003) maintained nerve cells for 15 days and Nadala *et al*. (1993) for 3 months such results were not found to have been reproduced by other workers for reasons not known.

Growth and multiplication of cells in SCCM were compared with those in other media based on MTT assay. The lymphoid cells grown in SCCM exhibited an increase of 107% growth in comparison with 2x L-15, and 59% and 82% in comparison with modified L-15 (Jose *et al.*, 2010) and Grace's insect medium respectively (p <0.05). Meanwhile, ovarian cells showed an increased growth of 45%, 37% and 36% (p <0.05) in SCCM in comparison with 2x L-15, modified L-15 (Jose *et al.*, 2010) and Grace's insect medium respectively. However, increase in growth and multiplication of cells from heart, hepatopancreas and haemolymph in SCCM was less than 20% compared to the same in the other media.

The passaging of primary shrimp cell cultures and their survival have been found to be the most difficult tasks (Fraser and Hall, 1999; Chun-Lei *et al.*, 2003). However, we could passage lymphoid cell culture twice using shrimp dissociation cocktail. But only about 40% cells were found surviving after the passage as evidenced by visual observation. Even though, lymphoid cell culture were reported to be passaged 3 times (Chen and Wang, 1999) to 44 times (Tapay *et al.*, 1995), neither confirmation of their results nor adoption of methods by other shrimp research labs could be found reported elsewhere.

From the difficulties experienced by researchers over the last few decades, it is obvious that several untold hindrances stand on the way of the development of shrimp cell lines, for that matter crustaceans altogether. The major unsettling fact was that the media used for the study were mostly by the modification of commercially available ones which were designed mainly for supporting cells from mammalian origin or mostly for the terrestrial animals. Virtually, application of none of the media could end up with a lead in cell line development specific for shrimp cell culture. Availability of growth medium and optimal conditions for supporting cell cultures could be one of the major possible ways to tackle the issue of immortalization. In this context the medium developed here gives the lead to work towards attainment of immortality of shrimp cell culture especially from lymphoid tissue. However, it may require further improvisation.

In conclusion, through this study, a seawater based novel cell culture medium for the development of shrimp cell culture has been formulated and validated with various tissues of *P. monodon*. The success in extending longevity, metabolic activity and the passaging efficiency of the cells grown in SCCM *in vitro* suggests that this medium should help researchers in the development and establishment of shrimp cell lines for various applications.

Table 1. Fatty acid profile of *P.monodon* haemolymph.

S.No	Retention time (RT)	Carbon atom	Fatty acid	Percentage
1.	2.808	10:0	Capric acid	0.07
2.	4.302	12:0	Lauric acid	0.17
3.	6.682	14:0	Myristic acid	1.28
4.	8.166	15:0	Pendadecylic acid	0.48
5.	9.782	16:0	Palmitic acid	29.30
6.	11.476	17:0	Margaric acid	1.95
7.	13.208	18:0	Stearic acid	13.84
8.	12.807	18.1 ω-9	Oleic acid	17.96
9.	12.719	18.2 ω -6	Linoleic acid	20.53
10.	12.485	18.3 ω -6	Linolenic acid	0.16
11.	14.939	19:0	Nonadecyclic acid	0.53
12.	16.651	20:0	Arachidic acid	0.46
13.	16.250	20:1 ω -9	Eicosenoic acid	0.34
14.	16.184	20.2 ω -6	Eicosadienoic acid	1.31
15.	15.605	20.4 ω -6	Arachidonic acid	2.58

Table 2. Comparison of the metal ion composition of *P. monodon* haemolymph with *P. stylirostris*, *P. aztecus* and seawater (27‰).

S.No	Parameter analyzed	Seawater 27‰ (mg l^{-1})	Concentration in haemolymph (mg l^{-1})		
			P. monodon	*P. stylirostris* (Shimizu *et al.*, 2001)	*P. aztecus* (Najafabadi *et al.*, 1992
1.	Sodium	8075.5±260.92	6784.34±785.83	8411.1±549.7	6188.00±795.6
2.	Potassium	512.935±73.16	524.535±157.88	328.44±50.8	281.52±62.56
3.	Calcium	443.72±63.10	488.84±107.93	439.2±44.3	410±70
4.	Copper	BDL (<0.01)	148.595±34.50	81.6±13.9	
5.	Zinc	0.035±0.02	39.415±2.88	11.4±1.9	
6.	Barium	0.015±0.01	15.29±14.27	NA	
7.	Iron	0.053±0.01	12.98±6.63	0.1±0.0	
8.	Strontium	7.05±0.44	7.27±1.90	6.3±0.6	
9.	Manganese	BDL (<0.01)	0.325±0.10	NA	
10.	Boron	4.87±0.65	8.42±7.68	0.4±0.3	
11.	Osmolality (mOsm kg^{-1})	810±20	730.5±51.2	784±36	625±46

Table 3. Effect of different combinations of Shrimp Cell Culture Medium (SCCM) on primary cell culture of ovary (OV), lymphoid organ (LY), heart (HT), hepatopancreas (HP), haemocytes (HC), Nerve cord (NC), Testis (TS), nauplii (N), eyestalk (ES) and muscle (MS). BASE: All ingredients except FBS (Fetal bovine serum) and seawater. ASW: Artificial seawater; SW: Seawater (natural). The confluence of primary cell cultures was categorized as: + tissues/cells attached; ++ attached tissues/cells started multiplying; +++ and ++++ started monolayer formation, +++++ completed monolayer formation, – no attachment.

Exp.No	Media combinations pH 6.8, Osmolality 720±10 mOsm kg^{-1}	Primary cell culture growth and monolayer formation									
		LY	HT	HC	OV	HP	NC	TS	N	ES	MS
I	BASE + ASW+0% FBS	-	-	-	-	-	-	-	-	-	-
	BASE + ASW+1% FBS	-	-	-	-	-	-	-	-	-	-
	BASE + ASW+2.5% FBS	-	-	-	-	-	-	-	-	-	-
	BASE + ASW+5% FBS	-	-	-	-	-	-	-	-	-	-
	BASE + ASW+7% FBS	+	+	+	-	-	-	-	-	-	-
	BASE + ASW+10% FBS	++	+	+	+	+	-	-	-	-	-
	BASE + ASW+15% FBS	++	+	+	+	+	-	-	-	-	-
	BASE + ASW+20% FBS	++	+	+	-	-	-	-	-	-	-
II	BASE + SW+0% FBS	-	-	-	-	-	-	-	-	-	-
	BASE + SW+1% FBS	-	-	-	-	-	-	-	-	-	-
	BASE + SW+2.5% FBS	+	+	-	-	-	-	-	-	-	-
	BASE + SW+5% FBS	+	+	-	-	-	-	-	-	-	-
	BASE + SW+7% FBS	++	++	-	-	-	-	-	-	-	-
	BASE + SW+10% FBS	+++++	++++	+++	++++	+++	+	+	+	+	+
	BASE + SW+15% FBS	++++	++++	++	++++	+++	+	+	+	+	+
	BASE + SW+20% FBS	++++	++++	++	+++	++	+	+	+	+	+
III	BASE + SW 5‰ + 10% FBS	+++	+++	++	+	+	-	-	-	-	-
	BASE + SW10‰ + 10% FBS	+++	+++	++	+	+	-	-	-	-	-
	BASE + SW 15‰ + 10% FBS	+++	+++	++	+	+	-	-	-	-	-
	BASE + SW 20‰ +10% FBS	+++	+++	++	+	+	+	+	+	+	+
	BASE + SW 27‰ +10% FBS (SCCM)	+++++	++++	+++	++++	+++	+	+	+	+	+
	BASE + SW 30‰ +10% FBS	+++	+++	++	+	+	+	+	+	+	+
	BASE + SW 35‰+10% FBS	+++	+++	+	+	+	-	-	-	-	-
IV	SCCM	+++++	++++	+++	+++	+++	+	+	+	+	+
	SCCM+ Inorganic salts & trace elements	+++	++	+	++	+	+	+	+	+	+
	SCCM+ Supplement A	+++++	++++	+++	++++	++	+	+	+	+	+
	SCCM+ Supplement B	+++++	++++	+++	++++	++	+	+	+	+	+
	SCCM+ Supplement C	+++++	++++	+++	++++	++	+	+	+	+	+
	SCCM+ Supplement D	+++++	++++	+++	++++	++	+	+	+	+	+
	SCCM+ Supplement E	+++++	++++	+++	++++	++	+	+	+	+	+

Table 4. Effect of different cell dissociation agents on passaging primary cell cultures. Ovary (OV), lymphoid organ (LY), heart (HT), hepatopancreas (HP), haemocytes (HC). The confluence of primary cell cultures was categorized as: + <10% of cells passaged, ++ 10% of cells passaged, +++ 20% of cells passaged, ++++ 30% of cells passaged, +++++ >30% of cells passaged, – no survival of cells.

Exp.No	Cell dissociation agent	Cell type	Cells survived	Passage no
I	Collagenase type V	LY	++	1
		HT	++	1
		HC	+	1
		OV	++	1
		HP	++	1
II	Trypsin	LY	+	1
		HT	+	1
		HC	-	-
		OV	+	1
		HP	+	1
III	Accutase	LY	-	-
		HT	-	-
		HC	-	-
		OV	+	1
		HP	-	-
IV	Non enzymatic solution-I	LY	-	-
		HT	-	-
		HC	-	-
		OV	-	-
		HP	-	-
V	Non enzymatic solution-II	LY	-	-
		HT	-	-
		HC	-	-
		OV	-	-
		HP	-	
VI	"Cocktail"	LY	+++++	2
		HT	++++	1
		HC	+	1
		OV	++++	2
		HP	+++	1

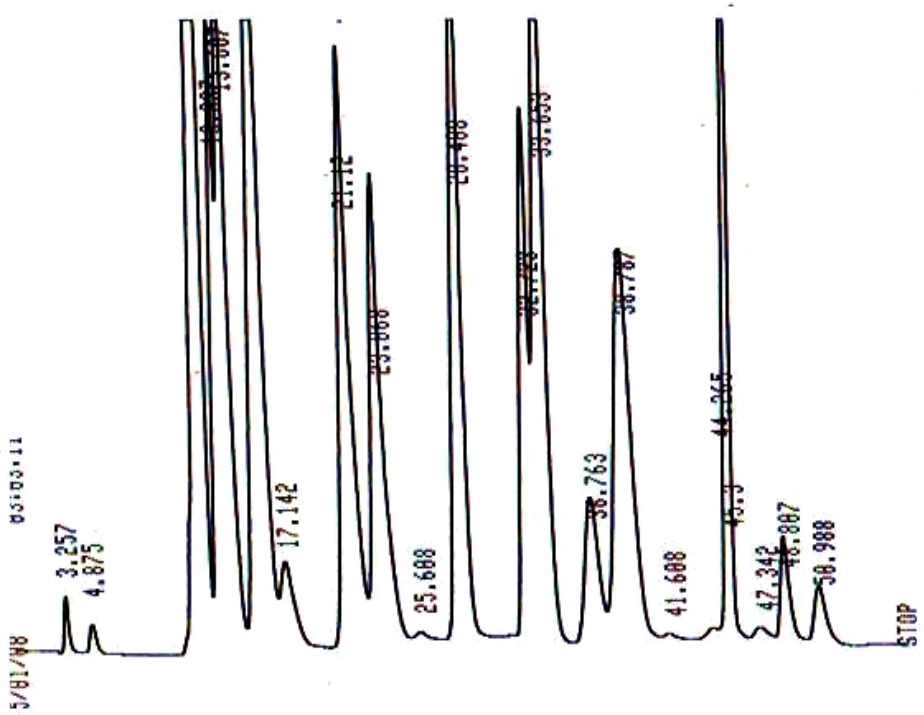


Fig. 1. Chromatogram of free amino acids from *P. monodon* haemolymph. Retention time (Rt) and the respective amino acids: 11.887-Aspartic acid, 13.037-Threonine, 13.067-Serine, 15.602-Glutamic acid, 17.142-Proline, 21.12- Glycine, 23.068-Alanine, 25.608-Cysteine, 28.408-Valine, 32.723- Isoleucine, 33.653-leucine, 36.763-Tyrosine, 38.763-Phenyl alanine, 45.3- Histidine, 48.807-lysine, 50.988-Arginine.

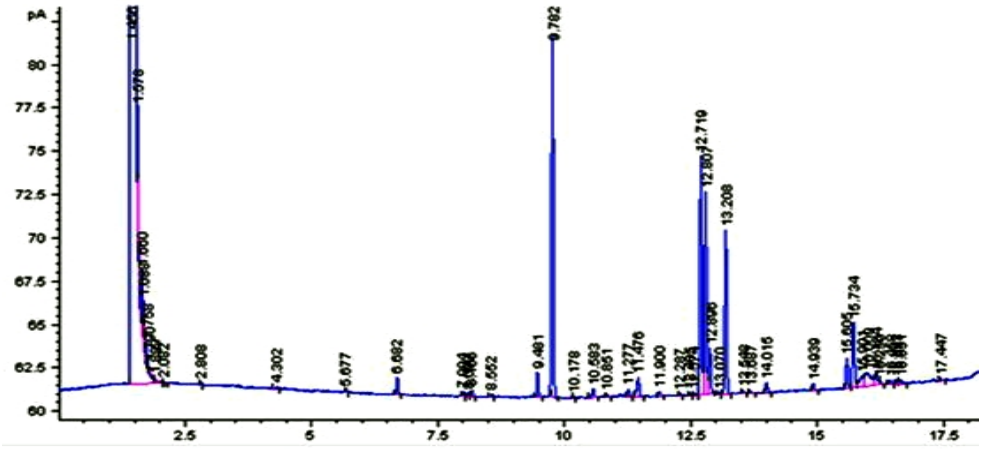


Fig. 2. Gas chromatogram of haemolymph fatty acid from *P. monodon*.

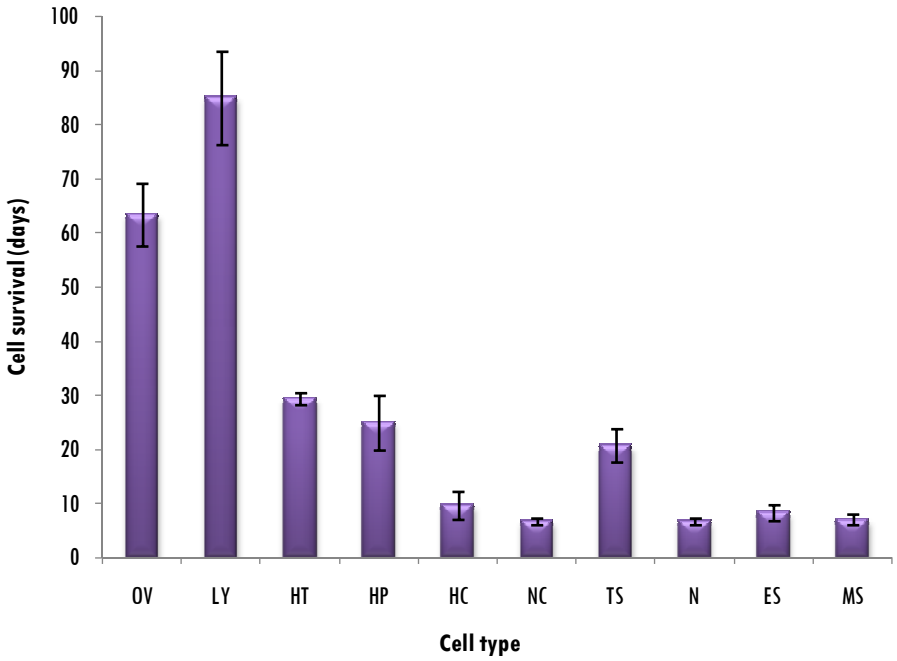


Fig. 3. Survival of primary cell cultures of ovary (OV), lymphoid organ (LY), heart (HT), hepatopancreas (HP), haemocytes (HC), Nerve cord (NC), Testis (TS), nauplii (N), eye stalk (ES) and muscle (MS) of *P. monodon* cultured in SCCM supplemented with 10% FBS.

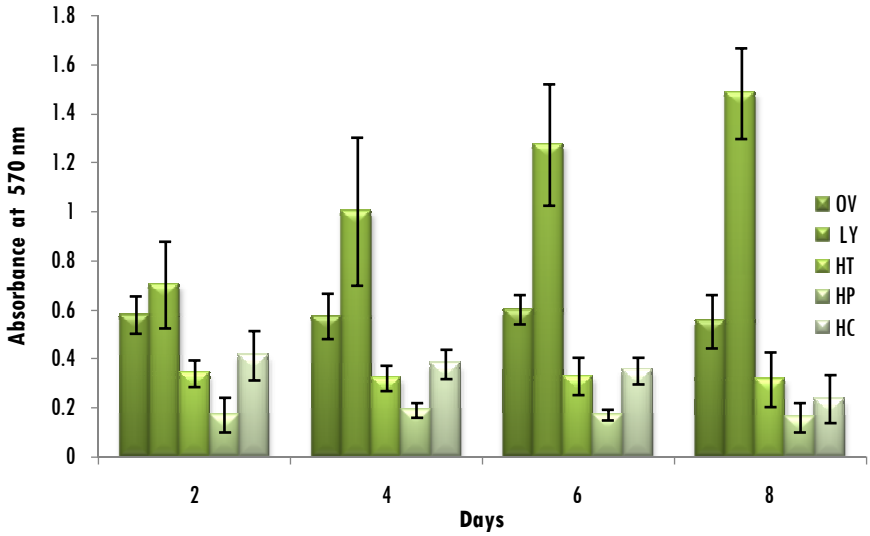


Fig. 4. Viability (in terms of MTT assay) of primary cultures of ovary (OV), lymphoid organ (LY), heart (HT), hepatopancreas (HP) and haemocytes (HC), of *P.monodon* cultured in SCCM supplemented with 10% FBS.

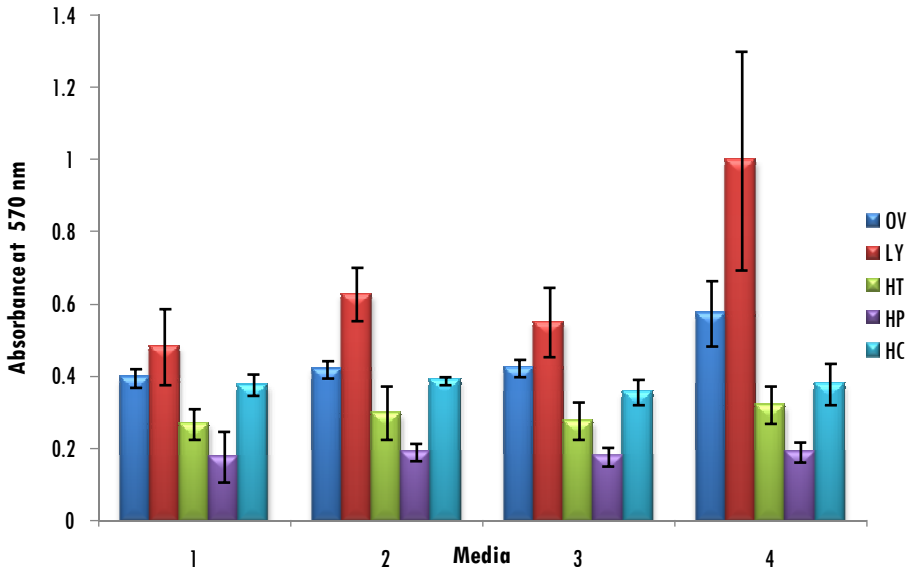


Fig. 5a. Effect of different media on *in vitro* growth (4th day results by MTT assay) of different cell types from *P. monodon*. 1: L-15+10% FBS; 2: Modified L-15; 3: Grace's insect medium +10% FBS; 4: SCCM+10% FBS. OV: ovary, LY: lymphoid organ; HT: heart; HP: hepatopancreas and HC- haemocytes.

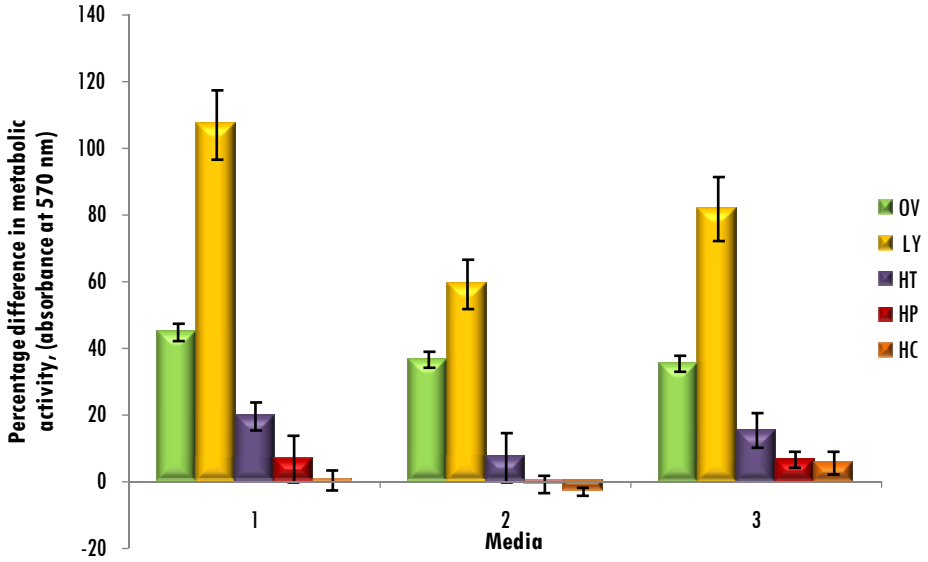


Fig.5b. Percentage difference in the metabolic activity (4th day results by MTT assay) of various cell types grown in SCCM in comparison with selected media. 1: L-15+10% FBS; 2: Modified L-15; 3: Grace's insect medium +10% FBS. OV: ovary; LY: ymphoid organ; HT: heart; HP: hepatopancreas and HC- haemocytes.

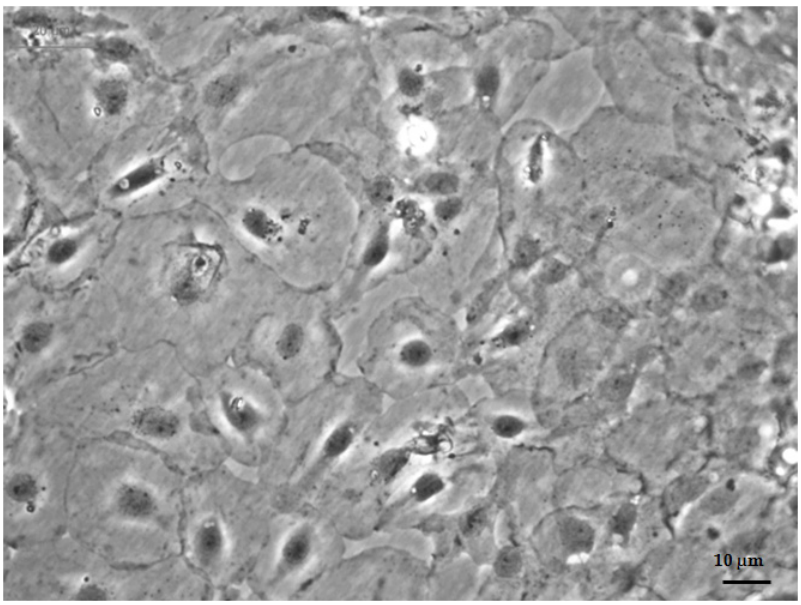


Fig.6. Primary cell culture developed from *P. monodon* lymphoid cells in SCCM.

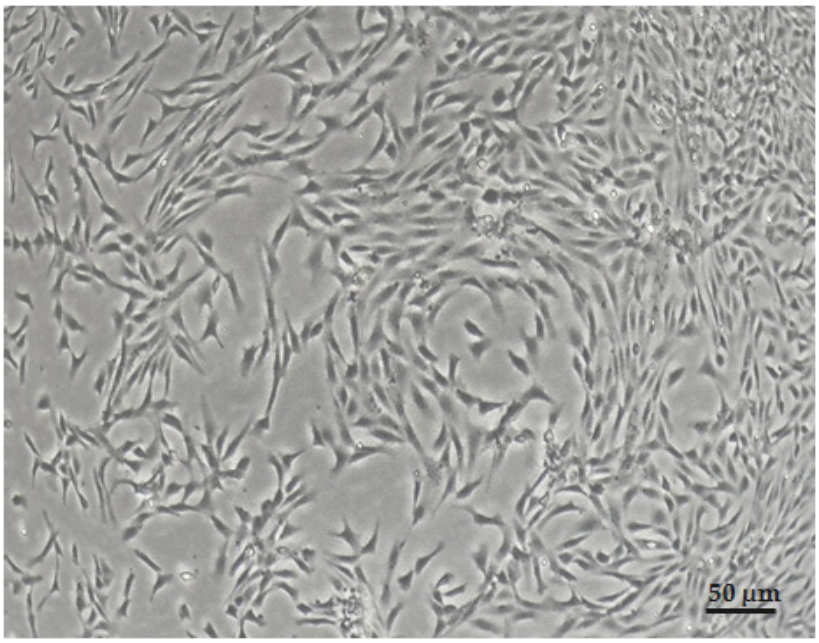


Fig.7. Primary cell culture from *P. monodon* haemocytes grown in SCCM.

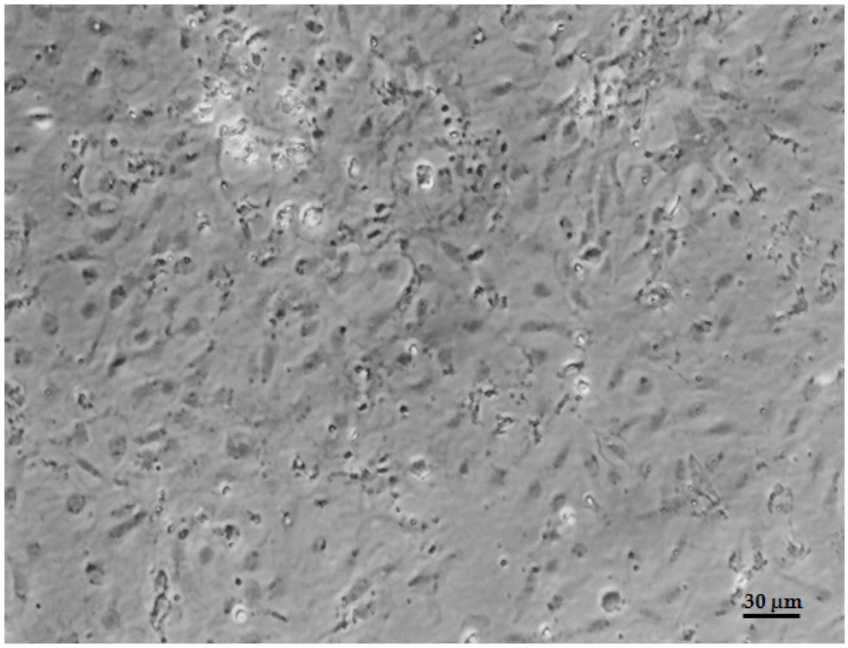


Fig. 8. Primary cell culture developed from *P. monodon* heart cells in SCCM.

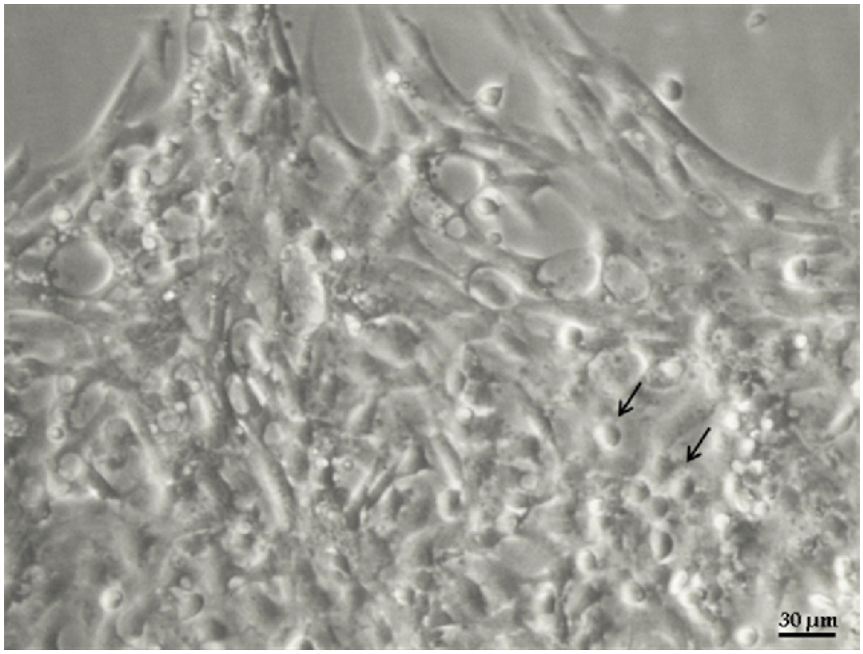


Fig. 9. Primary cell culture in SCCM developed from ovarian cells of *P. monodon*. Proliferating fibroblastic and round cells (black arrow) can be seen.

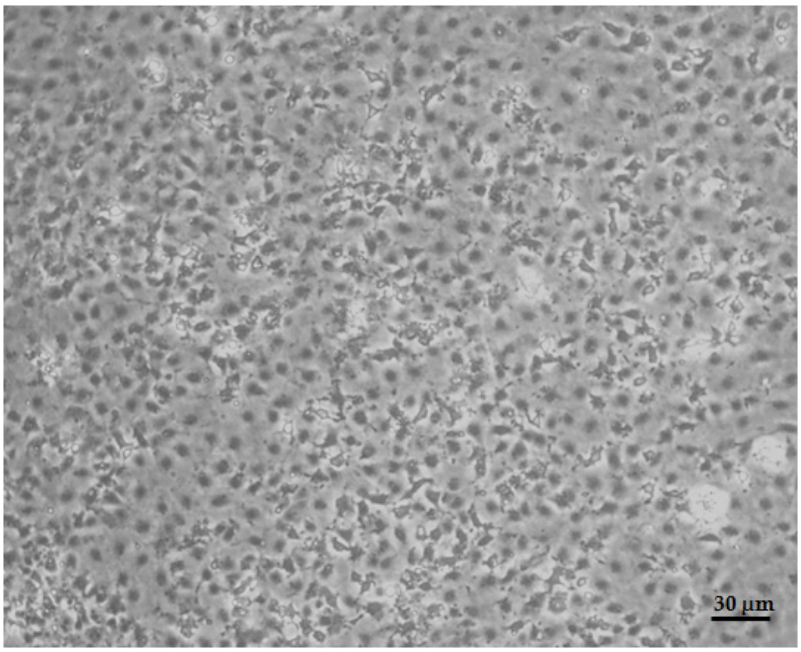


Fig. 10. Primary cell culture developed from hepatopancreatic cells of *P. monodon* in SCCM.

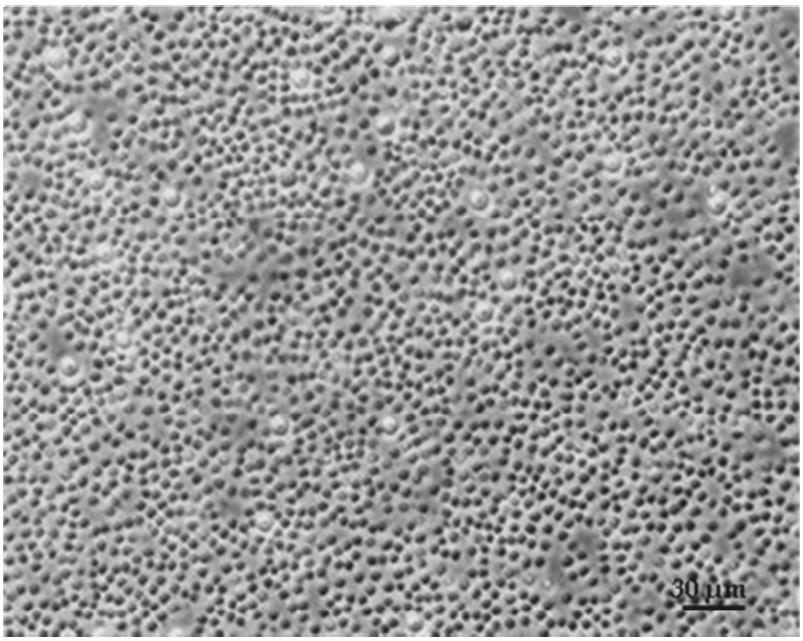


Fig. 11. Primary cell culture developed from *P. monodon* testicular cells in SCCM.

Chapter 3

Screening and optimization of growth factors and their potential impacts on lymphoid cell culture: Cellular activity and viral susceptibility

3.1. Introduction

Growth factors are protein or steroid hormone with cell stimulating property on growth, proliferation and differentiation, and have been considered as the choice of ingredients in shrimp cell culture medium for rapid cellular growth and proliferation (Nadala *et al.*, 1993; Hsu *et al.*, 1995; Fan and Wang, 2002). In addition, the growth factor - co induction has been considered as another option for establishing cell lines, alternative to transgenic technology with introduced oncogenes (Fan and Wang, 2002). Besides, the membrane receptors for each growth factor being the major limiting factor for the stimulation of signal transduction (Fan and Wang, 2002), single and the multifactorial interaction between the growth factors and their potential impacts on shrimp cell cultures have great importance for their optimization. In addition, each cell type requires a specific condition for growth and proliferation (Freshny, 2000); therefore it is necessary to optimize the growth factor requirements for each cell type with different lineage. Considering the above, application of growth factors has been considered as a promising tool for developing shrimp cell lines (Jayesh *et al.*,

2012). Lymphoid cell culture has been chosen taking in to account its importance as platform for studies on white spot syndrome virus (Jose *et al.*, 2012).

The development, formulation and preparation of the shrimp cell culture medium (SCCM) has been explained in the previous chapter (Chapter 2). However, as the cells with different lineage requires specific growth factors for cellular growth and proliferation (Freshny, 2000), a thorough investigation on growth factors is vital to achieve improved growth and multiplication of lymphoid cells *in vitro*.

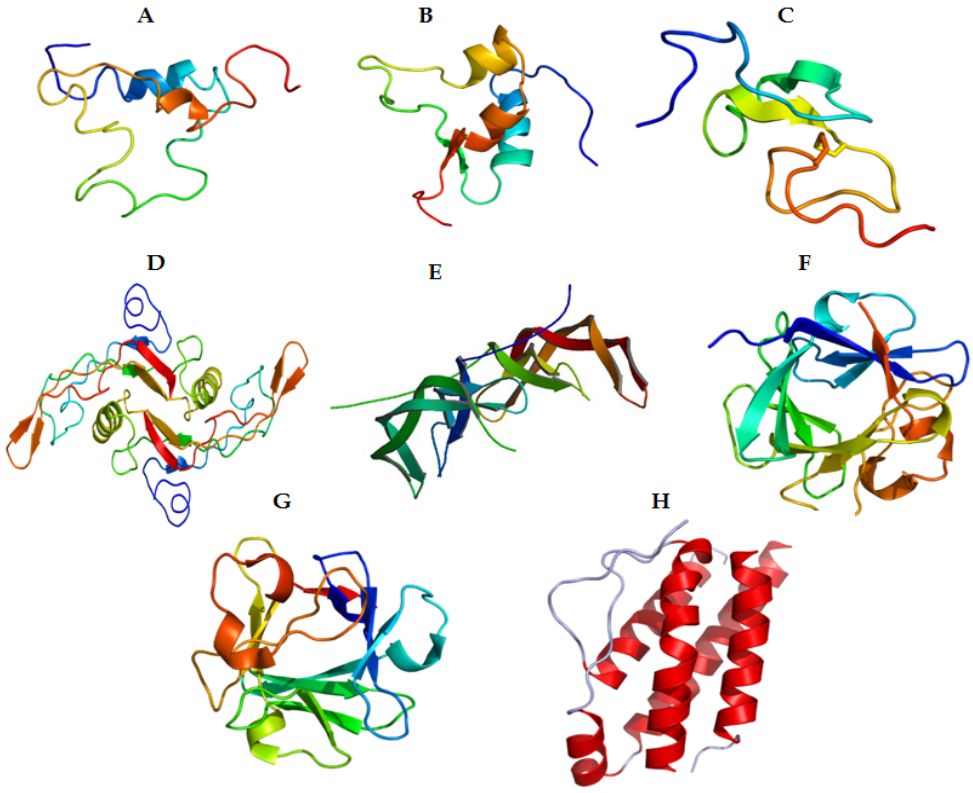


Fig. 1. Protein structure of growth factors used for this study. A: Insulin like growth factor-I (IGF-I); B: Insulin like growth factor-II (IGF-II); C: Epidermal growth factor (EGF); D: Transforming growth factor-β1 (TGF-β1); E: Platelet derived growth factor (PDGF); F: Fibroblastic growth factor-4 (FGF-4); G: Fibroblastic growth factor-basic (bFGF); H: Interleukin-2 (IL-2) (results from protein data bank).

There are two ways by which selection of appropriate growth factors for a growth medium could be addressed: a) classical and b) statistical. Classical experimental design requires only one growth factor being changed in the growth medium at a time to determine its contribution in cellular activity. Even though the classical way of screening is tedious and time-consuming, considering the importance to study the effect of individual growth factor on lymphoid cell culture, classical screening procedure of the selected nine growth factors has been performed in the present study, followed by the statistical screening. In the statistical screening protocol the widely accepted medium optimization tool, Plackett-Burman design (Plackett and Burman, 1946) was used as multifactorial statistical design (Stanbury *et al.*, 1986) that efficiently screened the important factors among a large number of variables and accounted for the interactions between the variables. (Srinivas *et al.*, 1994; Yu *et al.*, 1997; Krishnan *et al.*, 1998; Son *et al.*, 1998; Reddy *et al.*, 1999; Preetha *et al.*, 2007). Till date, the application of Plackett - Burman design for screening growth factors for enhanced growth and proliferation of cells *in vitro* has not been applied elsewhere.

After finding the critical components among the nine growth factors through Plackett- Burman design, the next step was to optimize the concentrations of these growth factors in the medium. Response surface methodology (RSM) using a central composite design (CCD) (Box and Wilson, 1951) was used to optimize the concentration of growth factors for formulating the growth medium (Preetha *et al.*, 2007). Growth factors induced metabolic activity during the classical as well as statistical screening was evaluated using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay. The performance of mitotic activity of the optimized medium was evaluated using DNA synthesis markers (5-Bromo-2'-deoxyuridine) and that was compared with the control (SCCM without growth factors) as well as routinely used commercial medium (L-15).

After optimizing the growth factors, activity of lymphoid cells at cellular and molecular level was evaluated to confirm the potential utility of the medium for immortalization, and as a tool for WSSV isolation. The molecular and cellular studies were, observation of mitotic events, DNA synthesis, cell cycle genes expression profiling (partial), cytoskeleton studies, and assay for metabolic activity which included XTT assay, besides glucose assimilation and protein synthesis. Moreover, the virus susceptibility test using WSSV was also performed to confirm its application as a platform for such studies.

The ability to coordinate environmental sensing with appropriate cell-fate decisions is a pre-requisite for the successful growth and survival of each cell *in vitro*. The response of lymphoid cells cytoskeleton (actin) with the nutrient medium (SCCM) was studied, because the actin microfilaments involved has potential link between nutritional sensing machinery of cells and the medium (Leadsham *et al*., 2010). Moreover, besides its normal structural function such as providing cell shape, cell movement, and cell– cell and cell–substratum interactions (Gotlieb and Lee, 1999; Searles *et al*., 2004) and the contractile force for tissue and cells (Kreis and Birchmeier, 1980; Pellegrin and Mellor, 2007), these microfilament bundles have been proven to undergo dynamic changes in response to physiological and pathological stresses, and are involved in cytoplasmic mRNA metabolism including transportation and its localization (Hesketh, 1996; Searles *et al*., 2004). In this line, the cytoskeleton (F-actin) organization in lymphoid cells grown in the novel SCCM medium was observed by using Phalloidin TRITC staining techniques.

Evaluation of cellular metabolic activity is an important index for assessing the quality of the medium, as they grow on the solid-liquid (medium) interface on culture flask. In this study, for the accomplishment of evaluating the metabolic activity, mitochondrial dehydrogenase activity was measured by reduction of 2, 3-bis [2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT)

(Scudiero *et al.*, 1988), protein synthesis by affinity of electrostatic binding of protein with Suforhodamine B dyes (Vichai and Kirtikara, 2006) and the cell metabolism by determining glucose assimilation were carried out. Since the cell cycle events occur under a favorable condition, the genes involved in cell cycle such as gene controlling transcription elongation factor, cell division cycle 2 protein, cyclin A, cyclin B, mitotic check point protein and β-actin gene (as control) were analysed along with the evaluation of mitotic events and DNA synthesis (S-Phase entry) in primary lymphoid organ cell culture (*in vitro*).

As the lymphoid organ probably is a prime target and site for replication of most systemic viruses (Rusaini and Owens, 2010), development of shrimp cell line from this organ has much significance. In addition, lymphoid organ cell culture system has been claimed as the best option for *in vitro* growth of several pathogenic viruses (Jayesh *et al.*, 2012). In this study, the susceptibility of lymphoid cell culture to white spot syndrome virus (WSSV) was tested and confirmed by the cytopathic effect (CPE) , and further confirmed by detecting 28 and 18 kDa WSSV proteins by immunofluorescence detection using WSSV C-38 monoclonal antibody (Anil *et al.*, 2002).

In spite of 25 years of research (Jayesh *et al.*, 2012) on shrimp cell culture, and despite the development of primary cell cultures from various tissues and organs (Chen *et al.*, 1986; Jose *et al.*, 2012), a permanent cell line from shrimp could not be accomplished yet by the global scientific community. Undoubtedly, a stable cell line is a prime requirement to address viral infections in shrimp at cellular and molecular level. In the previous chapter (Chapter 2), descriptions were given of a novel shrimp cell culture medium (SCCM) designed and developed, and found that the lymphoid cells had performed better in this medium with respect to their proliferation, survival and longevity. In this context, a study was undertaken to improvise the medium, SCCM, based on growth factor requirements of lymphoid cells to grow in to cell culture leading to its possible *in vitro* transformation in to a cell line.

3.2 Materials and methods

3.2.1. Experimental animals

Shrimps for the experiments were maintained in recirculation aquaculture system (RAS) integrated with nitrifying Bioreactor (Kumar *et al*., 2009, 2011) maintained at 27‰. Post larvae, nested PCR negative to WSSV were stocked in the system and reared for three months maintaining water quality parameters within a narrow range (pH 6.8-7.8; total ammonia <1 mg l^{-1}; nitrite <0.1 mg l^{-1}; total alkalinity ($CaCO_3$) 75-125 mg l^{-1}; total hardness > 5000-6000 mg l^{-1}) fed on pelleted feed containing 40% protein, 3% fat, 12% fiber, 18% ash and 12% moisture. Shrimps weighing 15-20 g were used as the donor animals for lymphoid cells (Jose *et al*., 2012).

3.2.2. Development of primary lymphoid cell cultures

Lymphoid organ, consisted of two distinct lobes located dorso-anterior to the ventral side of hepatopancreas (Bell and Lightner, 1988), found exclusively in penaeids (Rusaini and Owens, 2010) was dissected-out for the experiment. Prior to dissection, the animals were chilled in ice, surface disinfected by immersion in 800 mg l^{-1} sodium hypochlorite solution in chilled seawater for 10 min followed by washing in sterile seawater. Lymphoid organ was removed aseptically and collected to holding medium of 720 mOsm kg^{-1} (SCCM without FBS), washed three times with PBS and minced in to very small pieces using sterile surgical blade. The clumps of tissue were separated using cell dissociation sieve (CD-1, Sigma) with a 60 mesh screen (Mulford *et al*., 2001); the suspension was mixed thoroughly with the medium and seeded on to 96 well plates/ 25 mm^2 culture flask/dishes (Greiner Bio-One) depending on the experiments to be followed, incubated at 25 °C as an open system without CO_2 in the atmosphere (Jose *et al*., 2011, 2012). With the primary cell culture thus developed, the growth factors were screened based on the metabolic activity. Moreover, performance of the primary

cell culture in the novel medium (SCCM), novel medium supplemented with growth factors and modified L-15 medium (Jose *et al*., 2011) were compared using MTT assay (Mosmann, 1983).

3.2.3. Experimental design for screening and optimization of growth factors

Primary screening (one-variable-at-a-time) of growth factors was carried out by measuring its contribution on metabolic activity or cell respiration followed by the statistical screening and optimization. One-factor-at-a-time classical experimental design required experimenting one growth factors at a time to determine its effect. All growth factors were dissolved in various solvents as per manufacture's instruction (Sigma), diluted with shrimp cell culture medium (SCCM) to get the desired final concentrations. The details of growth factors and their preparation are given in the section below (section 3.2.3.1.). After incubation MTT assay was carried out and the metabolic activity (as absorbance) was compared with that of the control. Primary screening was followed by statistical screening using Plackett-Burman mutifactorial design and the maximum and the minimum concentrations of growth factors that contributed to the metabolic activity of the cells were selected for Central Composite Design (CCD) in Response Surface Methodology (RSM).

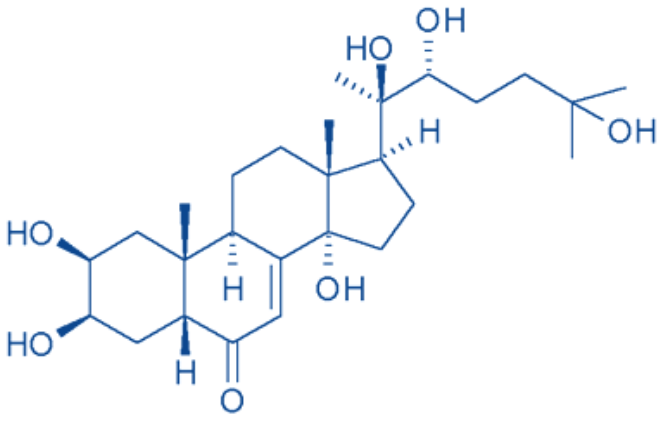


Fig. 2. Chemical structure of steroid hormone, 20-Hydroxyecdysone (20HE).

3.2.3. 1. Growth factors and their preparation

Recombinant human Insulin like growth factor-I and II (IGF-I and IGF-II) expressed in *E. coli* with a molecular weight of 7.6 kDa, epidermal growth factor (EGF) with a molecular weight of 6 kDa from mouse sub-maxillary glands, transforming growth factor-β1 (TGF-β1) from porcine platelets, platelet derived growth factor (PDGF) with a molecular weight of 28-31 kDa from human platelets, recombinant human fibroblast growth factor-4 (FGF-4) expressed in *E. coli* with a molecular weight of 19 kDa, fibroblast growth factor-basic (bFGF) with a molecular weight of 16-18 kDa from bovine pituitary glands, recombinant human interleukin-2 (IL-2) expressed in *E. coli* with a molecular weight of 15.5 kDa and the insect ecdysteroid hormone, 20-Hydroxyecdysone (20HE) were used for this study (Fig. 1 & 2). All growth factors were purchased from Sigma Aldrich, USA. Primary stock solutions of bFGF and EGF were prepared in the growth medium containing 10% FBS, while IGF-I and FGF-4 were prepared in phosphate buffered saline (PBS), IGF-II in 10 mM acetic acid containing 0.1% bovine serum albumin (BSA), interleukin-2 in 100 mM acetic acid, TGF-β1 and PDGF in 4 mM HCl containing 0.1% BSA and 20HE in ethyl alcohol (Jose *et al.*, 2012).

3.2. 3. 2. Primary screening of growth factors - One-factor-at-a-time (Classical method)

The nine growth factors were dissolved in appropriate solvent as per the details given above (3.2.3.1.). Subsequently all of them were diluted with shrimp cell culture medium to get the final concentration of 2, 4, 6, 8, 10, 25, 50, and 100 ng ml^{-1}. The Platelet derived growth factor was diluted up to 10 ng ml^{-1} (2, 4, 6, 8, 10 ng ml^{-1}). An aliquot of 100 µl growth factor containing 2x concentration growth factor was added to 96 well plate seeded with 100 µl lymphoid cell suspension. The plates were incubated at 25°C for 48 h. After incubation, the medium was changed and added 50 µl MTT solution (5 mg ml^{-1}) prepared in PBS (720 mOsm kg^{-1}) and kept for incubation (in dark) at 25 °C for 5 h. The entire medium was

removed and added 200 µl dimethyl sulphoxide (DMSO, HiMedia, Mumbai). Mixed the wells using pipette and the formazan crystals were dissolved, absorbance measured at 570 nm using microplate reader (Infinite M-200 Tecan, Austria) and the results obtained were compared with control wells and depicted.

3.2.3.3. Statistical screening and optimization of growth factors by Plackett-Burman factorial design and central composite design using response surface methodology (RSM)

Response Surface Methodology (RSM) is an empirical statistical modeling technique employed for multiple regression analysis using quantitative data obtained from properly designed experiments to solve multivariable equations simultaneously (Preetha *et al*., 2007), and was first described by Box and Wilson (1951). It has been used for the optimization of a particular response that is influenced by significant (multiple) variables, effect of individual variables and interaction effects between the variables (Beg *et al*., 2003; Bas and Boyaci, 2007; Preetha *et al*., 2007; Wang *et al*., 2007). Moreover, this experimental methodology generates a mathematical model which describes the chemical or biochemical processes (Myers and Montgomery, 1995; Anjum *et al*., 1997; Bas and Boyaci, 2007). The model used in RSM is generally a full quadratic equation and second order model can be written as follows:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\, \beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j,$$

where Y represents the response variable, β_0 represents the interception coefficients, β_i is the coefficient of linear effect, β_{ii} is the coefficient of quadratic effect and β_{ij} is the coefficient of the interaction effect (Bas and Boyaci, 2007; Jian and Nian-fa, 2007; Venil *et al*., 2009). The relationship between the response and the input is given in the equation

$$\eta = f(x1, x2 \ldots\ldots x_n) + \varepsilon$$

where η is the response, *f* is the unknown factor of response, x1,x2…..xn denotes independent variables, n is the number of independent variables and finally ε is the statistical error that represents other source of variability not accounted for by *f*. These sources include the effects such as measurements error. It is generally assumed that ε has a normal distribution with mean zero and variance (Bas and Boyaci, 2007).

Statistical screening experiments were used to identify the independent parameters of growth factors using factorial designs. After identification of important growth factors that contribute to the metabolic activity of cells, next step was to determine the level of parameters important for a successful optimization. Moreover, all the parameters should be normalized before regression analysis. In such case, each variable was coded within a range from -1 to +1 for more evenly response irrespective of the parameters used. Commonly used equation for coding is represented as follows:

$$X = \frac{x - [x_{max} + x_{min}] / 2}{x_{max} - x_{min} / 2}$$

Where, x is the natural variable, X is the coded variable and x_{max} and x_{min} are the maximum and minimum values of the natural variable (Bas and Boyaci, 2007). Moreover, the design was based on the following first order model:

$$Y = \beta_0 + \sum \beta_i x_i$$

Where, Y represents the yield (metabolic activity), β_0 is the model intercept, β_i is the linear coefficient, *xi* is the level of the independent variable (Liu *et al.*, 2010; Mukherjee and Rai, 2011).

Plackett-Burman is useful in decreasing the number of variables and number of experiments in further optimization step (He *et al.*, 2009; Fakhfakh-Zouari *et al.*, 2010; Liu *et al.*, 2010; Tiwary and Gupta, 2010; Mukherjee and Rai,

2011). As the membrane receptors were considered to be the limiting factor for stimulation of signal transduction (Fan and Wang, 2002), the use of Plackett-Burman allows selection of the most significant growth factors that contribute to metabolic activity of lymphoid cells and elimination of unwanted growth factors.

A central composite design (CCD) of RSM is used to estimate coefficients of quadratic models and consists of three groups of design points: a) Two-level factorial or fractional design points - all possible combinations of the +1 and -1 levels of the factors (2^k), b) Axial points or star points - all of the factors set to 0, the midpoint, except one factor, which has the value +/- alpha. However, in the case of axial points of face centered central composite design, all the factors are set to 0 (midpoint), except one factor, which is at the +1/-1 value (i.e., the star points are set at the face of the cube portion on the design), c) Center points - points with all levels set to coded level zero (midpoint) (Preetha *et al*., 2007; Oskouie *et al*., 2008).

RSM also involves the graphical representation of the model equation and determination of optimal concentration of the variable to be used. The predicted model equation can be obtained by the response surface plot or contour plot. The response surface plot is the three dimensional graphical representation showing the relationship between the response and the independent variables (Wang and Lu, 2004). The two dimensional display of the surface plot is called contour plot and in the contour plot, lines of constant responses are drawn in the plane of the independent variables and helps to visualize the shape of a response surface. Accordingly, when the contour plot displays ellipses or circles, the centre of the system refers to a point of maximum or minimum responses. Moreover, in the case of hyperbolic or parabolic shaped contours, the stationary point is called the saddle point and is neither a maximum nor a minimum point. Even though these plots give useful information about the model fitted, they may not represent the true behavior of the system (Myers and Montogomery, 1995).

After selecting the various growth factors by testing their significance in one - at - a time screening, Plackett-Burman statistical screening (Plackett and Burman, 1946) was employed to find out the most significant growth factor components that contributed to cellular metabolism. Insulin-like growth factor-I and II (IGF-I and IGF-II), interleukin-2 (IL-2), epidermal growth factor (EGF), transforming growth factor -β1 (TGF-β1), basal fibroblast growth factor (bFGF), fibroblast growth factor-4 (FGF-4) and the arthropod specific growth hormone (20-hydroxy ecdysone) were used to apply Plackett-Burman factorial design. Platelet derived growth factor (PDGF) was not included in this screening due to its inadequacy and the little contribution in the metabolic activity of the cells compared to the other growth factors tested. The above 8 growth factors were included for the screening with each one (variable) represented at two levels, high (+1) and low (-1). The response was measured as metabolic activity in terms of MTT assay. All the trials were carried out in triplicate and the average metabolic activity (MTT assay) of each trial was used as the response variable. Regression analysis determined the variables that had significant (p <0.05) effect on metabolic activity and those variables were evaluated for further optimization.

Response surface approach using Central Composite Design (CCD) was applied to find out the optimum levels of IGF-I and IGF-II and the effects of their interaction on the metabolic activity of lymphoid cells. The design provided 13 combination of IGF-I and IGF-II in which each run was performed in triplicate and the average metabolic activity in terms of MTT assay was taken as the experimental value of the dependent variable or response (Y), while predicted values of the response were obtained from quadratic model fitting. A multiple regression analysis of the data was carried out to define the response in terms of independent variables. The response surface graphs were obtained to understand the effects of variables individually and in combination and to determine their optimum levels for maximum activity. The data on induced metabolic activity was

subjected to analysis of variance (ANOVA). The soft ware, Design Expert (version 6.0.9, Stat-Ease Inc, Minneapolis, MN) was used for the experimental design, data analysis and quadratic model building. In this optimization process, the statistical results gave a contour plot and three dimensional surface responses with the predicted optimal value of the growth factors IGF-I and IGF-II to be used in the medium.

3.2.3.4. Validation of the model

The statistical model was validated with respect to metabolic activity contributed by the selected growth factors under the concentrations predicted by the model. Metabolic activity of the lymphoid cells was determined by MTT assay and the experiments were carried out in triplicates and compared with the predicted values and with the control. Cell viability depends on an intact mitochondrial membrane and the respiratory chain. MTT assay measures the mitochondrial dehyrogenase enzyme which reflects the metabolic activity of the cells. Succinate-tetrazolium reductase system which belongs to the mitochondrial respiratory chain reduces MTT ((3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) (Sigma-Aldrich Co.)) to water-insoluble formazan crystals (Xia and Laterra, 2006), solubilized in dimethyl sulphoxide (DMSO) yielding a purple-colored solution (Mosmann, 1983). Aliquots of 200 µl cell suspensions were seeded on to 96 well plates and after desired incubation period (24 h, 48 h, 72 h…etc), the medium was pipetted out and replaced with 50 µl MTT solution (5 mg $ml^{-1)}$ prepared in PBS (720 mOsm kg^{-1}), and the plate was kept for incubation (in dark) at 25 °C for 5 h. Removed the entire medium and added 200 µl DMSO, (HiMedia Laboratories, Mumbai) mixed well to confirm the dissolution of formazan crystals. Absorbance was measured at 570 nm in a microplate reader (Infinite M-200 Tecan, Austria) with reference wavelength at 690 nm.

3.2.4. Mitotic activity of the cells grown in growth factor optimized shrimp cell culture medium

5-Bromo-2'-deoxyuridine (BrdU) ELISA was performed to confirm the DNA synthesis in the lymphoid cells grown in growth factor optimized shrimp cell culture medium. The efficiency of the medium was compared with control as well as commercially available L-15 medium. An aliquot of 20 µl 10 mM BrdU solution was added to each well of 96 well plates containing primary lymphoid organ cell culture grown in basic SCCM, SCCM - growth factors optimized, and modified L-15 (Jose *et al*., 2010, 2011). ELISA was done after 48 h using the Cell Proliferation Biotrak ELISA System (Amersham Biosciences, UK). Medium was removed, fixed for 30 min and blocked using blocking buffer. An aliquot of 100 µl peroxidase conjugated anti BrdU solution was added to each well and incubated for 90 min at room temperature (RT). Wells were rinsed with washing buffer for three times and tetra methyl benzidene substrate was added to the wells immediately. After 30 min, reaction was stopped by adding 25 µl of 1 M sulphuric acid into each well and optical density was determined at 450 nm. Medium without cells but with BrdU and medium with cells but without BrdU were kept as controls.

3.2.5. Molecular cell biology of lymphoid cell culture grown in SCCM

3.2.5.1. Mitotic events in lymphoid cells *in vitro*

Mitotic events in the lymphoid cells grown in SCCM were evaluated by microscopic examination using Inverted phase contrast microscope (Leica, Switzerland) connected with time lapse imaging facility controlled by image acquisition software (LAS, Leica, Switzerland). This was mainly performed to establish some understanding that the new medium (SCCM) was supporting mitotic division of the cells *in vitro* (Lang *et al*., 2002a, 2002b). For the observation of mitotic events, epithelioid type cells emerged from lymphoid organ was selected because of its larger size and visibility of the nucleus compared to

fibroblast-like cells. Cultured cells were observed daily and the cell division processes in a single cell was tracked by marking on culture dish (Lang *et al*., 2002b) to take the image of the same cells accurately and quickly as possible. The photographs obtained were arranged with respect to the pattern of mitotic division in a eukaryotic cell.

3.2.5.2. Entry of lymphoid cells in to S-phase of cell cycle and DNA synthesis

Immunofluorescence detection of S-phase cells labeled with Bromo-2'-deoxyuridine (BrdU), a synthetic analog of thymidine that can be incorporated into deoxyribonucleic acid (DNA) during the S-phase of the cell cycle, has been used for analysis of DNA synthesis (Gratzner 1982). Primary lymphoid organ cultures grown in 96 well plates with 200 µl medium were selected for BrdU incorporation assay. A sample of 20 µl of l0 mM BrdU solution was added to each well and the ones without the addition of BrdU were kept as control. After 24 h incubation, the medium was removed, washed with PBS, fixed with 4% paraformaldehyde for 15 min and washed again with PBS, 2 M HC1 was added to each well, incubated for 20 min, neutralized with 0.1 M sodium borate (pH 8.5) for 2 min and washed with PBS. Cells were permeabilised with PBS containing 0.2% triton X-100 and 3% BSA for 5 min. After blocking with 3% BSA in PBS for 1 h, 1:1000 dilution (in 3% BSA) of the mouse monoclonal anti BrdU antibody (Sigma, USA) was added and incubated for 1 h. Cells were washed thrice with PBS for 5 min each, and incubated for 1 h with rabbit anti mouse FITC conjugate, 1: 40 dilution (Sigma, USA). Wells were washed with PBS, stained with DAPI (0.2 µg ml^{-1}) and observed under Inverted fluorescent microscope (Leica, Switzerland). DAPI and FITC were viewed under filters with excitation wavelength 360-370 nm and 470-490 nm respectively. Test wells were compared with the wells without BrdU (negative control). The images were processed and merged using the software, "Leica Application Suite" (Leica Microsystems, Switzerland).

3.2.5.3. Cell cycle gene (s) expression in lymphoid cell culture

To investigate the cell cycle progression and expression of cell cycle regulatory genes, the expression of gene involved in transcription elongation factor, cell division cycle 2 protein, cyclin-A, cyclin-B and mitotic check point protein in primary lymphoid cell culture (*in vitro*) were analysed along with β-actin as control genes. The expression profile was compared with its tissue counter parts (*in vivo*). The intensity of gene expression was quantified and depicted.

3.2.5.3.1. RNA isolation

Lymphoid cell cultures grown in 24 well plate containing SCCM were used for cell cycle gene expression studies. RNA isolation was done after the cells attained 80% confluent monolayer. For RNA isolation, the growth medium (SCCM) was removed, wells washed with ice cold PBS (720 mOsm kg^{-1}) and TRI reagent (Sigma, USA) was added to each well. Complete lysis of cells was allowed to take place by repeated pipetting and the reagent was collected in 1.5 ml MCTs. The medium removed from the wells containing detached cells was centrifuged at 400x g for 5 min, washed with ice cold PBS. Lymphoid organ from six animals were pooled and macerated in 1 ml TRI reagent to isolate RNA from the tissue (*in vivo*) counterpart. The samples were stored for 5 min at RT to ensure complete dissociation of nucleoprotein complexes. An aliquot of 0.2 ml chloroform was added to 1 ml TRI reagent, shaken vigorously for 15 sec (CM101, Cyclomixer, REMI), and allowed to stand for 15 min. The resulting mixture was centrifuged (3K30, Sigma) at 12,000xg for 15 min at 4 °C. Colorless upper aqueous phase was separated carefully from the three layers formed and transferred to a fresh tube. An aliquot of 0.5 ml isopropanol was added and stored for 10 min at RT and centrifuged at 12,000xg for 10 min. RNA was precipitated on the sides and bottom of the tube after centrifugation at 12,000xg for 10 min at 4 °C. The supernatant was discarded and the pellet washed twice with 75% ethanol. The pelleted RNA was air dried and dissolved in 20 µl DEPC treated sterile water by repeated pipetting at 55

°C. These RNA samples were subjected to DNase treatment with RNase-free DNase 1 (New England Biolabs). An aliquot of 0.2 units of the enzyme was added per µg of RNA and incubated at 37 °C for 10 min. The enzyme was inactivated at 75°C for 10 min. Concentration and quality of RNA was measured by taking the absorbance at 260/280 nm in a UV-Visible spectrophotometer (U2800, Hitachi).

3.2.5.3.2. RT-PCR of cell cycle genes

One µg RNA was subjected to cDNA synthesis with 20 µl of reaction mix containing M-MuLV reverse transcriptase (80 U), RNase inhibitor (8 U), Oligo $(dT)_{12}$ primer (40 pmoles), dNTP mix (1 mM), RTase buffer (1x) and $MgCl_2$ (2 mM) at 42 °C for 1 h. All reagents were purchased from New England Biolabs. Subsequently, cell cycle related genes of *P. monodon* were amplified by PCR using 2 µl cDNA with specific primer sets. For the gene amplification, the forward and reverse primer sequences for cyclin-A NP579F-5' CGT CAA TAG TGT GCG GGT TCT GG 3' and NP579R-5' CCA TTC TCA AGA TCT GCC CAA CT 3', for cyclin-B NP580F-5' AAC CAC CAC GCA TCT CAA CAG TA 3' and NP580R-5' GAA GCA GAG TGA AGC GGA GGT GT 3', for mitotic check point gene NP581F-5' CTC GCA TGG AAT TTC GGT TGA 3' and NP581R-5' GCC CTC TTT GCT ACA TCG TGA 3', for Cell division cycle 2 NP582F-5' GCA CGA GGC AGC TGA ATC AAG3' and NP582R-5' TTG GCT GGA CGA GTG GAC TTG 3', and for transcription elongation factor (ELF) 5' ATG GTT GTC AAC TTT GCC CC 3' and 5' TTG ACC TCC TTG ATC ACA CC 3' were used. Shrimp β-actin gene was also amplified as a reference using the forward and reverse primer sequence 5' CTT GTG GTT GAC AAT GGC TCC G 3' and 5' TGG TGA AGG AGT AGC CAC GCT C 3' respectively. The 25 µl PCR reactions contained 0.5 U of Taq DNA polymerase, 200 µM dNTP mix, 10 pmoles of each forward and reverse primer and 1x PCR buffer. The hot start PCR programme used for cell cycle genes was 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, annealing for 30 sec, 72 °C for 30 sec followed by final extension

at 72 °C for 10 min. Annealing temperatures were 54 °C for cyclin B, mitotic check point and cell division cycle-2, 58 °C for cyclin A, 60 °C for the transcription elongation factor (Loongyai *et al*., 2007) and control gene shrimp β-actin (Supungul *et al*., 2004). An aliquot of 10 µl from each PCR product was analysed by 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light, documented using a gel documentation system connected with Quantity One® software (Gel Doc XR^{+}, Bio-Rad, USA). The intensity of gene expressions was compared by quantifying the pixel intensity from gel image using Image J software (National Institute of Health (NIH), USA) and depicted as bar diagram of gene expression.

3.2.5.4. Actin cytoskeleton organization in lymphoid cells grown in SCCM

The widely used Phalloidin TRITC staining was slightly modified to see the organization of F-actin filaments in the primary lymphoid organ cells grown in SCCM. The staining was performed as per manufactures direction (Sigma, USA). Briefly, the lymphoid cells grown in 24 well plates were washed thoroughly with PBS (720 mOsm kg^{-1}) and fixed for 5 min in 3.7% formaldehyde in PBS. Washed thoroughly in PBS and the cells were dehydrated in acid alcohol for 1 min. Removed the dehydrating agent and the cells were permeabilized with PBS containing 0.1% Triton X-100 for 5 min, washed twice in PBS. Cells were stained with 50 µg ml^{-1} fluorescent phalloidin conjugate solution in PBS for 40 min at RT. Cells were washed several times with PBS to remove the unbound phalloidin conjugate, stained with DAPI (0.2 µg ml^{-1}) and observed under Inverted fluorescent microscope (Leica, Switzerland). DAPI and TRITC were viewed under filters with excitation wavelength 360-370 nm and 470-490 nm respectively. The images were processed and merged using the "Leica Application Suite" software (Leica Microsystems, Switzerland).

3.2.6. Metabolic activity and physiological status of lymphoid cells

3.2.6.1. Mitochondrial dehydrogenase enzyme activity

Cell viability depends on an intact mitochondrial membrane and the respiratory chain. The assay is a colorimetric method based on the determination of cell viability utilizing the reaction of a tetrazolium salt (2, 3-bis [2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide, XTT) with mitochondrial dehyrogenase enzyme of metabolically active cells. The reduction of the tetrazolium salt by mitochondrial dehydrogenase within the cells produces a water soluble formazan product. This reagent allows direct absorbance readings, therefore eliminating a solubilization step and shortening the assay procedure (Scudiero *et al*., 1988). Whilst the use of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) produces a non-soluble formazan compound which necessitated dissolving the dye in order to measure it (Mosmann, 1983)**,** the XTT was used in the place of MTT as the former produced water soluble crystals. Aliquots of 200 µl lymphoid cell suspension were seeded on to 96 well plates and after desired incubation period (24 h, 48 h, 72 h…etc), the medium was pipetted out and replaced with 50 µl pre-warmed XTT solutions (Xenometrix, Germany), and the plate was kept for incubation (in dark) at 25 °C for 5 h. After incubation, mixed the formazan formed in each well very carefully by pipeting, and the absorbance was measured at 480 nm in a micro plate reader (TECAN Infinite Tm, Austria) with a reference wave length at 690 nm.

3.2.6.2. Glucose assimilation by the cultured lymphoid cells

Cultured cells continually consume glucose from the culture media as a major source of energy and carbon (Stryer, 1995). The rate at which cells consume glucose can be measured and used to assess their physiological status and metabolic activity. Moreover, continuous monitoring of glucose concentration in the medium after the passage of cells should make it possible to draw conclusions

concerning their metabolic state (von Woedtke *et al.*, 2002). By knowing the initial glucose concentration in the medium and its rate of consumption in the presence of growth factors compared with that of a control where growth factors are not added, changes in their metabolic state induced by growth factors in the medium can be assessed. The assay performed here utilizes the coupled activities of glucose oxidase (GOD) and peroxidase enzymes (POD). An aliquot of 5 µl medium from the 96 well plates prepared for MTT assay were used for the measurement of glucose assimilation rate. Glucose consumption by lymphoid cells grown in shrimp cell culture medium (SCCM) was monitored at 24, 48 and 72 h of incubation against the initial concentration, using the glucose assay kit as per the manufactures direction (Xenometrics, Switzerland). In brief, 5 µl aliquots of the medium were diluted with 45 µl deionized water, added 100 µl reaction mixture containing substrate and the enzyme in the ratio 1:50, incubated for 30 min at 37 °C. After incubation, reaction was stopped by adding 100 µl 12 N H_2SO_4. Mixed well by pipetting and confirmed the absence of air bubbles; absorbance was measured at 540 nm in a microplate reader (InfiniteM-200 Tecan, Austria). The glucose consumption rate of lymphoid cells grown in SCCM was compared with that of control and modified L-15 (Jose *et al.*, 2010).

3.2.6.3. Protein synthesis in the cells *in vitro*

A modified version of the sulforhodamine B (SRB) colorimetric assay for the measurement of cellular protein was performed in accordance with Vichai and Kirtikara (2006). The assay is based on electrostatic binding of anionic SRB to basic amino acid residues. Protein Synthesis in lymphoid cells grown in shrimp cell culture medium (SCCM) was monitored at 24, 48 and 72 h of incubation against control cells. Briefly, lymphoid cells grown in 96 well plates were washed with PBS (720 mOsm kg^{-1}) and fixed in 10% TCA for 1 h at 4 °C and again washed with PBS. Added 50 µl SRB solution (Xenometrics, Switzerland) and incubated for 15 min at RT. Quickly washed the wells three times with 1% acetic acid and air

dried at RT. Added 200 µl Tris base solution (pH 10.5) to the wells and incubated for 30 min at RT, mixed gently using a multichannel pipette, absorbance measured at 540 nm in a microplate reader (InfiniteM-200 Tecan, Austria). Background absorbance was measured with a reference filter at 690 nm.

3.2.7. Viral susceptibility test

White Spot Syndrome Virus (WSSV) was used to test the viral susceptibility of lymphoid cells grown in SCCM.

3.2.7.1. Virus preparation

WSSV challenged *Penaeus monodon* was used for the virus preparation following the methodology described by Jose *et al*. (2012) with slight modifications. Briefly, gill tissue (500 mg) from WSSV infected shrimps was macerated in 10 ml ice cold shrimp cell culture Medium (SCCM) with mortar and pestle. The extract was centrifuged at 10,000xg for 10 min at 4 ºC and the supernatant filtered through 0.22 µm polyvinylidene fluoride (PVDF) membrane (Millipore, USA). The preparation was stored at -80 ºC until use. A 300-fold dilution from this preparation was used for the viral susceptibility test.

3.2.7.2 Inoculation and Immunofluorescence assay for the detection of WSSV

WSSV preparation diluted with SCCM (300 fold) was added on to lymphoid cell culture grown in 24 well plates and incubated till cytopathic effects (CPE) were observed (within a period of ~48 h). Cells were washed with PBS (720 mOsm kg^{-1}) and fixed for 5 min in 10% paraformaldehyde in PBS. Washed in PBS and added 70% ethanol for 10 min, blocked in 3% BSA in PBS and incubated for 1 h in a humidified chamber. The cells were washed in 0.01% Tween 20, incubated for 1 h with WSSV C-38 monoclonal antibody (Anil *et al*., 2002) and washed 3 times in PBS. The secondary antibody rabbit-anti-mouse FITC conjugate (Sigma Aldrich, USA), was added against WSSV C-38 at a dilution 1:40 and incubated for

1h. After incubation cells were stained with DAPI (10 µl, 0.2 µg ml^{-1}) for 3 min, rinsed in distilled water, air dried, mounted (Vectashield, USA) and observed under fluorescence microscope (Leica DMIL, Switzerland). DAPI and FITC were viewed under filters with excitation wavelength 360-370 nm and 470-490 nm respectively. Test wells were compared with the control wells without the addition of WSSV preparation (negative control). The images were processed and merged using the "Leica Application Suite" software (Leica Microsystems, Switzerland).

3.2.8. Statistical analysis

The results in the figures are average values of 3–6 replicates ± standard deviation. All data were subjected to analysis of variance (ANOVA and differences were considered significant at $p < 0.05$. The statistical screening and optimization of growth factors was performed by Plackett-Burman and Central Composite Design in Response Surface Methodology using the software, Design Expert version 6.0.9 (StatEase, USA).

3.3. Results

3.3.1. Development of primary lymphoid cell cultures

Lymphoid cells in culture were found getting attached to the culture vessel within 2 h of seeding. Two types of cells, epithelioid and fibroblastic were observed, the fibroblastic cells being prominent with elevated lifespan. Mixed cell population was used for screening growth factors and for metabolic activity determination, and fibroblastic cell type for virus susceptibility tests. Epithelioid type was used for studying molecular and cellular studies due to its larger size than the fibroblastic type.

3.3.2. Screening and optimization of growth factors and its validation

One-variable-at-a-time screening of growth factors using lymphoid cell culture was performed for the identification of the most effective concentrations,

which were chosen for further statistical screening using Plackett- Burman multifactorial design. Growth factors such as IGF-I, IGF-II, EGF and TGF-β1 with the concentration at 50 ng ml^{-1} to 100 ng ml^{-1} (in a range) was found to be significant (p <0.05) and produced elevated metabolic activity in lymphoid cell culture (Fig. 3.1-3.4). However, FGF-4 and bFGF were found to be effective (p <0.05) at lower concentration within a range of 2 ng ml^{-1} to 6 ng ml^{-1} and had negative effect at higher concentration (Fig. 3.6 & 3.7). Interleukin was found to be effective (p <0.05) at 6 ng ml^{-1} to 8 ng ml^{-1} (Fig. 3.8) whilst, insect steroid hormone 20HE at 2 ng ml^{-1} to 8 ng ml^{-1} with an elevated activity (p <0.05) at 2 ng ml^{-1} (Fig. 3.9). In both the cases, at higher concentration, negative impact was observed in metabolic activity of the cells. In addition, PDGF was not selected for further screening as it did not contribute to significant (p >0.05) cellular metabolic activity (Fig. 3.5). The concentration of growth factors that contributed to cellular activity as determined by MTT assay with maximum and minimum concentrations selected for statistical screening are given in Table 1 and Table 2. Plackett-Burman statistical screening reduced the number of variables from 9 to 6 with respect to its statistical significance in the interactions among components. Out of the 6 growth factors, IGF-1 (A) and IGF-II (B) were at statistical confidence greater than 95% with the p value 0.0077 (p <0.05) and 0.017 (p <0.05) respectively, suggesting these variables in the model significant (Table 3). Even though, the other four components such as EGF (C), TGF (D), bFGF (E) and 20-HE (H) did not have any a negative effect on metabolic activity of the cells, their interaction among components (p >0.05) were insignificant with p values 0.5766, 0.3169, 0.5640 and 0.4284 respectively. The coefficient of determination, R^2 in this experiment was 0.87, which meant that 87% of variability in the observed data could be explained by the selected polynomial equation. Moreover, the signal to noise ratio 7.5 implied that the model was adequate to proceed further as the ratio greater than 4 was desirable. The regression analysis of the model using ANOVA suggested an F-value of 5.56 along with the p value 0.0397 (p < 0.05) which

implied the model used was significant with a statistical confidence greater than 95% and could be used for further optimization (Table 3).

As the Plackett- Burman statistical screening used in the study to identify the most significant (effective) growth factor components for shrimp cells, growth factors with statistical confidence less than 95% were omitted, because of its insignificance, without even considering their narrow positive effects. Accordingly, the growth factors such as IGF-I (A) and IGF-II (B) with statistical confidence greater than 95% were selected for further optimization by response surface methodology using central composite design (CCD). Table 4 shows various combinations of IGF-I and IGF-II used and corresponding metabolic activity of the lymphoid cells in terms of MTT assay. The quantities of the remaining components in SCCM were kept constant and each combination was added to the medium used for developing the cell culture from lymphoid cells.

The central composite design of RSM provided the most suitable concentration of IGF-I and IGF-II, which contributed for the enhanced growth of lymphoid cells *in vitro*. Table 4 summarizes the metabolic activity of the cells for each combination along with the predicted response. The results obtained after CCD were analysed by standard analysis of variance (ANOVA), which gave the following regression equation (in terms of coded factors) of the metabolic activity (Y) as a function of IGF-I (A), IGF-II (B).

Metabolic activity of the cell = 0.38 + 0.059*A + 1.924x10E+003 *B + $0.077*A^2 + 0.015*B^2 + 0.042*A*B$

The regression analysis for response surface quadratic model using ANOVA suggested an F-value of 39.36 along with the p value < 0.0001 ($p < 0.05$) which implied the model used was highly significant with a statistical confidence greater than 95%. The linear and quadratic model terms for IGF-I such as A and A^2 were found significant ($p < 0.0001$) whilst, the model terms for IGF-II (B and B^2)

were insignificant (p >0.05) with a p value 0.7901 and 0.0841. However the combination AB (IGF-I and IGF-II) was found significant with the p value 0.0037 ($p < 0.05$), suggesting the combined effect of the growth factors IGF-I and IGF-II, despite the effect of IGF-I alone. This indicated that both IGF-I and IGF-II were required to improve the metabolic activity of the shrimp cells *in vitro*. The coefficient of determination, R^2 in this CCD experiment was 0.966, which meant that 96.6% of variability in the observed data could be explained by the selected model. Moreover, Predicted R^2 (Pred R^2) was found in reasonable agreement with the Adjusted R^2 (Adj R^2) and were 0.906 and 0.941 respectively. In addition, the model had an adequate signal to noise ratio (Adequate precision value) of 18.262 suggesting the model could be used to navigate the design space, as the ratio greater than 4 was suggested to be desirable. The model showed coefficient of variation (CV), standard deviation, mean and predicted residual sum of squares (PRESS) values of 4.47%, 0.020, 0.44, 7.409E+003 respectively, and the 'lack of fit' of this model was found to be 'not significant' with an F-value of 0.36 (Table 5). Altogether, the model used in the software (Design-Expert) was found to to be significant and the combination suggested by the model could be accepted for improving the metabolic activity of the cells. The optimum concentration suggested by the model for IGF-I and IGF-II were 100 ng ml^{-1} and 150 ng ml^{-1} respectively, and the predicted metabolic activity in terms of absorbance of MTT assay at 570 nm was 0.579. The regression equation represented in 3D response surface plot and 2D contour plot (Fig 4), determined the optimum concentration of growth factors IGF-I and IGF-II for the improved metabolic activity of the shrimp cells *in vitro*. Validation of the optimized concentration of growth factors given by the model, suggested that the experimental value of absorbance 0.581 at 570 nm was very close to the predicted value (0.579) in the MTT assay, despite an additional increase of 0.35% in the experimental value (Table 6). In addition, during validation, excellent growth of fibroblastic cells from lymphoid tissue was observed in SCCM with optimized growth factors IGF-I and IGF-II (Fig. 5).

3.3.3. Mitotic activity of the lymphoid cells

The results of BrdU incorporation assay showed that the mitotic activity of lymphoid cells grown in growth factor optimized shrimp cell culture medium was higher than (p <0.05) that in the basal medium (SCCM without growth factors) with 24.8% increase of BrdU incorporation. This value suggested that the growth factors such as IGF-I and IGF-II with a concentration 100 ng ml^{-1} and 150 ng ml^{-1} induced DNA synthesis that occurred in the cell cycle events (S-phase) of lymphoid cells *in vitro*. Moreover, 58.2% and 59.4% increase of BrdU incorporation was detected in SCCM than modified L-15 and 2xL15 (Fig. 6). However, an increase of 26.7 and 27.7% mitotic activity could be observed in the basal medium (SCCM without growth factors) while comparing the same in the modified L-15 and 2xL15 respectively, suggesting that the basal medium itself had the required potential to induce mitotic activity. Meanwhile, the additional increase of 24.8% in mitotic activity was contributed by the growth factors IGF-I and IGF-II.

3.3.4. Molecular cell biology of lymphoid cell culture

3.3.4. 1. Mitotic events in lymphoid cells *in vitro*

Epithelioid type lymphoid cells grown in SCCM were tracked and imaged with 400 times magnification using inverted phase contrast microscope (Leica, Switzerland) connected with time-lapse imaging facility controlled by image acquisition software (LAS, Leica, Switzerland). The photographs showed clear indication of mitotic division *in vitro* (Fig. 7). Even though more dividing cells could be found in fibroblastic cell culture of lymphoid cells grown as a monolayer in SCCM, for distinct and clear imaging, epithelioid cells were selected. During division the cells were found fully expanded out with many protrusions. Moreover, the cells were found firmly attached to the substratum (culture flask) and not elevated from the spread layer during division. Though the complete time

lapse image of mitotic events was not obtained, mitosis in lymphoid cells *in vitro* could be proved with the recorded image of cell cycle events.

3.3.4. 2. Entry of lymphoid cells into S-phase of cell cycle and DNA synthesis

The synthesis phase of the cells was confirmed by immunofluorescence detection of 5- bromo-2'-deoxyuridine (BrdU) incorporated nucleus of lymphoid cells. The synthetic analog of thymidine (BrdU) incorporated to the newly synthesized DNA during S-phase of the cell cycle was detected by allowing to react with mouse monoclonal anti BrdU antibody followed by the secondary antibody rabbit anti mouse FITC conjugate. The green positive fluorescence of FITC (fluorescein isothiocyanate) was observed in very few cells though most of the cells were negative with only blue signals from DAPI. The epithelioid type lymphoid cells with large nucleus producing fluorescence signals of FITC could be observed from 24 h old culture onwards (Fig. 8). In them 24±2% of the cells were found to be in S-phase after 48 h while 10±1% at 24 h interval. These results revealed that the culture medium SCCM supported active DNA replication in primary lymphoid organ cells.

3.3.4. 3. Cell cycle gene (s) expression in lymphoid cell culture

Cell cycle gene of *Penaeus monodon* such as transcription elongation factor, cell division cycle 2 protein, cyclin -A and cyclin-B, and the β-actin as control gene were detected from lymphoid organ (*in vivo*) and lymphoid cell culture (*in vitro*) by RT-PCR. Nevertheless, gene encoding mitotic check point protein was not detected in both the cases. For the gene expression profiling, each 8-bit tagged image file format (TIFF) of the gel image (grayscale) was transferred to Image J software to quantify the total brightness. This brightness was calculated as the quantity of amplicon produced from cell cycle gene during PCR, and graphically represented as differential expression (expression profile) of gene (s) in terms of brightness in gel image. From the expression profile results, it was clear

that, while comparing with the tissue counterpart, 19.7% increase in the expression of gene encoding transcription elongation factor was observed in lymphoid cells *in vitro*, suggesting that the growth factor induced transcriptional activation occurred in the cells. The cyclin A and cyclin B genes were expressed in similar pattern. Furthermore, cell division cycle 2 proteins (mitosis) of *Penaeus monodon* displayed elevated expression level with almost uniform expression pattern of β-actin gene (control) in the tissue and cell culture (Fig. 9).

3.3.4. 4. Actin cytoskeleton organization in lymphoid cells grown in SCCM

The red fluorescence of TRITC (tetramethyl rhodamine isothiocyanate) was observed from the cytoskeleton network of the cells, where the TRITC conjugated phalloidin bound F-actin filaments were seen. The nucleus of the cells produced blue signals from DAPI stain. Well established cytoskeleton net work (as F-actin) in cells could be observed particularly in epithelioid cells originated from lymphoid organ. F-actin filaments exhibited integrity and expanded net work inside the primary lymphoid cells grown in SCCM in comparison with modified L-15 medium (Fig. 10). Moreover, normally seen 'stress fiber formation' during cell proliferation in unfavorable conditions could not be seen in this medium. Even though it is not known about the supporting elements in SCCM that provide structural integrity of shrimp cells *in vitro*, this finding lends itself to the use of this medium for maintaining shrimp cells *in vitro* and for further immortalization.

3.3.5. Metabolic activity and physiological status of lymphoid cells

Mitochondrial dehydrogenase enzyme activity in lymphoid cells grown in growth factor (IGF-I and IGF-II) optimized SCCM was detected by XTT and compared it with that in control (SCCM without growth factor) and the modified L-15. Within 24 h of incubation with the presence of growth factors, the metabolic activity was increased by 19.4% compared to that of control and at 48 h and 72 h the differences were 16.5% and 17.3% ($p < 0.05$) respectively (Fig. 11). In protein

synthesis, observed increase of the activity was found to be 0.22%, 0.84%, 0.33% at 24 h, 48 h and 72 h time interval respectively (Fig. 12). In addition, the glucose consumption was increased by 19.5%, 17.5% and 53.6% (p <0.05) at time intervals of 24 h, 48 h and 72 h respectively and a corresponding decrease was observed in the medium (Fig. 13). While comparing this elevated activity in SCCM with modified L-15, the values for mitochondrial dehydrogenase was 49.7%, 57.6% and 81.1%, for protein synthesis 0.87%, 0.17% and 0.54%, and for glucose consumption 35.9%, 36.6% and 65.1% (p <0.05) at time intervals 24 h, 48 h and 72 h respectively. Even though there was a slight increase in protein synthesis, it was statistically insignificant (p >0.05). The observed results from the comparative analysis of SCCM with growth factors and without growth factors (control) testified the induced metabolic activity in lymphoid cells by IGF-I and IGF-II (Fig. 14). However, the results observed while comparing with modified L-15 suggested that the basal SCCM (control) itself could induce the metabolic activity of the lymphoid cells, confirming the suitability of the basal medium to support growth of lymphoid cells even without the induction caused by the growth factors IGF-I and IGF-II.

3.3.6. Susceptibility of lymphoid cells to WSSV

Cytopathic effect in lymphoid cells was visible within 12 h of inoculation with gill extracts from shrimp infected with WSSV (diluted 300 times) and it was more prominent at 24 h (Fig. 15). Infected cells were shrunken, slightly detached from the culture plate and lysed due to necrosis. Green positive signals with FITC conjugated monoclonal antibodies against WSSV (C-38) were observed (Fig. 15) from the nuclei of infected cells and no such positive signals were observed from the nuclei of control cells other than the blue signals from the nucleus stained with DAPI .

3.4. Discussion

The study was intended to develop a lymphoid cell culture platform, by improvising the SCCM by incorporating growth factors, for undertaking cellular and molecular studies, cultivation of viruses, and to strengthen the research on shrimp cell culture towards establishment of cell lines. From the previous chapter (Chapter 2), it was found apparent that the lymphoid cells remained stable for longer period of time in SCCM with consistent growth and proliferation. Moreover, it has been confirmed that the rapid monolayer formation, longevity and stability are the characteristics of lymphoid cells in culture (Nadala *et al.*, 1993), and accordingly the lymphoid tissue has been designated as the most preferred one (Jayesh et *al.*, 2012) for the development of shrimp cell culture (Chen *et al.*, 1989; Tapay *et al.*, 1995; Jose *et al.*, 2012).

Among the vertebrate growth factors and hormone screened by Plackett-Burman statistical screening, only IGF-I and IGF-II were found to have significant effect on lymphoid cell culture. This was further optimized by applying central composite design and obtained the most effective concentration as 100 ng ml^{-1} and 150 ng ml^{-1} of IGF-I and IGF-II respectively. Jose *et al.* (2012) suggested that, IGF-I enhanced the cell proliferation in lymphoid cells from *P. monodon* at a concentration of 10 ng ml^{-1}. Fan and Wang (2002) noticed enhanced growth and proliferation in embryonic cells of *P. chinensis* after the administration of IGF-II and bFGF. Hsu *et al.* (1995) reported that lymphoid cells from *P. monodon* treated with IGF-I was capable of sub-culturing. These findings support the results obtained from the present study. Moreover, the coefficient of determination, R^2 in the Plackett-Burman and CCD in this experiment were found to be 0.87 and 0.97 respectively. This meant that 87% and 97% of variability in the observed data could be explained by the polynomial equation used in this model. Normally, a regression model having an R^2 value higher than 0.9 is considered as having a very high correlation (Guilford and Fruchter, 1973; Haaland, 1989; Ahuja *et al.*, 2004)

and a model with an R^2 value between 0.7 and 0.9 is considered as having a high correlation (Guilford and Fruchter, 1973; Ahuja *et al*., 2004). In the present case, an R^2 value of 0.87 and 0.97 reflected a good fit between the observed and predicted responses, and it was reasonable to use the regression model to analyze the trends in the responses. In addition, the predicted response value and the experimental response value of metabolic activity (MTT assay) in lymphoid cell culture were found to be having 0.579 and 0.581 absorbance at 570 nm. These observations confirmed that the model used for screening and optimizations of growth factors were valid.

BrdU incorpotaion in the lymphoid cell culture of *Penaeus monodon* was first reported by Jose *et al*. (2012), and claimed that very few BrdU positive cells were observed from the culture grown in modified Leibovitz's L-15 medium. However, Braasch *et al*. (1999) and Jose *et al*. (2010) reported that the haemocyte culture was BrdU positive with 1-2% and 22±7% respectively from *P. vannamei* and *P. monodon*. In addition, Maeda *et al*. (2003) confirmed 35% positive cells in primary ovarian cell culture developed from *P. japonicus*. In accordance with these results, in the present study, 10±1% and 24±2% of the lymphoid cells were found to be BrdU positive after 24 h and 48 h intervals respectively. The lower rate in BrdU incorporation supports the observed mitotic events in lymphoid cells which were very few and happened late in culture. This supported the findings of van de Braak *et al*. (2002), who reported that a limited number of mitotic cells alone were observed in lymphoid cell culture in commercial media.

Insulin-like growth factor I (IGF-I) coordinates proliferation and differentiation in a wide variety of cell types (Barton *et al*., 2010) and its stimulatory effects on protein synthesis, growth promoting effects and glucose metabolism (Mathews *et al*., 1988) have been well documented in vertebrates. Baker and Carruthers (1980) suggested that the bovine insulin stimulates sugar transport in giant muscle fibers of the barnacle *Balanus nubilis*. In addition,

Richardson *et al*. (1997) reported that IGF-I induced glucose metabolism in the red claw crayfish *Cherax quadricarinatus*. Gutie´rrez *et al*. (2007) reported that the IGF-I induced, elevated *in vivo* glucose metabolism in *P. vannamei*. Similar to these findings, an elevated glucose assimilation and mitochondrial dehydrogenase activity were observed in the present study. In addition, the up-regulation of the gene encoding transcription elongation factor suggested the growth factor induced transcription in cells *in vitro*. However, as there is no similar study, this is considered to be the first comprehensive report on growth factor induced cell cycle gene expression and the metabolic activity in shrimp cells *in vitro*. However, Jose *et al*. (2012) reported enhanced proliferation of lymphoid cells *in vitro* after incorporating IGF-I to the modified L-15 medium at a concentration 10 ng ml $^{-1}$.

Actin represents one of the most abundant and extensively studied proteins found in eukaryotic cells (Leadsham *et al*., 2010). It has been identified as the cytoskeleton components in the initiation and inhibition of apoptotic processes and as part of the signaling mechanisms that link nutritional sensing to a mitochondrial dependent commitment to cell death (Leadsham *et al*., 2010). Moreover, the actin cytoskeleton has been shown to be intimately involved in the maintenance of endothelial integrity by providing the structural framework for cell shape, cell movement, and cell– cell and cell–substratum interactions (Searles *et al*., 2004; Gotlieb and Lee, 1999). In addition, it provides contractile force for cell migration (Kreis and Birchmeier, 1980; Simon *et al*., 1995) and play well-defined role generating static contractile forces in tissues (Pellegrin and Mellor, 2007). It has been shown that for some proteins, the sub-cellular localization and targeting of their mRNAs plays a significant role in determining efficient translation and proper protein localization (Hesketh, 1996). This has led to the concept that transport and localization of mRNA involves cytoskeleton components (Hesketh, 1996). Because mRNA stability is often closely linked to translation, cytoskeleton-mediated transport and localization of mRNA to appropriate polysomal fractions, it is

important to regulate its turnover (Searles *et al.*, 2004). The ability to coordinate environmental sensing with appropriate cell-fate decisions is a pre-requisite for the success of both unicellular and multicellular organisms. Interestingly, actin cytoskeletons are highly responsive to cellular stress and will rapidly de-polarize in response to a signal cue such as heat shock or entry into the diauxic phase of growth. Chen *et al*. (2011) reviewed that the culture medium and the cytoskeleton assembly were considered to be important factors in baculoviral gene delivery. The present study identified an expanded cytoskeleton network in lymphoid cells grown in SCCM, suggesting that the medium very much support the growth and proliferation of cells *in vitro*.

Development of shrimp cell line has been mainly focused on lymphoid tissue (Jayesh *et al*., 2012) as it was found to be susceptible to most of the shrimp viruses (Rusaini and Owens 2010), such as; Lymphoidal parvo like-virus (Owens *et al*., 1991), Spawner-isolated mortality virus (Fraser and Owens, 1996), White spot syndrome virus (Rodríguez *et al*., 2003), Yellowhead virus (Chantanachookin *et al*., 1993), lymphoid organ virus (Spann *et al*., 1995) , Taura syndrome virus (Hasson *et al*., 1999), Infectious myonecrosis virus (Tang *et al*., 2005) , Mourilyan virus (Rajendran *et al*., 2006), Laem-Singh virus (Sritunyalucksana *et al*., 2006), rhabdovirus of penaeid shrimp (Nadala *et al*., 1992), and lymphoid organ vacuolization virus (Bonami *et al*., 1992). In addition, Wang *et al*. (2000) have proved the susceptibility of lymphoid primary cell culture to WSSV. As observed in the present study, many researchers reported that shrinkage, rounding and detachment of infected cells were the common cytopathic effects in the event of WSSV infection (Wang *et al*., 2000; Maeda *et al*., 2004; Jiravanichpaisal *et al*., 2006). Furthermore, Jose *et al*. (2012) reported that the accumulation of refractile granules also is an indication of cytopathic effect (CPE) in lymphoid cell culture. Monoclonal antibody C38 (MAb C38) developed by Anil *et al*. (2002) was used for immunofluorescence detection of the WSSV protein in infected cells, which

strongly reacted with the 28 kDa but weakly with 18 kDa envelope proteins of WSSV. Using these MAbs we could observe strong positive signals from the infected nuclei after 48 h of virus administration. This is in accordance with the observation of Jiang *et al*. (2005), who has performed immunodetection utilizing monoclonal antibodies against WSSV to confirm the WSSV infection.

In conclusion, considerable progress has been made in establishing a shrimp cell culture system for the study of viruses which exacerbated the stress on 'shrunken' shrimp aquaculture industries. Even though primary cell cultures from *P. monodon* could be developed and maintained in the commercially available media, they all were with limitations at varying levels. SCCM is the first seawater based shrimp cell culture medium developed exclusively for the development of primary cell culture system especially from lymphoid organ (Refer Chapter 2). In our experiment we could maintain 'stable' primary lymphoid cell culture system for more than 3 months in SCCM. Development of a cell culture system with longevity is the prime requirement towards immortalization. Moreover, this will be helpful in the successful study of host viral interaction, viral acquisition, replication and transmission at molecular level. Investigations on viral susceptibility, expression of cell cycle genes, mitotic events, and cytoskeleton networks were successfully investigated using the lymphoid cell culture developed in the novel medium. This will prove to be invaluable in immortalization and study on viral morphogenesis. Moreover, statistical screening and optimization of growth factors and the study on their interaction with the cells could yield valuable information about the growth factor receptors and signal molecules on th cells which might lead to the development of pseudotyped vectors and molecules for gene delivery. Furthermore, the development of the primary lymphoid cell culture may provide the much-needed *in vitro* system for cytotoxic and genotoxic investigations. However, much more work needs to be done to identify the molecular blocks which are to be removed to develop a continuous cell line from *P. monodon*.

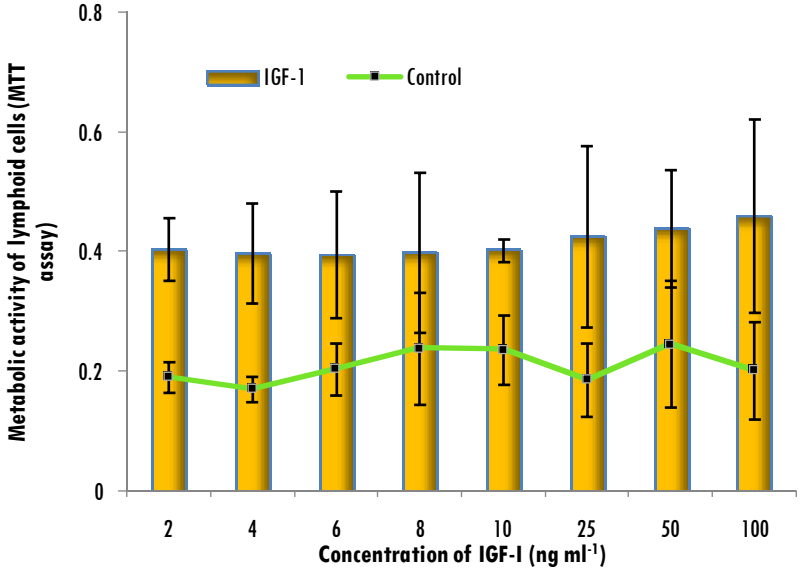


Fig.3.1. Insulin-like growth factor-I induced metabolic activity in lymphoid cells (n=6).

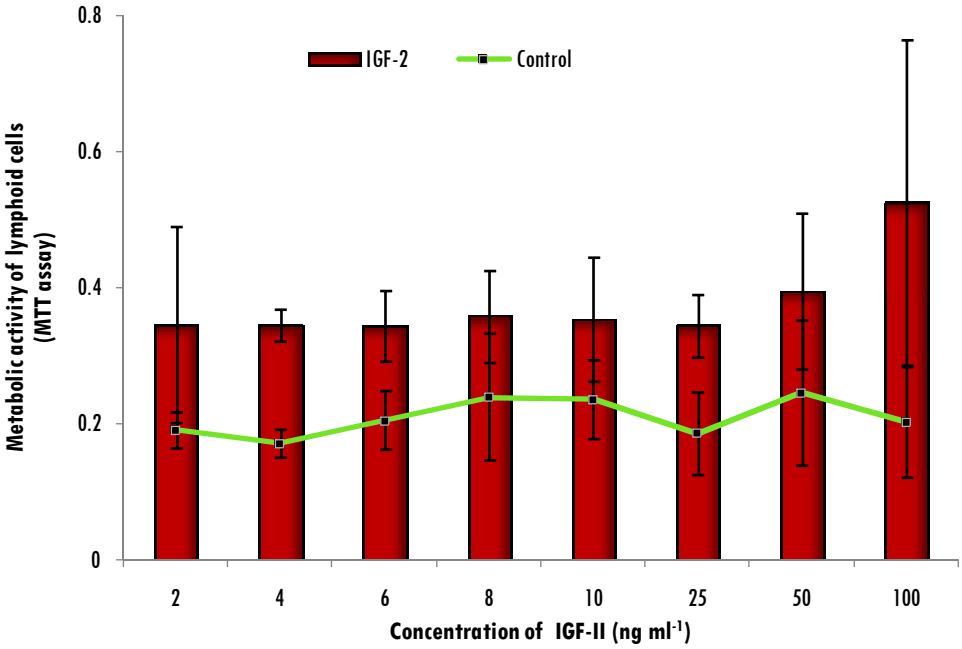


Fig.3.2. Insulin-like growth factor-II induced metabolic activity in lymphoid cells (n=6).

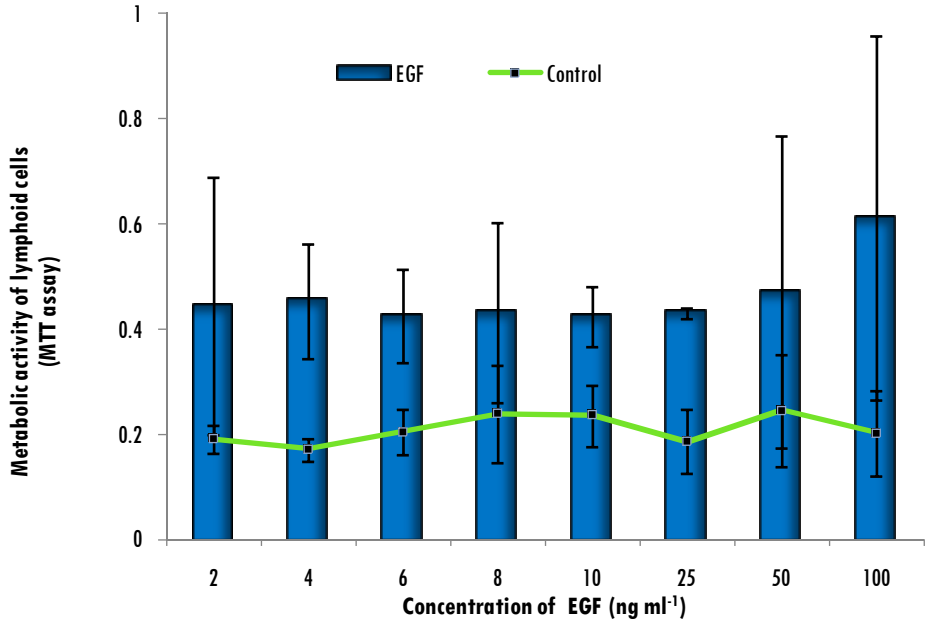


Fig.3.3. Epidermal growth factor induced metabolic activity in lymphoid cells (n=6).

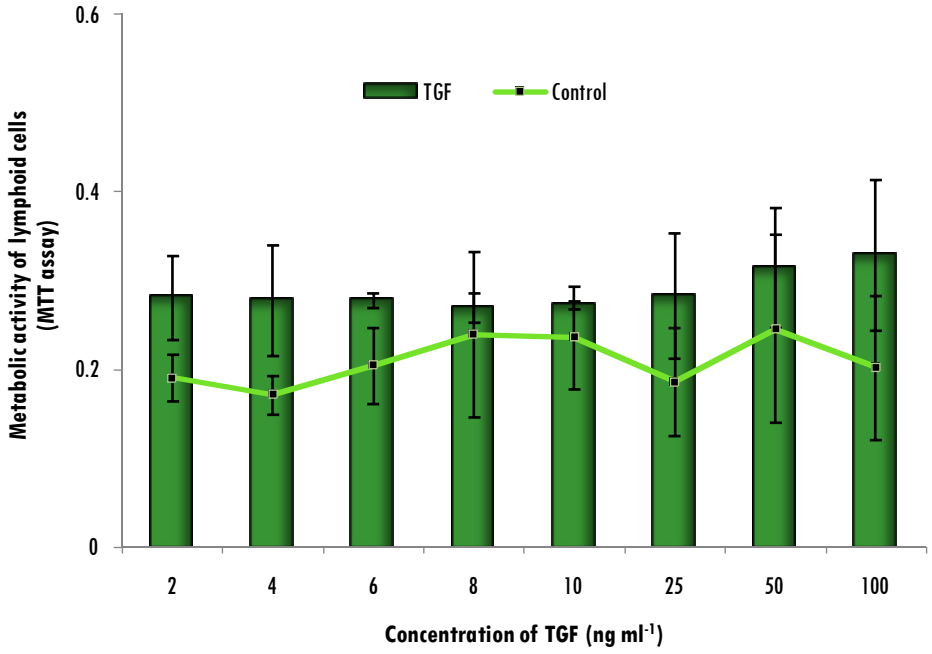


Fig.3.4. Transforming growth factor induced metabolic activity in lymphoid cells (n=6).

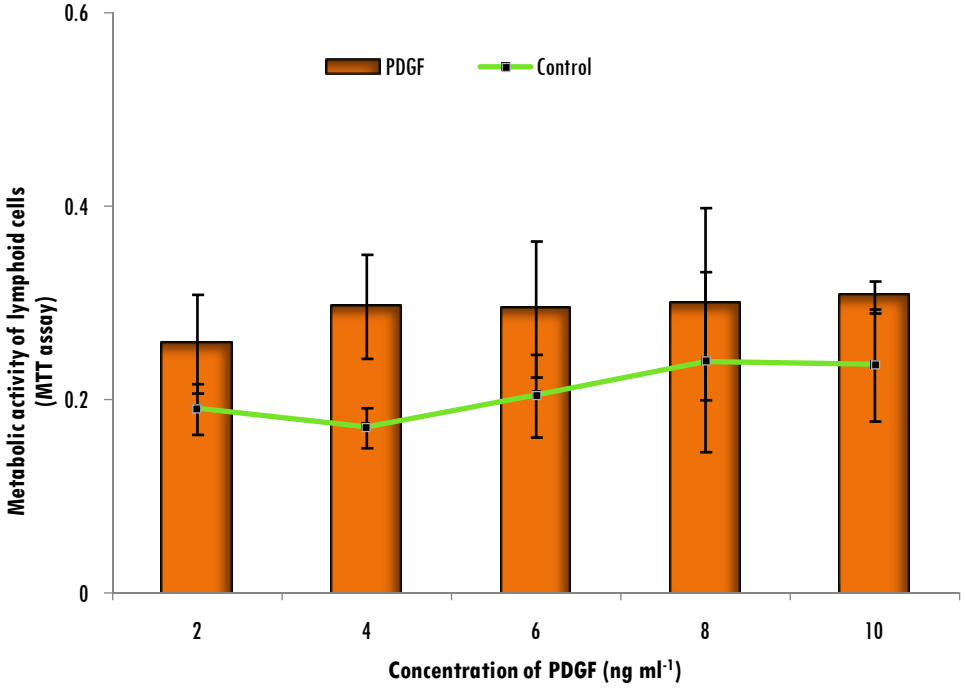


Fig.3.5. Platelet derived growth factor induced metabolic activity in lymphoid cells (n=6).

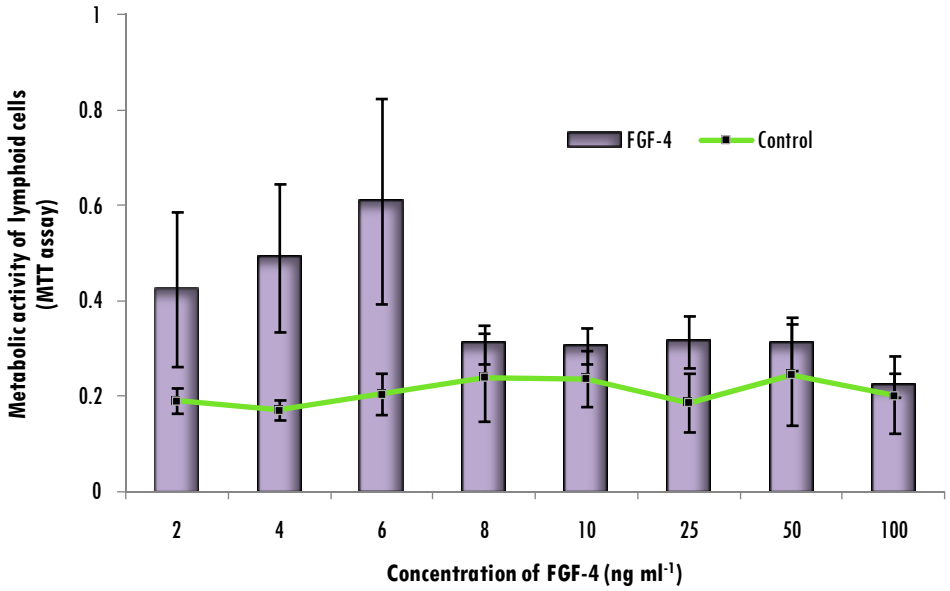


Fig.3.6. Fibroblastic growth factor 4 induced metabolic activity in lymphoid cells (n=6).

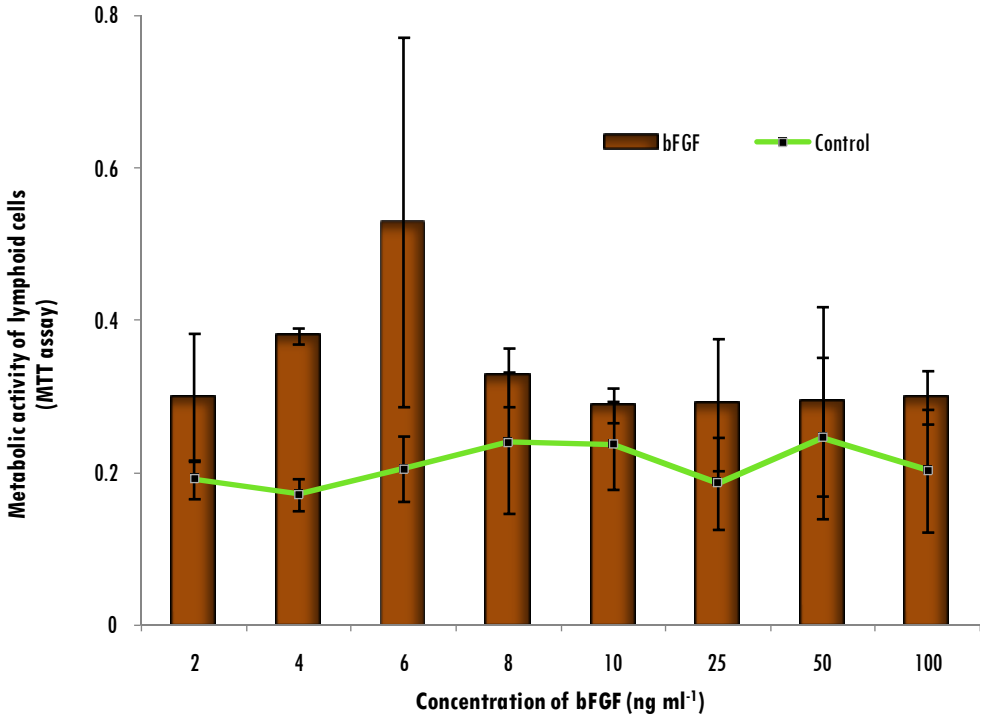


Fig.3.7. Basic fibroblastic growth factor induced metabolic activity in lymphoid cells (n=6).

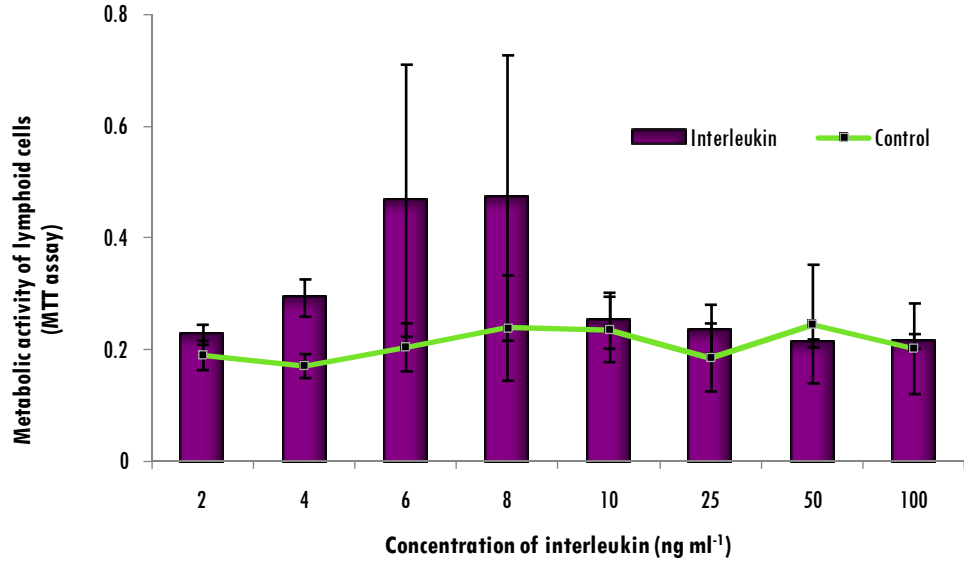


Fig.3.8. Interleukin induced metabolic activity in lymphoid cells (n=6).

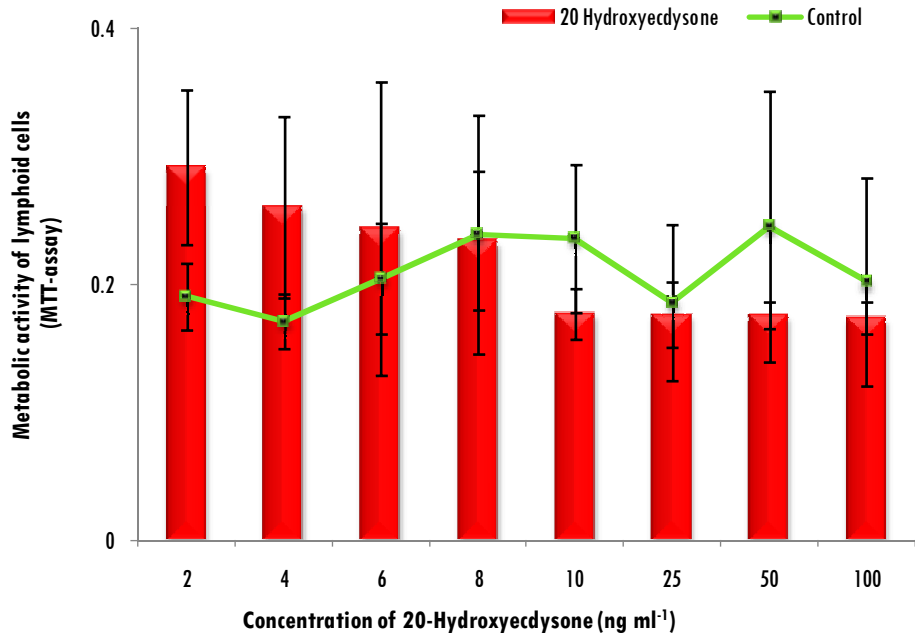


Fig.3.9. 20-hydroxyecdysone induced metabolic activity in lymphoid cells (n=6).

Table1. Growth factors and their two levels used in the Plackett-Burman design.

Factor	Name	Low actual (ng ml $^{-1}$)	High actual (ng ml $^{-1}$)	Low coded	High coded
A	IGF-I	50.00	150.00	-1.000	1.000
B	IGF-II	50.00	150.00	-1.000	1.000
C	EGF	50.00	150.00	-1.000	1.000
D	TGF	50.00	150.00	-1.000	1.000
E	bFGF	4.00	8.00	-1.000	1.000
F	FGF	2.00	6.00	-1.000	1.000
G	Interleukin	4.00	8.00	-1.000	1.000
H	20-HE	0.000	2.00	-1.000	1.000
J	Dummy	0.000	1.00	-1.000	1.000
K	Dummy	0.000	1.00	-1.000	1.000
L	Dummy	0.000	1.00	-1.000	1.000

IGF: Insulin like growth factor; EGF: Epidermal growth factor; TGF: Transforming growth factor-β1; PDGF: Platelet derived growth factor; bFGF: Fibroblastic growth factor-basic; FGF: Fibroblastic growth factor-4; 20-HE: 20-hydroxyecdysone.

Table 2. Plakett-Burman design matrix of the variables along with the experimental (n=3) and predicted values of impact of growth factors on cellular metabolism.

Run order	Factors											Metabolic activity	
	A	B	C	D	E	F	G	H	J	K	L	Experimental value	Predicted value
1	1	-1	1	-1	-1	1	1	1	0	0	0	0.425433	0.324833
2	1	1	-1	-1	-1	-1	1	1	0	0	0	0.475400	0.555278
3	-1	1	1	1	-1	-1	-1	1	0	0	0	0.901467	0.827089
4	1	-1	1	-1	1	-1	-1	-1	0	0	0	0.388833	0.408289
5	1	1	-1	1	-1	1	-1	-1	0	0	0	0.652667	0.666622
6	1	1	1	1	1	-1	1	-1	0	0	0	0.677800	0.667789
7	-1	1	1	-1	1	1	-1	1	0	0	0	0.704100	0.799200
8	-1	-1	1	1	-1	1	1	-1	0	0	0	0.608500	0.678933
9	-1	-1	-1	1	1	-1	1	1	0	0	0	0.696167	0.698845
10	1	-1	-1	1	1	1	-1	1	0	0	0	0.458767	0.456089
11	-1	1	-1	-1	1	1	1	-1	0	0	0	0.986033	0.881489
12	-1	-1	-1	-1	-1	-1	-1	-1	0	0	0	0.639167	0.649878

Table3. Analysis of variance (ANOVA) for the selected factorial model of growth factor induced metabolic activity in lymphoid cell culture

Source	Sum of Squares	Degrees of freedom	Mean square	F value	Prob>F
Model	0.32	6	0.053	5.56	0.0397*
A	0.18	1	0.177	18.50	0.0077*
B	0.12	1	0.116	12.16	0.0175*
C	3.403E+003	1	3.403E+003	0.36	0.5766
D	0.012	1	0.012	1.24	0.3169
E	3,642E+003	1	3,642E+003	0.38	0.5640
H	7.089E+003	1	7.089E+003	0.74	0.4284
Residual	0.048	5	9.554E+003		
Cor Total	0.37	11			

R^2= 0.8697 , Adjusted R^2= 0.7134, Coefficient of Variation (CV) = 15.4%, standard deviation =0.098, mean = 0.63, Predicted residual sum of squares (PRESS) = 0.28

* indicates significant model terms. IGF-I (A) and IGF-II (B) are significant terms

Table 4. Central Composite Design (CCD) matrix of the variables (IGF-I and IGF-II) along with the experimental (n=3) and predicted values of impact of growth factors on cellular metabolism.

Standard order	Factors		Metabolic activity	
	IGF-I (ng ml^{-1})	IGF-II (ng ml^{-1})	Experimental value	Predicted value
1	25	50	0.4636	0.4570
2	100	50	0.4811	0.4905
3	25	150	0.3810	0.3767
4	100	150	0.5669	0.5786
5	9.47	100	0.4458	0.4545
6	115.53	100	0.6348	0.6209
7	62.5	29.29	0.4120	0.4110
8	62.5	170.71	0.4206	0.4164
9	62.5	100	0.3991	0.3837
10	62.5	100	0.3494	0.3837
11	62.5	100	0.3994	0.3837
12	62.5	100	0.3699	0.3837
13	62.5	100	0.4007	0.3837

IGF: Insulin like growth factor

Table 5. Analysis of variance (ANOVA) for the fitted quadratic polynomial model of growth factor (IGF-I and IGF-II) induced metabolic activity in lymphoid cell culture

Source	Sum of Squares	Degrees of freedom	Mean square	F value	Prob>F
Model	0.076	5	0.015	39.36	<0.0001*
A	0.028	1	0.028	71.48	<0.0001*
B	2.963E+005	1	2.963E+005	0.076	0.7901
A^2	0.041	1	0.041	106.49	<0.0001*
B^2	1.569E+003	1	1.569E+003	4.05	0.0841
AB	7.087E+003	1	7.087E+003	18.29	0.0037*
Residual	2.712E+003	7	3.875E+004		
Lack of Fit	5.715E+004	3	1.905E+004	0.36	0.7888
Pure Error	2.141E+003	4	5.352E+004		
Cor Total	0.079	12			

R^2= 0.9657 , Adjusted R^2= 0.9411, Predicted R^2= 0.9062, Coefficient of Variation (CV) = 4.47%, standard deviation =0.020, mean = 0.44, Predicted residual sum of squares (PRESS) = 7.409E+003
* indicates significant model terms

Table 6. Solution for the model with predicted response and the experimental response from the model validation

Solution	Growth factors		Metabolic activity		
	IGF-I	IGF-II	Predicted response	Experimental response	
	(ng ml^{-1})	(ng ml^{-1})			
1	100	150	0.578577	0.5811 0.5801 0.5829	0.581367 *

*Average value of absorbance (570 nm) from MTT assay during model validation (n=3). IGF: Insulin like growth factor.

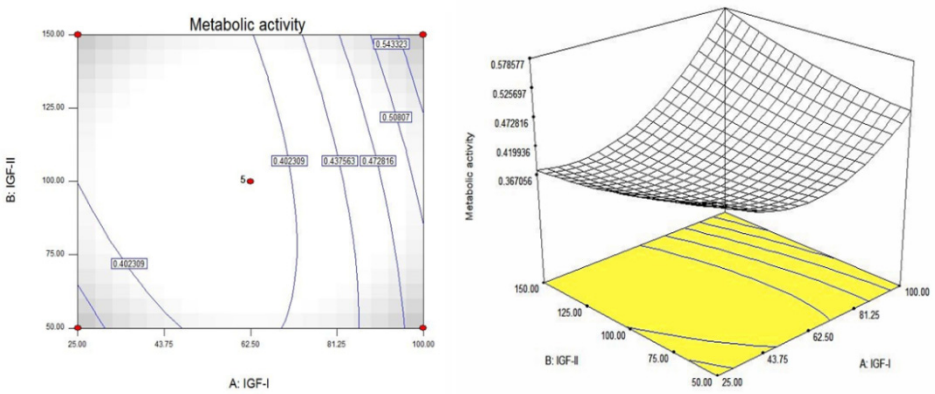


Fig. 4. Contour and Response Surface Plots showing the relative effects of growth factors IGF-I and IGF-II on the metabolic activity of lymphoid organ cell culture grown in SCCM.

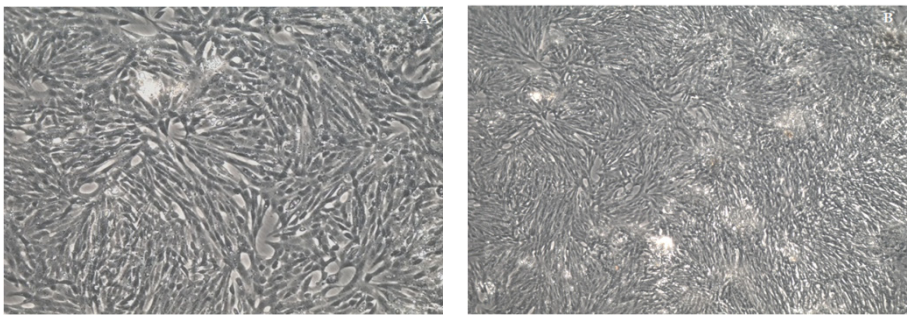


Fig.5. Lymphoid cells from *Penaeus monodon* grown as confluent monolayer in growth factor optimized shrimp cell culture medium (SCCM) *in vitro*. A: Fibroblastic cell type with 20x magnification, and B: the same image with 10x magnification.

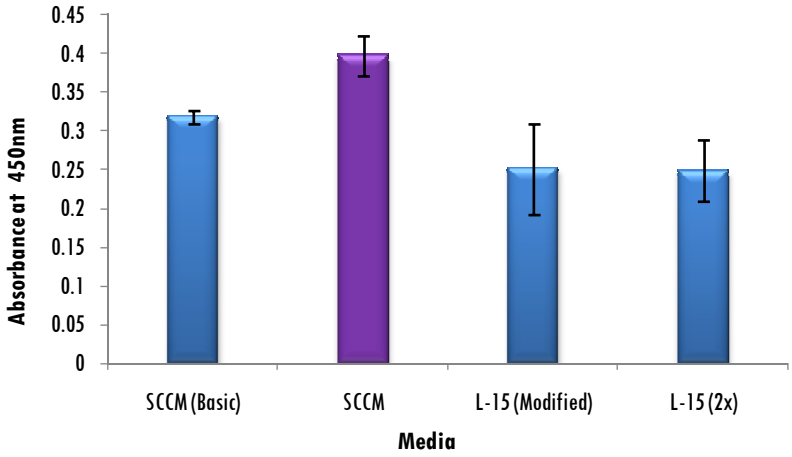


Fig.6. Mitotic activity of lymphoid cells grown in various media. BrdU incorporation assay shows an elevated synthesis phase in lymphoid cells grown in SCCM than other medium (n=3).

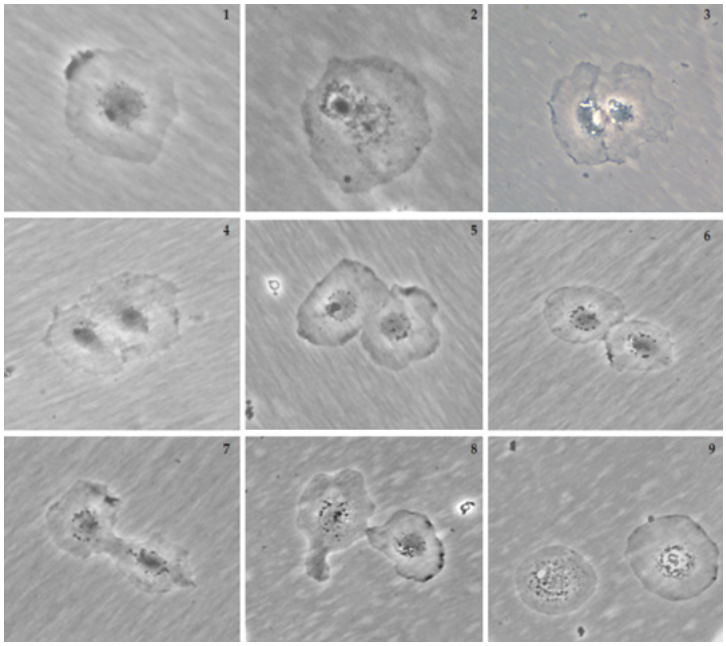


Fig.7. Lymphoid cells *in vitro* grown in SCCM shows mitotic division. Images shows various mitotic events recorded from epithelioid type cells (40 x magnification with focus enlarged images).

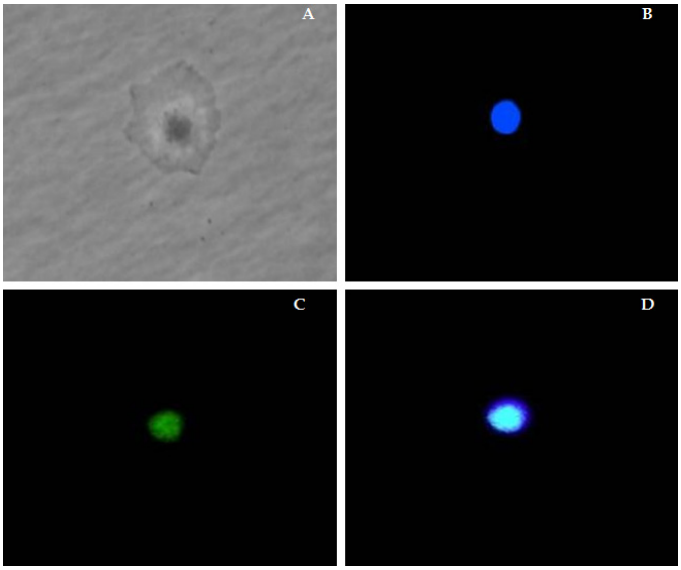


Fig.8. Immunofluorescence detection of synthesis phase of cell cycle in lymphoid cells recorded by BrdU incorporation (40 x magnifications). A: Phase contrast image of lymphoid cell; B: DAPI stained nucleus; C: FITC stained nucleus indicating BrdU incorporation; D: Merged image of B and C.

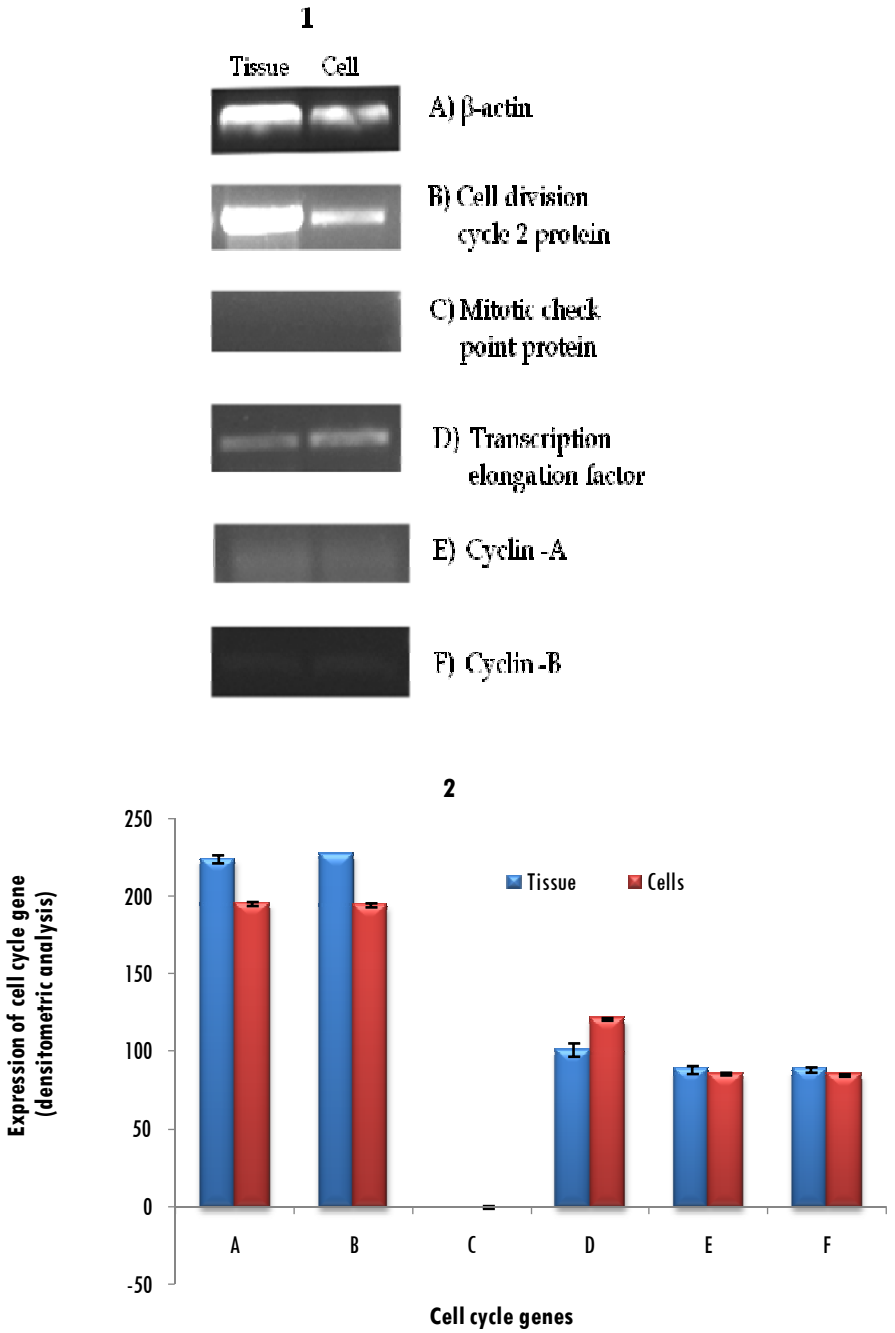


Fig. 9. Cell cycle gene expression in lymphoid cells *in vivo* and *in vitro*. 1: agarose gel image shows PCR amplified cell cycle genes in lymphoid cells of *P. monodon*, 2: Calculated intensity of gene expression as expression profile using Image J software.

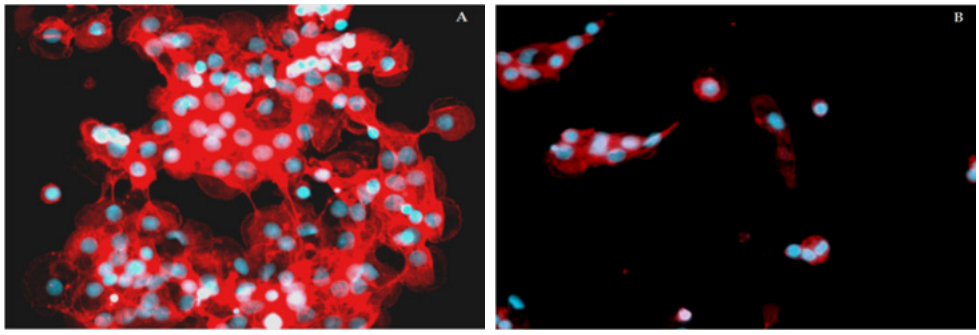


Fig.10. Lymphoid cells from *Penaeus monodon* grown *in vitro* shows actin filaments (Cytoskeleton) stained with phalloidin conjugated with TRITC. A: Picture shows typical 'stress free' petaloid structure of cytoskeletal F-actin filaments (red colour) in lymphoid cells grown in SCCM, and B: the F-actin filaments of lymphoid cells grown in modified L-15 medium. The blue DAPI stained nucleus also seen (40 x magnification).

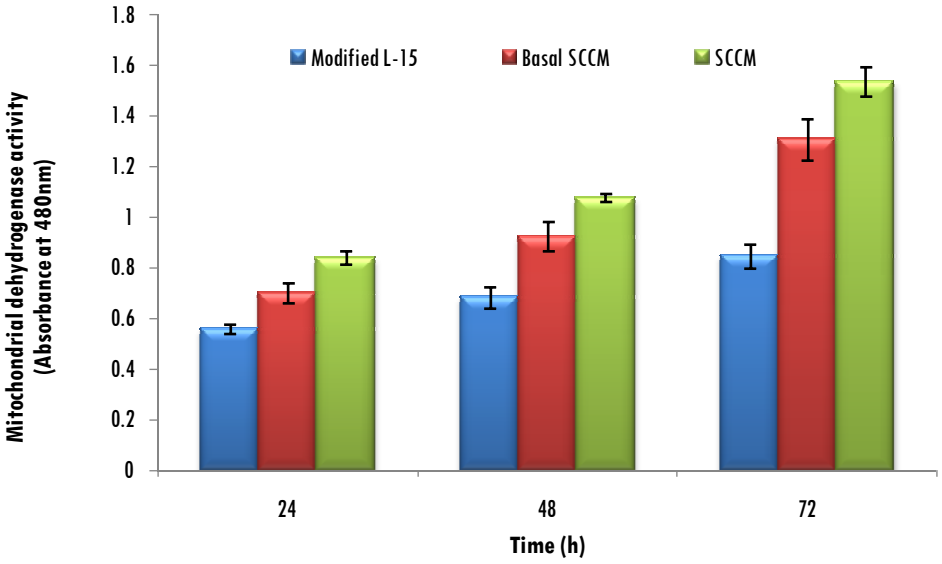


Fig. 11. Growth factor (IGF-I and IGF-II) induced mitochondrial dehydrogenase enzyme activity in lymphoid cell culture. Modified L-15 used as the control medium, while Basal SCCM (without growth factors) for comparing the growth factors induced metabolic activity of cells grown in SCCM (n=3).

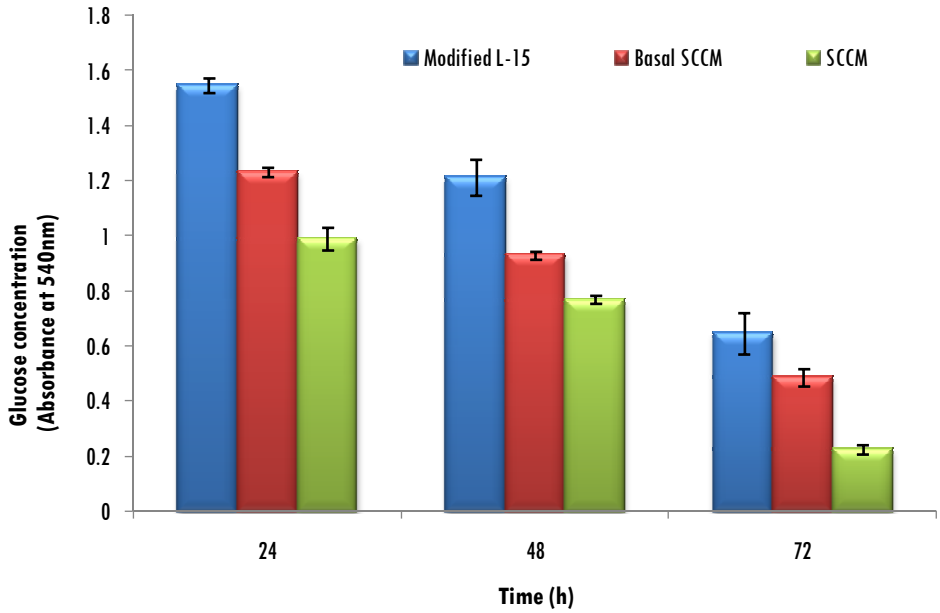


Fig. 12. Growth factor (IGF-I and IGF-II) induced glucose assimilation in lymphoid cell culture. Modified L-15 used as the control medium, while Basal SCCM (without growth factors) for comparing the impact of growth factors in SCCM on lymphoid cell culture (n=3).

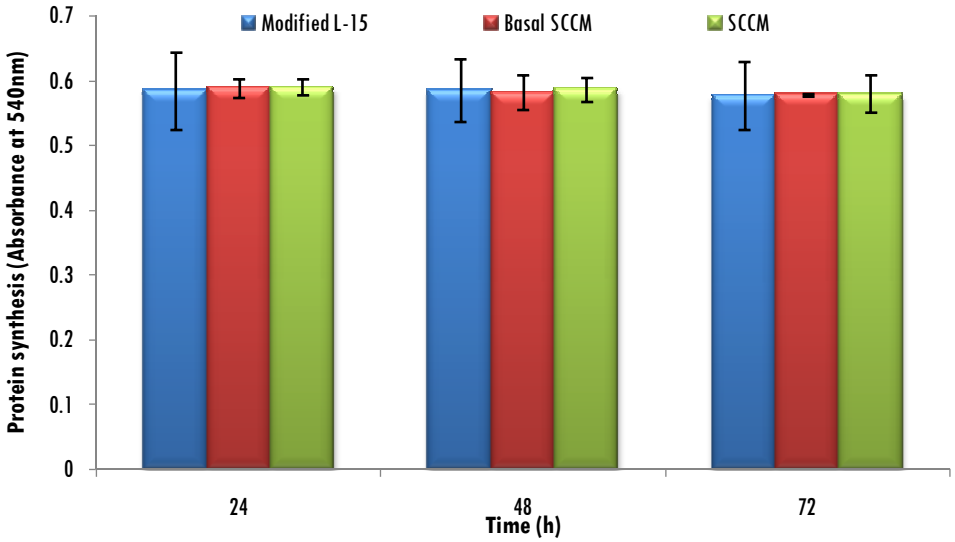


Fig. 13. Growth factor (IGF-I and IGF-II) induced protein synthesis in lymphoid cell culture. Modified L-15 used as the control medium, while Basal SCCM (without growth factors) for comparing the impact of growth factors from SCCM on lymphoid cell culture (n=3).

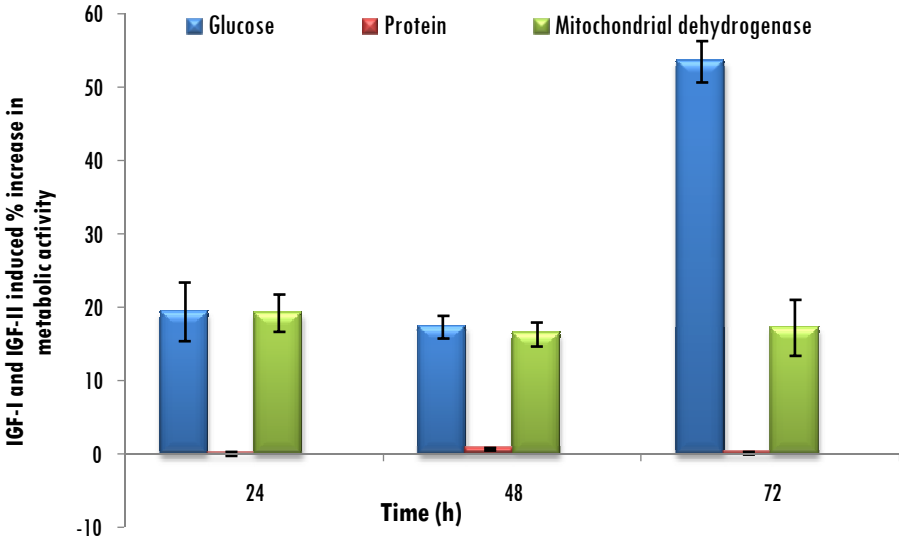


Fig. 14. Growth factors (IGF-I and IGF-II) induced metabolic activity in lymphoid cell culture from *Penaeus monodon*. The percentage increase was calculated while comparing the metabolic activity of lymphoid cell culture grown in SCCM without growth factors (Control).

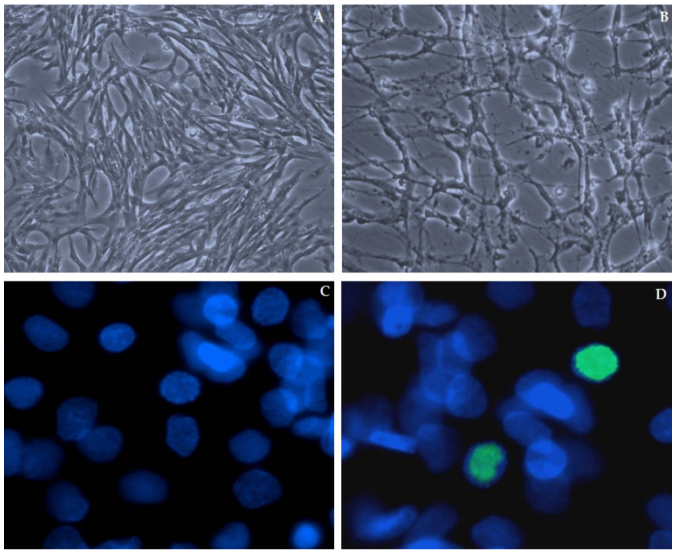


Fig. 15. Virus (WSSV) susceptibility of lymphoid cells grown in shrimp cell culture medium (SCCM). A: control cells, B: 24 h post inoculated lymphoid cells shows typical cytopathic effect. C and D: results from immunofluorescence determination of WSSV protein in cells after 48 h post infection. Green fluorescence from the nucleus (D) confirms the presence of WSSV protein while blue signals show control (C) and uninfected cells.

Chapter 4

Differential expression of telomerase in various tissues and primary lymphoid cell culture, and identification and partial sequencing of telomerase reverse transcriptase *(TERT)* gene in *Penaeus monodon*

4.1. Introduction

Aging is a characteristic of normal cells as a result of their limited proliferative capacity, after attaining their finite life span; the normal cells cease to divide and enter into a state of senescence (Bibby, 2002). In 1961, Hayflick and Moorhead (Hayflick and Moorhead, 1961) reported that diploid fibroblastic cells divide only a finite number of times in culture (the Hayflick limit) and undergo replicative senescence, as described below, (Hayflick, 1965), due to low or absence of telomerase activity and subsequent progressive shortening of telomere (Harley, 1991; Allsopp *et al.*, 1992). With each cell division, telomeres shorten due to the inability of DNA polymerases to replicate the ends of linear DNA molecules (Morin, 1997; Nakamura *et al.*, 1997) and when telomeres reach a critical length, cells enter a non-dividing stage termed senescence (Harley *et al.*, 1990; Cerni, 2000; Lang *et al.*, 2004). Further, the role of telomere shortening in cellular senescence has been observed in different cell lineage (Harley *et al.*, 1990; Allsopp *et al.*, 1992), and confirmed that immortalized cells should have telomerase activity and stable telomeres (Kim *et al.*, 1994; Harley, 1991; Olovnikov, 1973).

Accordingly, germ cells and immortal cell lines express telomerase and maintain telomere length through countless cell divisions (Bibby, 2002).

Telomerase are specialized ribonucleoprotein complex required for the synthesis of telomere terminal repeats through telomere terminal transferase activity that have an important role in chromosome structure and function. The essential components required for this activity are telomerase reverse transcriptase (*TERT*), the catalytic component (Fig.1), and telomerase RNA (TR or TERC: telomerase RNA component) which is the template for DNA repeat synthesis (Bryan and Cech, 1999; Blackburn, 1991, 1994, 2001).

Even though the molecular mechanism of telomerase activation that lead to cell immortalization is not known, the association of telomerase activation in cells during cellular immortalization and expression in tumor growth has been illustrated (Counter *et al*., 1992; Balasubramanian *et al*., 1999). Moreover, telomerase is shown to have a correlation with cell cycle progression and the abnormal expression of regulatory molecules such as cyclins, cyclin dependent kinases (cdks), and cyclin dependent kinase inhibitors (cdkis) may cause alterations in cell cycle with uncontrolled cell growth (Balasubramanian *et al*,. 1999). In addition, telomerase reverse transcriptase (*TERT*) has been considered as the limiting factor for telomerase activity in most normal cells. Further, it has been proved that its ectopic expression or activation has led to the extended replicative lifespan of many cell types (Bodnar *et al*., 1998; Vaziri and Benchimol, 1998; He *et al*., 2009) and their immortalization as well (Venin *et al*., 2000).

Like vertebrates (Forsyth *et al*., 2002), and plants (Fitzgerald *et al*., 1996), the distribution of telomerase activity has been studied in insects (Sasaki and Fujiwara, 2000) and other invertebrates including sponges (Koziol *et al*., 1998), lobster (Klapper *et al*., 1998a), shrimp (Lang *et al*., 2004), molluscs (Owen *et al*., 2007), sea squirt (Sköld *et al*., 2011) corals and algae (Zielke and Bodnar, 2010). Subsequent to the detection of telomerase activity by Lang *et al*. (2004) in *P.*

japonicas, there have been no further studies in this direction. Even though the enzymatic activity of telomerase has been well studied, very little is known about the telomerase genes and their expression profile in many of the cells including those of shrimp.

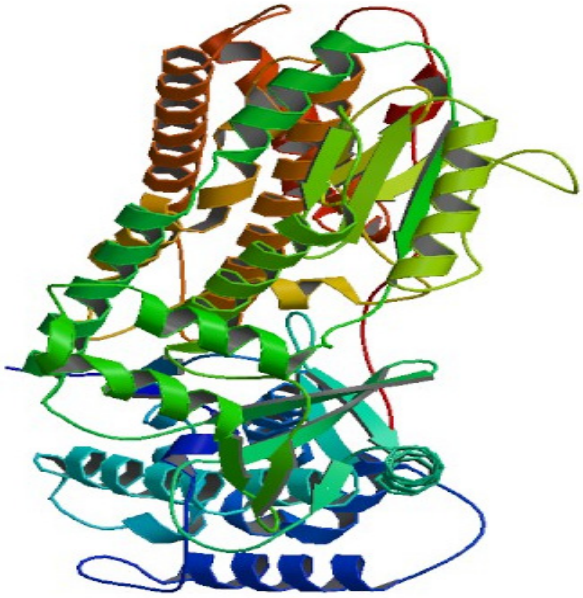


Fig.1. Structure of the *Tribolium castaneum* telomerase catalytic subunit telomerase reverse transcriptase (TERT) (Gillis *et al.*, 2008)

The highly sensitive PCR-based telomerase activity assay, telomeric repeat amplification protocol (TRAP, Kim *et al*., 1994; Kim and Wu, 1997) is the widely accepted technique used to evaluate telomerase activity in tissues or cell extracts

The identification of *P. monodon Telomerase Reverse Transcriptase* (*PmTERT*) gene and its expressed enzyme may be used to discover the telomerase inhibitors (Gomez *et al*., (2002) and the activation of telomerase in cells for the development of *PmTERT* induced immortalized shrimp cells.

In this study, a hypothesis was drawn that the existence of shortened telomeres and the inadequate expression of telomerase activity might be the blocks that resisted the proliferative capacity, spontaneous neoplastic transformation and

immortalization of shrimp cells. To test this hypothesis we measured telomerase in terms of telomerase terminal transferase activity in various stages of life cycle of *P. monodon,* its different tissues, organs and the primary lymphoid cell culture. An attempt was also made for the identification (amplification and sequencing) of shrimp telomerase reverse transcriptase (*PmTERT*) gene from the same.

4.2. Materials and methods

Methodology in this study was designed to test three objectives such as 1: Detection of telomerase activity in various tissues and organs, and the developed lymphoid primary cell culture; 2: Identification of telomeric repeats; and 3: Identification of the *PmTERT* gene. These objectives were identified based on the hypothesis that inadequate telomerase activity in shrimp cell culture *in vitro* might be the reason for preventing continuous proliferation and subsequent spontaneous immortalization. The study envisaged that over expression of *PmTERT* in primary cell cultures might remove the block in immortalization of the cells.

4.2.1. Detection of telomerase activity by telomeric repeat amplification protocol (TRAP)

The TRAP assay was first described in 1994 by Kim *et al*. (1994) to measure telomere terminal transferase activity of telomerase present in tissues or cell extracts. The TS primer (5'-AATCCGTCGAGCAGAGTT-3') in this protocol, acted as substrate (artificial chromosome end) for telomerase-mediated addition of repeats (telomere) by the telomerase present in the extracts which contains the RNA template. The addition of repeats on TS primer occurs at the time of elongation process in this assay, after which PCR is used to amplify the extended products. The TS primer served as forward primer while CX-ext-Shrimp (5'-GTGTAACCTAACCTAACC-3', Lang *et al*., 2004) and CX-ext-Lobster (5'-GTGCCTTCCTTCCTTCCTTCCTTCCTA-3', Klapper *et al*., 1998a) as the reverse primers for the PCR of extended telomere (*in vitro*) by the action of

shrimp tissues/organs and cell culture extracts, while using artemia and C6/36 cell line extracts as the controls. The reverse primer CX-ext-HeLa (5'-CCCTTACCCTTACCCTTACCCTAA-3', Kim *et al*., 1994) was used for PCR amplification of the extended telomere repeats (*in vitro*) on TS primer by the action of HeLa extract as one of the controls. The amplified products were electrophoresed on a 15% nondenaturing polyacrylamide gel, stained with ethidium bromide or SYBR green and photographed using Gel Doc™ XR+ imaging system. (Bio-Rad, USA). The internal amplification standard (ITAS) of 138-bp size was used as control.

4.2.1.1. Experimental animals

White spot syndrome virus negative *P. monodon* larvae obtained from a local hatchery were stocked and reared in a recirculation aquaculture system (RAS) for shrimp with 15 g l^{-1} salinity integrated with nitrifying bioreactors (Kumar *et al*., 2009). Detritus in the system was managed by the addition of Detrodigest™ - (National Centre for Aquatic Animal Health (NCAAH), India) and Enterotrophotic™ - (NCAAH) to control *Vibrio* at the rate of 100 ml m^{-3} of water. The larvae were fed with commercially available pelleted feed (Higashimaru). They were confirmed negative to WSSV by nested PCR after a period of 3 months when they grew to 8–12 g and used as the donor animals for various tissues (Jose *et al*., 2012).

4.2.1.2. Preparation of internal amplification standard (ITAS) as internal control for TRAP assay

For the quantitative TRAP assay in the detection of telomerase activity in shrimp, and as the internal control for PCR amplification, ITAS was prepared and introduced in to the reaction mixture after *in vitro* addition of telomeric repeats on TS-primer by the tissue or cell extracts (telomerase). Cloned 16S rRNA gene of *pseudomonas aeruginosa* was used to get ITAS template (Fig. 2).

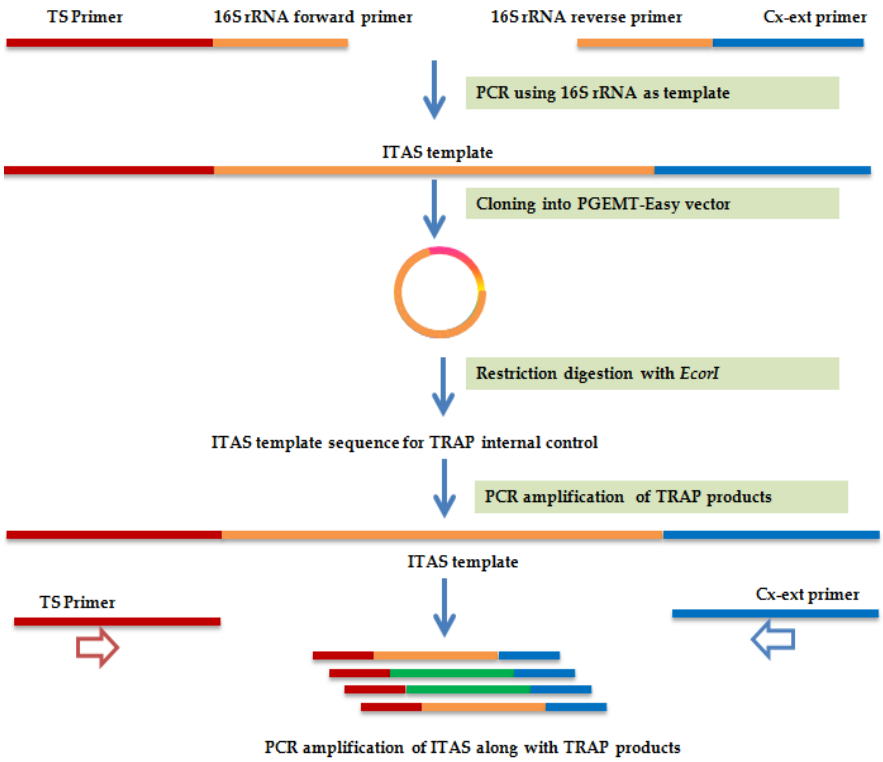


Fig.2. Schematics of the preparation of ITAS template

4.2.1.2.1. Primers designed to construct ITAS and PCR amplification

The primers used were a fusion of TS primer (Bold) and *P. aeruginosa* MCCB 103 16S rRNA gene (underlined) as the forward primer (NP214-F 5'-**AAT CCG TCG AGC AGA GTT** AGC GGC GGA CGG GTG AGT AA-3') and CX-ext of shrimp (Bold) and *P. aeruginosa* MCCB 103 16S rRNA gene (underlined) as the reverse primer (NP214-R5'-**GTG TAA CCT AAC CTA ACC** CCC CCA CTT TCT CCC TCA GG-3') which yielded a 138-bp product. TS and CX-ext primers were selected from literature (Lang *et al.*, 2004) where as the gene sequence moiety from *P. aeruginosa* designed using GeneTool Lite 1.0 software. Specifically, *P. aeruginosa* MCCB 103 16S rRNA gene sequence (Fig. 3a) was taken from the GenBank submission by National Centre for Aquatic Animal Health (Accession No: EF053508). For the synthesis of the forward primer, sequence of *P.*

aeruginosa MCCB 103 16S rRNA gene was added at 3' end of TS primer and the reverse sequence was added at 3' end of Cx-ext shrimp primer. These fused primers were custom made and synthesized by Sigma, USA. The PCR amplification of ITAS was performed in 25 µl reaction mixture containing 2.5 µl 10x buffer, 2.5 µl dNTP (2.5 mM), 1 µl Taq polymerase (0.5 U μl^{-1}), 0.2 µl plasmid DNA (75 ng) containing *P. aeruginosa* MCCB 103 16S rRNA gene sequence, 1 µl of each NP214-F (forward) and NP214-R (reverse) primer (10 pmol μl^{-1}) and the mixture was made up to 25 µl with MilliQ. PCR was performed in Mastercycler personal™ (eppendorf, Germany) and the programme used for the amplification was 95 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 sec, extension at 72 °C for 60 sec, followed by final extension at 72 °C for 10 min. Ten µl of PCR products was analyzed by 1% Agarose gel electrophoresis, stained with ethidium bromide, visualized and documented using Gel Doc™ XR+ imaging system (Bio-Rad, USA).

4.2.1.2.2. Cloning of ITAS with pGEM-T Easy vector and transformation

After PCR amplification of ITAS template, a 138-bp (Fig. 4) was recovered and ligated with pGEM-T Easy Vector (Promega, USA). The 10 µl ligation mixture containing 0.5 µl pGEM-T Easy vector, 3.5 µl PCR product, 5 µl ligation buffer, 0.5 µl ligase and MilliQ was incubated at 4 °C, overnight. The vector containing cloned ITAS template was transformed into *E. coli* JM109 host. Competent cells of the host, *E. coli* JM109 were thawed by placing on ice for 5-10 min. An aliquot of 10 µl ligation reaction mixture was added to a sterile 15 ml culture tube kept on ice, transferred 50-100 µl of thawed competent cells into the same tubes. The tube was gently flicked to mix and placed them on ice for 20 min followed by heat shock for 90 sec in a water bath at exactly 42 °C. Immediately returned the tubes to ice for 2 min, and added 600 µl SOC medium. The mixture was incubated for 2 h at 37 °C with shaking at 220-230 rpm. An aliquot of 100 µl

transformed culture was plated onto LB/ampicillin/IPTG/X-gal plates in triplicate and incubated for overnight (16-18 h) at 37 °C.

4.2.1.2.3. Confirmation of insert in cloned vector and propagation of the confirmed colony

The white colonies were screened and patched on ampicillin/IPTG/X-gal plates to reconfirm the transformation. All the individually streaked colonies were subjected for colony PCR using vector primers designed from either side of the multiple cloning site of the vector so that whatever be the product formed primer could amplify it from either side. The 25 µl reaction mixture containing 2.5 µl 10x buffer, 2.5 µl dNTP (2.5 mM), 1 µl Taq polymerase (0.5 U μl^{-1}), pinch of colony, 1 µl of T7 and SP6 primers each and the mixture was made up to 25 µl with MilliQ. PCR was performed in Mastercycler personal™ (eppendorf, Germany) and the programme used for the amplification was 95 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 sec, extension at 72 °C for 60 sec, followed by final extension at 72 °C for 10 min. Ten µl of PCR products was analyzed by 1% Agarose gel electrophoresis, stained with ethidium bromide, visualized and documented using Bio-Rad gel Documentation system. After confirmation, the transformed *E. coli* JM10 containing cloned vector was propagated in 10 ml LB ampicillin (100 µg μl^{-1}) medium at 37 °C at 230 rpm.

4.2.1.2.4. Extraction and purification of plasmid containing template for ITAS

Plasmid extraction was done using GenElute™ HP Plasmid Miniprep kit (Sigma, USA). An aliquot of 2 ml culture after overnight incubation was pelletised at 12,000 x g for 1 min. The pellet was resuspended in 200 µl resuspension solution containing RNase A and lysed by adding 200 µl lysis buffer. An aliquot of 350 µl neutralization solution was added and centrifuged at 12,000 x g for 10 min to remove the cell debris. Lysate was loaded into GenElute HP Miniprep binding column inserted into a microcentrifuge tube and centifuged at 12,000 x g for 1 min. Plasmid DNA bound to the column was washed twice with wash solution to remove the endotoxins,

salt and other contaminants. To elute the plasmid DNA, the column was transferred to a fresh collection tube, added 100 µl 10 mM Tris-Cl and centrifuged at 12,000 x g for 1 min and stored at -20 ºC. Purity of the plasmid DNA obtained was analysed by agarose gel electrophoresis and by determining the ratio of the absorbance spectrometrically reading at 260/280 nm in a UV-VIS spectrophotometer (U2800, Hitachi, Japan) and fluorometrically using Qubit® flourometer (invitrogen™, USA) .

4.2.1.2.5. Restriction digestion to release ITAS template and purification

The extracted plasmid was restriction digested with *EcoR* I (New England Biolabs) enzyme to use the product as ITAS template during PCR amplification of the extended telomeric repeats (telomere) on TS primer. An aliquot of 20 µl reaction mixture containing 5 µl plasmid, 0.5 µl enzyme (50,000U ml^{-1}), 2 µl reaction buffer (10X) and 12.5 µl MilliQ water was incubated at 37 ºC for 1 h followed by heat inactivation at 65 ºC for 20 min. The restriction digestion was confirmed by 1% agarose gel electrophoresis. The restricted, released ITAS template was gel purified using GenElute™ Gel Extraction kit (Sigma, USA). Briefly, the agarose gel that contain DNA fragment of appropriate size was excised using X-tracta gel extraction tool (Sigma, USA). The excised gel slice was taken in a 1.5 ml tube, weighed and added 3 gel volumes (~450 µl) of gel solubilization solution and incubated at 60 ºC for 10 min with repeated vortexing in every 2 min. After incubation, added 1 gel volume (~150 µl) of 100% isopropanol, mixed gently until it became homogenous. This solubilized gel solution was loaded into the binding column that was pre treated with column preparation solution, centrifuged at 12,000 x g for 1 min. Added 700 µl wash solution and centrifuged for 1 min at 12,000 x g , repeated the centrifugation and residual wash solution was removed. The binding column was transferred to a fresh collection tube (2 ml MCT) and added 50 µl of preheated (at 65 ºC) 10 mM Tris-HCl (pH 9.0), centrifuged at 12,000 x g for 1 min, stored at -20 ºC. The concentration of DNA was measured spectrometrically at 260/280 nm in a UV-VIS spectrophotometer (U2800, Hitachi, Japan) and

fluorometrically using Qubit® flourometer (invitrogen™, USA) . Approximately 18 fg template DNA of purified template of ITAS was used in TRAP assay as control.

4.2.1.3. Preparation of telomerase extracts from tissues and cell cultures

Telomerase extract from tissues and cell cultures were prepared by following the methodology of Lang *et al*. (2004) with slight modification. The extracts were prepared from testis, ovary, lymphoid tissue, heart, hepatopancreas, muscle, eyestalk, nerve cord and post larvae (PL-5) of *P. monodon* and from artemia as control. Approximately 50 mg of each sample was taken in 200 µl of CHAPS lysis buffer contained 10 mM Tris-HCl (pH 7.5), 1 mM EGTA, 0.1 mM benzamidine, 5 mM 2-mercaptoethanol, 0.5% CHAPS (3[(3-Cholamidopropyl) dimethylammonio]-propanesulfonic acid), and 10% glycerol (v/v), crushed with sterile micropestles and inserted in to crushed ice for 30 min. The mixture was centrifuged for 30 min at 20,000xg at 4 ºC; the supernatant was transferred into fresh 1.5 ml micro centrifuge tubes and frozen in liquid nitrogen. The samples were stored at -80 ºC till use (Lang *et al*., 2004).

Lymphoid cell cultures along with the control cell lines HeLa (Human cervical cancer cell line, derived from the cells of a cancer patient, Henrietta Lacks in 1951) and C6/36 (mosquito cell line from *Aedes albopictus* clone no. C6/36) were used to extract telomerase. Lymphoid cell culture was developed in growth factors optimized medium, SCCM, following the methodology explained in the previous chapter (Chapter 3). HeLa cell line was used as control as it had been widely used as telomerase positive control (Bryan *et al*., 1995; Kim *et al*., 1994). HeLa (Human-) cell line and mosquito cell line (C6/36) were collected from the cell repository of National Centre for Cell Science (NCCS), Pune, India. The cell lines, HeLa and C6/36 were maintained in minimum essential medium (MEM, Eagle's) with 2 mM L-glutamine and Earle's balanced salt solution adjusted to contain 0.35 g l^{-1} sodium bicarbonate, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate supplemented with 10% fetal bovine serum and antibiotic

mixture containing 100 µg ml^{-1} streptomycin and 100 IU ml^{-1} penicillin at 37 °C (Heraeus-BBK6220, Germany) and at 28 °C (SANYO MCO-17 A, Japan) respectively. The growth media were changed twice in a week. Cultured cells were washed first with ice-cold PBS then twice with ice-cold wash buffer contained 10 mM HEPES-KOH (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, cells were removed with cell scrapers (Greiner Bio-One, Germany), centrifuged for 5 min at 1000xg at 4 °C. Cell pellets were resuspended in ice-cold wash buffer, pelleted again, and resuspended in ice-cold CHAPS lysis buffer. Telomerase extracts were prepared as described above. The protein concentration of each extract was determined by BCA (bicinchoninic acid) protein assay kit (Sigma).

4.2.1.4. Elongation of telomeric repeats on TS primer by telomerase activity

The addition of telomeric repeats on TS primers by the activity of telomerase takes place as the elongation steps in the TRAP assay. The 50 µl reaction mixture for elongation reaction was composed of 20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.05% Tween 20, 1 mM EGTA, 0.01% BSA, 0.5 mM each dNTP, 1 µM TS primer, and tissue extracts or cell extracts that contained 6 µg protein. The reaction mixture was incubated at 30 ºC for 60 min and followed by incubation at 90 ºC for 90 sec to inactivate telomerase.

4.2.1.5. PCR amplification of extended telomeric repeats (telomere) on TS primer

After elongation step, 5 µl sodium acetate (1 M) and 100 µl ethanol were added to the elongation reaction mixture and kept overnight at 4 ºC for precipitation. Each precipitate was suspended in 50 µl PCR mixture consisted of 5 µl 10x Taq Buffer, 0.2 mM each dNTP, 1 µM CX-ext primer, and 2 U Taq enzyme (New England Biolabs, UK). PCR was performed in Mastercycler personal™ (eppendorf, Germany) and the programme used for the amplification was 95 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 sec, extension at 72 °C for 60 sec, followed by final extension at 72 °C for

10 min. Approximately 18 fg template DNA of ITAS was supplemented into every 50 µl of telomerase PCR reaction mixture as control (Lang *et al*., 2004).

4.2.1.6. Preparation of nondenaturing acrylamide gel and electrophoretic analysis of amplified telomere on TS primer

For preparing nondenaturing acrylamide gel for polyacrylamide gel electrophoresis, acrylamide monomer was prepared first, by mixing 0.8 g N,N′-methylene bisacrylamide to 30 g acrylamide and dissolved in 60 ml distilled water and made up to 100 ml, filtered through Whatman No.1 filter paper. An aliquot of 25 ml acrylamide monomer was mixed with 12.5 ml Tris-Cl (pH 8.8) and 500 µl ammonium persulfate solution (10% w/v) into 11.965 ml distilled water mixed well and added 35 µl N, N,N′,N′-Tetramethyl ethylene diamine (TEMED, #T9281 Sigma). Mixed well and dispensed into 20 x 20 vertical gel plate of Dcode™ Universal Mutation System (Bio-Rad, USA). The whole PCR product was (50 µl) mixed with 3 µl of gel loading dye and analyzed on 15% nondenaturing acrylamide gels. Electrophoresis was performed in 0.5x Tris-borate EDTA (pH 8.3) buffer at a voltage of 155V for 5-6 h (PowerPac™ HV, Bio-Rad, USA), the gel was stained in ethydium bromide solution and SYBR Green (Invitrogen) for 30 min and photographed using Gel Doc™ XR+ imaging system. (Bio-Rad, USA). Relative telomerase activity and the histogram of the telomerase expression were measured from the gel image using Image J software (National Institute of Health, USA). The telomerase activity observed from nerve cord was defined as 1 for calculating the relative telomerase activity as it was the lowest telomerase activity recorded.

4.2.2. Identification of telomere repeats of *Penaeus monodon* by sequencing the TRAP products

A part of the TRAP products from lymphoid tissues (from among the TRAP products obtained from all the tissues the one from lymphoid was chosen for

further work) used for the PAGE analysis was subjected to purification, amplification and sequencing. The sequenced product was analyzed for identifying the telomeric repeats of *P. monodon* that was added on TS primer by lymphoid tissue extracts containing telomerase enzyme.

4.2.2.1. Extraction of TRAP products

TRAP products of lymphoid tissues extract from *P. monodon* were separated on 15% nondenaturing acrylamide gels and the DNA fragments ranging from 100 to 700-bp were recovered from the gel using the 'crush and soak' method (Maxam and Gilbert, 1980). Briefly, the DNA fragment from acrylamide gel was excised into 1.5 ml tube using X-tracta gel extraction tool (Sigma, USA), crushed using small plastic pestle and soaked in 2 gel volume of diffusion buffer (pH 8.0) containing 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 10 mM magnesium acetate and 500 mM ammonium acetate. Incubated overnight at 50 °C, centrifuged at 12,000x g for 1 min and collected the supernatant in a fresh tube. To the supernatant, 3 volumes of gel solubilization solution was added , loaded into DNA binding column used in the GenElute™ Gel Extraction kit (Sigma, USA) and extraction method was followed as explained elsewhere (section 4.2.1.2.5. in this chapter) . The extracted TRAP product was stored at -20 °C (SANYO, Japan) till use.

4.2.2.2. PCR amplification of TRAP products, purification and sequencing

To get the required DNA concentration for sequencing, PCR amplification was carried out using extracted TRAP products as template. The 50 µl PCR mixture containing 5 µl 10x buffer, 5 µl dNTP (2.5 mM), 2 µl Taq polymerase (2U), 2 µl extracted TRAP products and 2 µl (1 µM) of each TS primer (forward) and CX-ext-shrimp (reverse) primer and the mixture was made up to 50 µl with MilliQ. The PCR programme used for the amplification was 95 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 sec, extension at 72 °C for 1 min, followed by final extension at 72 °C for 10

min. The whole PCR product was subjected for gel purification using GenElute™ Gel Extraction kit (Sigma, USA). Briefly, the agarose gel that contain DNA fragment of appropriate size (~700-bp) was excised using X-tracta gel extraction tool (Sigma, USA). Further methodology followed for gel extraction was explained elsewhere. The gel purified PCR product was subjected for further purification using GenElute™ PCR Clean-Up kit (Sigma, USA) to remove the excess salt. Briefly, PCR product from 2 reaction tubes was pooled to get 100 µl volume and added to 500 µl of binding solution. The mixture was transferred to silica binding column, centrifuged at 12,000 x g for 1 min. Added 500 µl wash solution to the colum and repeated the centrifugation to remove residual wash solution. . Replaced the binding column into the collection tube (2 ml MCT) and added 50 µl MilliQ, centrifuged 12,000 x g for 1 min. This colum purified PCR product was subjected for sequencing at SciGenom Labs Pvt. Ltd., Cochin, India.

4.2.2.3. Sequence analysis and identification of telomeric repeats from *P. monodon*

The sequence of TRAP products was subjected for the identification of telomeric tandem repeats of *P. monodon* by Fourier transformation using the spectral repeat finder (SRF) bioinformatic tool (Sharma *et al.*, 2004) at http://www.imtech.res.in/raghava/srf/. Contiguous telomeric sequence and its position were analyzed along with the identification of interstitial occurrence of telomeric repeats and its association with other sequences in *P. monodon*.

4.2.3. Identification of *Penaeus monodon* telomerase reverse transcriptase gene (*PmTERT*)

As gene sequence of *P. monodon* Telomerase Reverse Transcriptase (*TERT*) is not available, telomerase gene (*TERT*) from closely related species published in available databases (Telomerase database) were chosen. Primers were designed using GeneTool software. The species selected for designing primers

included *Bombyx mori* (Silkworm), *Tribolium castaneum (*Red flour beetle), Danio *rerio* (Zebra fish), *Apis mellifera* (Honeybee), *Ciona intestinalis* (Sea squirt), and Human *telomerase reverse transcriptase* (*hTERT*) were used as the control (Table 1). The PCR amplification of *PmTERT* could not be achieved from any of the primers which were designed from the available *TERT* sequence from the database. By the time, the first crustacean (*Daphnia pulex*) whole genome was found deposited in 2011 by Colbourne *et al.* (2011) in GenBank. Based on the sequence of *Daphnia pulex TERT* genes a primer could be designed for the amplification of TERT sequence of *P. monodon* as given below.

4.2.3.1. Identification of *Daphnia pulex TERT* genes and designing primers to amplify *TERT* sequence of *P. monodon*

As the genome sequence of *P. monodon* was not available, the identification of TERT sequences in *P. monodon* using available TERT protein sequence from various organisms was found difficult. To tackle the issue, we hypothesized that the TERT sequence of a crustacean might show some similarity with that of *P. monodon* and the available genome sequence of a crustacean was selected to test this hypothesis. The only available crustacean whole genome sequence was from the species *Daphnia pulex* (Colbourne *et al*., 2011) which was used as the representative crustacean genome. The available TERT protein sequences of invertebrates and vertebrates were collected from the database http://telomerase.asu.edu/ (Podlevsky *et al.*, 2007). With this protein sequences as query the *Daphnia pulex* genome database (Assembly version *Daphnia pulex* v1.0) at http://genome.jgi-psf.org was searched using the TBLASTN algorithm. The protein sequence and their GenBank accession numbers used for the TBLASTN search were *Ciona intestinalis* (EF077623), *Ciona* savignyi (EF514225), *Apis mellifera* (NM001040681), *Bombyx mori* (DQ467676), *Bombyx mandarina* (DQ467677), *Tribolium castaneum*, (NM001040706), *Caenorhabditis elegans*, (NM059972) and *Caenorhabditis remanei* (NM 059973).

4.2.3.2. Designing a primer sequence for the amplification of *PmTERT* gene by using complementary sequence from *Daphnia pulex*

TBLASTN search of *Daphnia pulex* whole genome database (http://genome.jgi-psf.org) was accomplished using *Ciona intestinalis* (EF077623) TERT protein sequence as query. The gene sequence present in the scaffold #47 of *Daphnia pulex* whole genome was found to have sequence identity with the *Ciona intestinalis* (EF077623) TERT protein sequence. The complimentary gene sequence present in the scaffold was used for designing the primer using the software GeneTool Lite™ (version 1.0) to amplify *PmTERT* from *P. monodon*. The oligonucleotide sequence NP593F-5' CGC TCG CCA TTA GTG GTC AGA TAA AGG AAA 3' and NP593R-5' TGA AGA AAA TTC GTC TGG CAT CCA GTG ATG 3' were designed and synthesized (Sigma, USA) as the forward and reverse primers respectively.

4.2.3.3. Amplification of *PmTERT* gene from *P. monodon*

4.2.3.3.1. Experimental animal

Penaeus monodon larvae used in this study were obtained from a shrimp hatchery at Cochin, India (Abad shrimp hatchery, Cochin). Approximately 2000 nauplii were used for RNA extraction. The nauplii were collected through a sieve and kept in RNAlater overnight at 4 °C, drained off the RNAlater and stored at -80 °C till use. This sample was directly used for RNA extraction using TRI reagent (Sigma, USA).

4.2.3.3.2. Total RNA extraction from the larvae of P. monodon

Approximately 50 mg nauplii were used and the total RNA extraction was performed according to the manufactures protocol. Briefly, nauplii were taken into 1.5 ml tube, macerated in 500 µl TRI reagent (Sigma, USA) using RNAse free plastic pestle followed by the addition of additional 500 µl TRI reagent to make the final volume 1000 µl. The sample was kept for 5 min at RT to ensure complete

dissociation of nucleoprotein complexes. To this 1000 µl TRI reagent mixture, 0.2 ml chloroform were added and shaken vigorously for 15 sec and allowed to stand for 15 min at RT followed by centrifugation at 12000x g for 15 min. From the three layers formed after centrifugation, colorless aqueous phase (upper) containing total RNA was transferred carefully to a fresh tube. An aliquot of 0.5 ml isopropanol was added and stored for 10 min at RT and centrifuged at 12000xg for 10 min at 4 °C. RNA was found precipitated on the sides and bottom of the tube after centrifugation. Discarded the supernatant and the pellet was washed twice in 74% ethanol. The pelleted RNA was air dried and dissolved in 20 µl DEPC (Diethyl pyrocarbonate) treated water by repeated pipetting at 55 °C. The extracted RNA was subjected to DNase treatment with RNase free DNase 1 (New England Biolabs, UK) by adding 0.2 U of enzyme to 1 µg of RNA and incubated at 37 °C for 10 min followed by 75 °C for 10 min for inactivation. RNA concentration and quality were determined by optical density (OD 260/280 nm) measurement using a UV-Visible spectrophotometer (Hitachi, Japan).

4.2.3.3.3. cDNA synthesis

An aliquot of 5 µg RNA was subjected to cDNA synthesis. The 20 µl reaction mix containing M-MuLV reverse transcriptase (200 U), RNase inhibitor (8 U), Oligo $(dT)_{12}$ primer (40 pmoles), dNTP mix (1 mM), RTase buffer (1x) and $MgCl_2$ (2 mM) and the synthesis was performed by incubating at 42 °C for 1 h. All reagents were purchased from New England Biolabs, UK.

4.2.3.3.4. RT-PCR of PmTERT gene from P. monodon

The telomerase reverse transcriptase gene (*PmTERT*) was amplified from *P. monodon* by PCR using the primers NP593 F-5' CGC TCG CCA TTA GTG GTC AGA TAA AGG AAA 3' and NP593R -5' TGA AGA AAA TTC GTC TGG CAT CCA GTG ATG 3' designed from the *TERT* gene of *Daphnia pulex*. An aliquot of 25 µl PCR reaction mix contained 1 µl cDNA, 0.5 U of Taq DNA polymerase, 200 µM dNTP mix, 10 pmoles each of forward and reverse primers

and 1x PCR buffer. The PCR programme used for the amplification of *PmTERT* was 95 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 65 °C for 30 sec, extension at 72 °C for 1 min, followed by final extension at 72 °C for 10 min. An aliquot of 10 µl PCR products was analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light and documented using Gel Doc™ XR+ imaging system (Bio-Rad, USA).

4.2.3.3.5. Cloning and sequencing of PmTERT gene

After PCR amplification, the partial sequence of *PmTERT* gene was cloned in to pGEM-T Easy vector (Promega, USA) and transformed into *E. coli* DH5α. The cloned plasmid was used for sequencing and the sequencing was performed at SciGenome Labs Pvt. Ltd, Cochin, India. The detailed methodology has already been given elsewhere in this chapter. The sequence was subjected for BLAST search in various genome/proteome databases such as European Nucleotide Archive (ENA) http://www.ebi.ac.uk/ena/, *Daphnia pulex* genome database in http://genome.jgi-psf.org. Protein Knowledge base (UniProtKB) http://www.uniprot.org/uniprot/ and the sequence similarity identification was performed (Altschul *et al*., 1997).

4.3. Results

4.3.1. Detection of telomere terminal transferase activity (telomerase) in various tissues and lymphoid cell culture from *P. monodon*.

The results of the polymerase chain reaction (PCR) based telomeric repeat amplification protocol (TRAP) showed the presence/absence of typical DNA ladder formation by telomerase activity confirming the presence/absence of telomere terminal transferase activity in the extracts of various tissues and organs of *P. monodon*, post larvae, *Artemia* nauplii, primary lymphoid cell culture, cell lines such as HeLa, C6/36, as presented in Fig. 3a and 3b. The internal

amplification standard (ITAS) was used to normalize the peak to perform the densitometric analysis and to confirm the PCR amplification of the product with TS and Cx-est primers (Fig. 4). In this experiment, telomerase activity was detected in the tissues of *P. monodon* such as testis, ovary, lymphoid organ, heart, hepatopancreas, muscle, eyestalk, nerve cord and from the post larvae, with slight variation in its expression. The histogram of telomerase activity of the extract was measured by densitometric analysis of the gel image using Image J software (Fig. 5). Moreover, the relative telomerase activity of various tissues of *P. monodon* was calculated with the value for nerve cord 1±0.08; heart 1.23± 0.18; eyestalk 1.83±0.21; muscles 2±0.05; post larvae (PL) 2.07±0.15; hepatopancreas 2.57±0.26; ovary 3.5±0.15; testis 3.5±0.14 and lymphoid organ 3.5±0.07 (Fig. 6). As shown in the Fig. 6, telomerase is expressed in all tissue that were analysed, with the highest relative telomere terminal transferase activity in ovary, testis and lymphoid organ. Moderate levels of activity were detected in eyestalk, muscles, hepatopancreas and tissues from the post larvae. Even though excellent activity and DNA ladder formation were observed from the HeLa cell extract, only limited activity was observed from C6/36 cells (Fig. 3b). The extract from artemia nauplii was also given a typical ladder formation in the gel due to telomere terminal transferase activity of telomerase. However, telomerase activity was found to be negative or inadequate in the primary lymphoid cell culture with repeated experiments. Moreover, results from control cells, tissues and the amplified internal amplification standard (ITAS) confirmed that the experimental protocols were valid without any ambiguity.

4.3.2. Identification of canonical telomeric repeats added by the telomere terminal transferase activity (telomerase) of lymphoid tissue extract from *P. monodon*

Sequencing results of the telomeric repeats added on the artificial chromosome end (TS primer) by the action of telomere terminal transferase

activity (telomerase) of lymphoid tissue extract revealed that the pentameric TTAGG repeats were the canonical telomeric repeats of *P. monodon*. Meanwhile, the results from spectral repeat finder (SRF, Fig. 7) suggested that in addition to the known TTAGG pentameric repeats of insects and invertebrates, telomere terminal transferase activity of *P. monodon* added two other pentameric repeats GGTTA and AGGTT. Further, the sequence analysis of the PCR products from telomerase activity showed the interstitial occurrence of TTAGG and association with certain unrelated sequences and low-copy repeats. However, among the repeats the TTAGG was contiguous (Fig. 7) with 32 repeats, $(TTAGG)_{32}$ suggesting the highest possibility as telomeric repeats of *P. monodon*.

4.3.3. Identification and cloning of *P. monodon TERT* (*PmTERT*) genes

Direct PCR amplification of *PmTERT* gene from *P. monodon* using the primers designed from the *TERT* gene sequence of various invertebrates and vertebrates including *Bombyx mori, Tribolium castaneum, Danio rerio, Apis mellifera, Ciona intestinalis* and Human *telomerase reverse transcriptase* (Table 1) were found unsuccessful. Meanwhile, the TBLASTN search of *Daphnia pulex* genome using the *Ciona intestinalis* (EF077623) and *C. savignyi* (EF514225) TERT protein sequences as query successfully identified the putative exons that were homologous with *TERT* gene sequence of *Daphnia pulex*. However, TERT protein sequence used from other invertebrate species such as *Apis mellifera* (NM001040681), *Bombyx mori* (DQ467676), *Bombyx mandarina* (DQ467677), *Tribolium castaneum* (NM001040706), *Caenorhabditis elegans* (NM059972) and *C. remanei* (NM 059973) did not give any results from the TBLASTN search indicating its divergence with crustacean *TERT* sequence. Even though *Ciona intestinalis* and *Ciona savignyi* TERT protein sequences were homologous with *Daphnia pulex* whole genome at the scaffold #47, *C. intestinalis* TERT protein sequence was used for further analysis. Moreover, the *Daphnia pulex* TERT protein was found to be homologous with *C. intestinalis* TERT located in the

scaffold #47 region in whole genome at 332981-333070 and at 333746-333883 DNA base pairs (Fig. 8), and this region was used for designing gene-specific primers to amplify partial cDNA fragments of *P. monodon TERT* gene and designated as *PmTERT*.

The primers NP593F -5' CGC TCG CCATTA GTG GTC AGA TAA AGG AAA 3' and NP593R-5' TGA AGA AAA TTC GTC TGG CAT CCA GTG ATG 3' were designed from the region of scaffold #47 and PCR amplification was successfully carried out; a 68-bp sized product (*PmTERT*) was obtained from *P. monodon* (Fig. 9) and sequenced (Fig. 10). *PmTERT* gene sequence was further converted to amino acid sequence and the resulted amino acid sequence (Fig. 10) was used for BLAST analysis (Fig. 11).

The BLAST search in European Nucleotide Archive (ENA) resulted in 100% identity with EMBL-CDS: EFX76361.1: *Daphnia pulex* telomerase reverse transcriptase, and the scaffold #47 of the *Daphnia pulex* genome database in http://genome.jgi-psf.org. Further to this identity, the sequence similarity identification revealed that PmTERT protein sequence (partial) shared 100% identity with the TERT sequence of *Daphnia pulex* (which are recently submitted in GenBank with accession no. E9GVZ2), 27% sequence identity with *Strongylocentrotus purpuratus* (Purple sea urchin) and only 24-25% with *Danio rerio* (Zebra fish) confirming its divergence (Fig.12).

4.4. Discussion

The results from telomerase activity demonstrate that telomerase is constitutively active in all tissues tested from *P. monodon*. The typical DNA ladder formation on using the tissue extract during TRAP assay was confirmed by the extension of TTAGG oligonucleotide repeats added by the presence of complimentary sequence on RNA components of telomerase enzyme. This implies that telomerase-dependent telomeric lengthening has been taking place in *P.*

monodon and the adult tissues were retaining this capacity throughout their lifespan. The need for this activity can be explained by the continuous growth of *P. monodon* tissues, which must be based on active cell proliferation in various stages of its life cycle including regeneration of exoskeleton during molting, suggesting maintenance of stem cells throughout life (Vogt, 2012). Such constitutive activity of telomerase has been reported from lobsters (Klapper *et al*., 1998a), bivalves (Owen *et al*., 2007), shrimp (Lang *et al*., 2004), fishes (Klapper *et al*., 1998b; Anchelin *et al*., 2011) and insects (Sasaki and Fujiwara, 2000; Mohan *et al*., 2011). Moreover, telomerase activity has been considered as cell proliferation and cell aging markers (Vogt, 2011; Belair *et al*., 1997). Contradictory to this, most of the human somatic tissues lacks telomerase activity (Lang *et al*., 2004) and was detected only in germline and tumor cells (Kim *et al*., 1994; Wright *et al*., 1996).

Lang *et al*. (2004) suggested that among various tissues tested from the shrimp *P. japonicus*, consistent telomerase expression was observed in ovary and testis, and explained that this was due to the presence of germline cells and low differentiated cells along with the active cell division in reproductive organs. In the same line, the present study support the findings of Lang *et al*. (2004) with highest (relative) telomerase activity detected in ovary and testis along with lymphoid organ from *P. monodon*. Meanwhile, Klapper *et al*. (1998a) reported a highest telomerase activity in hepatopancreas and heart tissue of the lobster *Homarus americanus*. In contradictory to the findings of Lang *et al*. (2004), in the present study, despite the telomerase activity in tissue (*in vivo*) counterpart, telomerase activity was not detected from lymphoid cell culture *in vitro* with repeated experiments. These findings support the hypothesis that the inadequate telomerase activity or gene expression might be the reason that prevents neoplastic transformation and spontaneous immortalization of the cells *in vitro*.

Interestingly, the sequencing results showed that the pentameic repeats, TTAGG were added to the TS primers by the telomeric terminal transferease

activity (telomerase) present in the lymphoid tissue extract from *P. monodon*. These repeats were added based on the complimentary RNA sequence present in the telomerase of *P. monodon*, suggesting that the TTAGG repeats were synthesized by telomerase of *P. monodon*. Moreover, the sequence analysis of added telomere repeats on TS primers and the results from spectral repeat finder revealed the interstitial occurrence of the telomere repeats. This supports the findings of Mohan *et al*. (2011) on the interstitial occurrence of telomeric repeats and its association with certain unrelated low-copy repeats in mealy bug, *Planococcus lilacinus*.

The synthesize of canonical TTAGG telomeric sequence by telomerase has been reported from many species such as lobsters (Klapper *et al*., 1998a), shrimp (Lang *et al*., 2004), and from many insects (Sasaki and Fujiwara, 2000; Vitkova *et al*., 2005; Monti *et al*., 2011) and studied the phylogenetic distribution of TTAGG telomeric repeats in insects (Frydrychová *et al*., 2004). Besides, the telomerase-independent mechanism of telomere maintenance, such as the alternative lengthening of telomeres (ALT) has been reported in *Drosophila* (Pardue *et al*., 1996) yeasts (Lundblad and Blackburn, 1993), and human (Bryan *et al*., 1995, 1997; Henson *et al*., 2002;). In this context the telomerase-dependent telomere maintenance has been considered as a conserved mechanism for maintaining long-term cell proliferation capacity (Kapper *et al*., 1998a). The telomere sequence type $(TTAGGG)_n$ is conserved among many phyla in the Animalia, including Vertebrata (Meyne *et al*., 1989), Mollusca (Wang *et al*., 2001; Gallardo-Escarate *et al*., 2005; Sakai *et al*., 2005) Annelida (Jha *et al*., 1995), Platyhelminthes, (Joffe *et al*., 1996) Onychophora, (Vitkova *et al*., 2005) and Echinodermata (Okazaki *et al*., 1993). However, other telomere types, namely $(TTAGGC)_n$ in the Nematoda (Muller *et al*., 1991) and $(TTAGG)_n$ in the Arthropoda (Okazaki *et al*., 1993; Sahara *et al*., 1999; Frydrychová *et al*., 2004; Vitkova *et al*., 2005) including shrimp (Lang *et al*., 2004) and lobster (Klapper *et al*., 1998a), are found in the kingdom Animalia (Sakai *et al*., 2007). More recently,

Gomes *et al*. (2010) concluded that with the exception of Nematodes and Arthropods, the $(TTAGGG)_n$ sequence is conserved in most Metazoa. Precisely, the results from this study emphasize the conclusion that the distribution patterns of TTAGG repeats are essentially consistent with telomerase-dependent telomeric maintenance in Phylum Arthropoda (Okazaki *et al*., 1993; Klapper *et al*., 1998a; Sahara *et al*., 1999; Sasaki and Fujiwara, 2000; Lang *et al*., 2004; Vitkova *et al*., 2005; Monti *et al*., 2011).

Telomerase reverse transcriptase (TERT) is the catalytic subunit of telomerase, that synthesize telomeric DNA $(TTAGG)_n$ (in the case of Arthropods) or $(TTAGGG)_n$ (in the case of most Metazoa with the exception of Arthropods) directly onto chromosome ends. Various studies have proved that the ectopic expressions of TERT in cells induce the telomerase activity and lengthen telomeres in human (Bodnar *et al*., 1998; Counter *et al*., 1998; Vaziri *et al*., 1998; Perrem *et al*., 2001) dogs, (Techangamsuwan *et al*., 2009) and mouse (Armstrong *et al*., 2000). Meanwhile, immortalization of cells was successfully performed with the ectopic expression of TERT in epithelial cells of human (Morales *et al*., 1999; Piao *et al*., 2005) and goat (He *et al*., 2009). As a corollary, identification of gene that encoding TERT in *P. monodon* (*PmTERT*) is of great importance as the envisaged immortalization of shrimp cells could be possible with the ectopic expression of *PmTERT*. The *TERT* cDNA has been isolated and characterized from yeast (Counter *et al*., 1997) and vertebrates such as fish (Rao *et al*., 2011), frog, (Kuramoto *et al*., 2001), chicken (Delany and Daniels, 2004), mouse (Greenberg *et al*., 1998), hamster (Guo *et al*., 2001), dog (Nasir *et al*., 2004), and human (Meyerson *et al*., 1997); invertebrates such as ascidians (Li *et al*., 2007), protozoan (Bryan *et al*., 1998), and insects (Gillis *et al*., 2008; Osanai *et al*., 2006). However the telomerase reverse transcriptase has not yet been identified from *P. monodon*. In the present study a partial sequence of *P. monodon TERT* gene, *PmTERT* was identified and characterized. Moreover, the BLAST analysis suggests its similarity with *TERT* of *Daphnia*, zebra fish and sea squirt. As the *TERT* gene sequence was

not available, the partial gene sequence identified from this study is vital for the characterization the *PmTERT* from *P. monodon*.

In conclusion, telomerase activity was detected in all tissues tested and the TTAGG repeats identified as the canonical telomeric repeats from *P. monodon*. However, telomerase activity was not detected from the primary lymphoid cell culture indicating the inactivation of telomerase gene or inadequate telomerase in cells *in vitro*. Additionally, partial putative TERT region of *P. monodon* was identified, sequenced and named as *PmTERT*. The partial sequence of *PmTERT* and eventual reconstitution of active telomerase reverse transcriptase gene and its expressed enzyme may be used to discover the telomerase inhibitors (Gomez *et al.*, 2002) in *P. monodon* cell culture *in vitro*, which in turn will permit the activation of telomerase in cells with an envision of *PmTERT* induced immortalized shrimp cells in future.

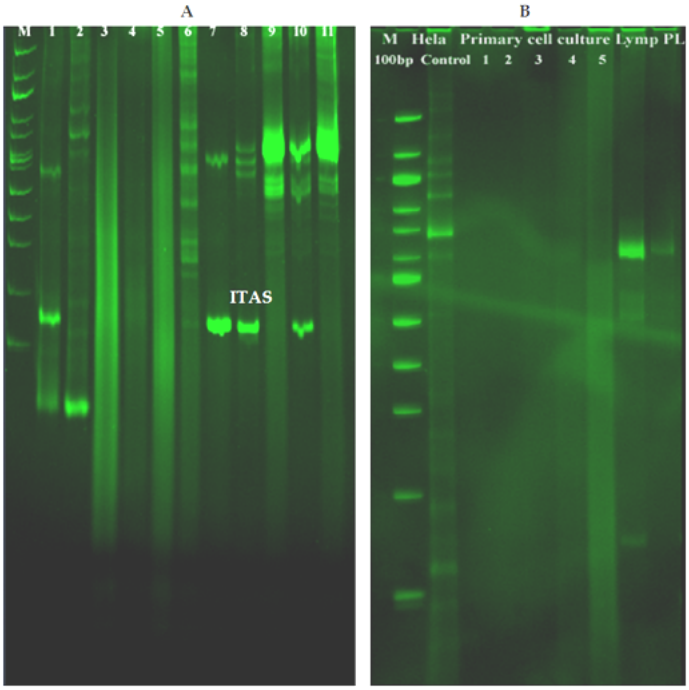


Fig. 3a. A: TRAP assay results: M- 100-bp Marker, 1,2-HeLa cells (+ve control), 3-Hepatopancreas, 4-Heart, 5-Post Larvae, 6- Artemia, 7-Muscles, 8-Nerve cord, 9- Ovary, 10-Testis, 11-Eyestalk, ITAS-internal amplification standard, B: TRAP assay results: M-100-bp Marker, HeLa cells (+ve control), 1, 2, 3, 4, 5: Primary lymphoid cell culture, Lymp-Lymphoid organ, PL-Post larvae

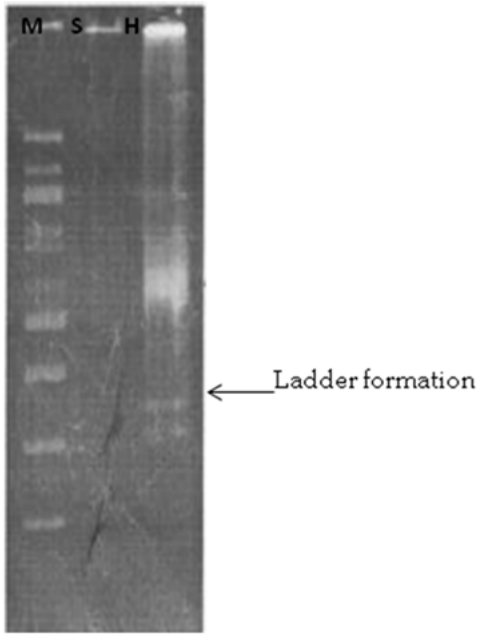
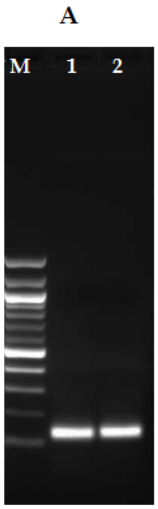


Fig.3b. TRAP assay results: M-Molecular marker (100-bp), S- Shrimp primary lymphoid organ culture, H-C6/36 insect cell line



B

GAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGATGAAGGGA
GCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGATAGTAGGGGATAACG
TCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTAT
CAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGT
CTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAA
TATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGC
ACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACC
GGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAA
GCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACT
ACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGG
AACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACA
GGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGT
GGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGAC
GGGGGCCCGCACAAGCGGTGGAGCACGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGA
CATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCAC
CTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCA
TGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGG
AGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGC
TAGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCAT
GGGAGTGGGTTGCTCCAGAAGTAGCTAGTCTAACCGCAAGGGGGACGGTTACCACGGAGTGATTCATGAC
TGGGGTGAAGTCGTAACAAGGTAGCCGT

Fig. 4. TRAP internal amplification standard (ITAS). A: Gel image of PCR amplified TRAP internal control ITAS designed from the 16S rRNA gene sequence of *Pseudomonas aeruginosa*, TS and Cx-ext primer sequence, M: 100-bp molecular marker, 1 and 2: amplified ITAS of 138-bp size, B: *P. aeruginosa* MCCB103 16srRNA gene sequences (GenBank accession No: EF053508) used for constructing ITAS. The marked region is the 104-bases from 16srRNA gene contributed in ITAS.

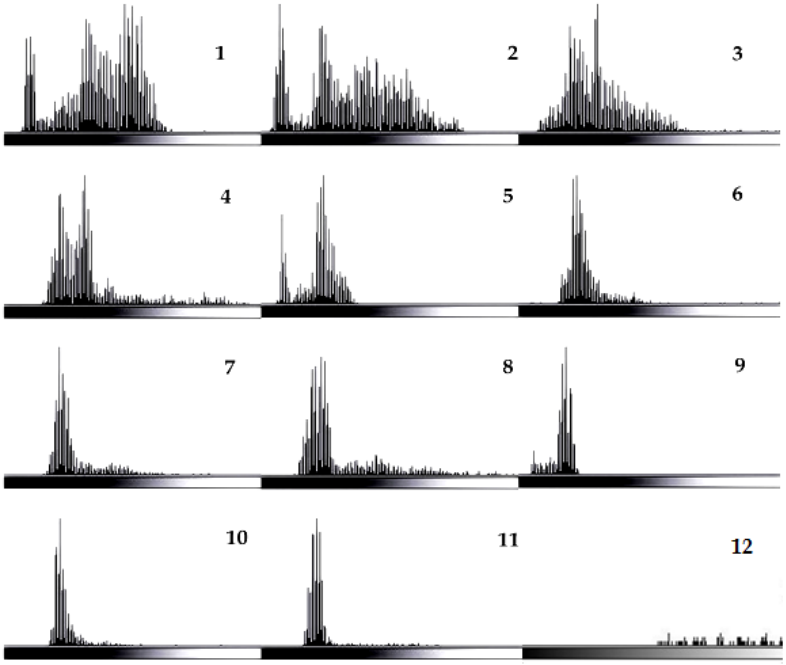


Fig.5. Telomerase activity scored as histogram using Image J software. 1) HeLa cell line 2) artemia 3) ovary 4) testis 5) lymphoid 6) hepatopancreas 7) eyestalk 8) muscle 9) post larvae 10) heart 11) nerve cord 12) primary lymphoid cell culture.

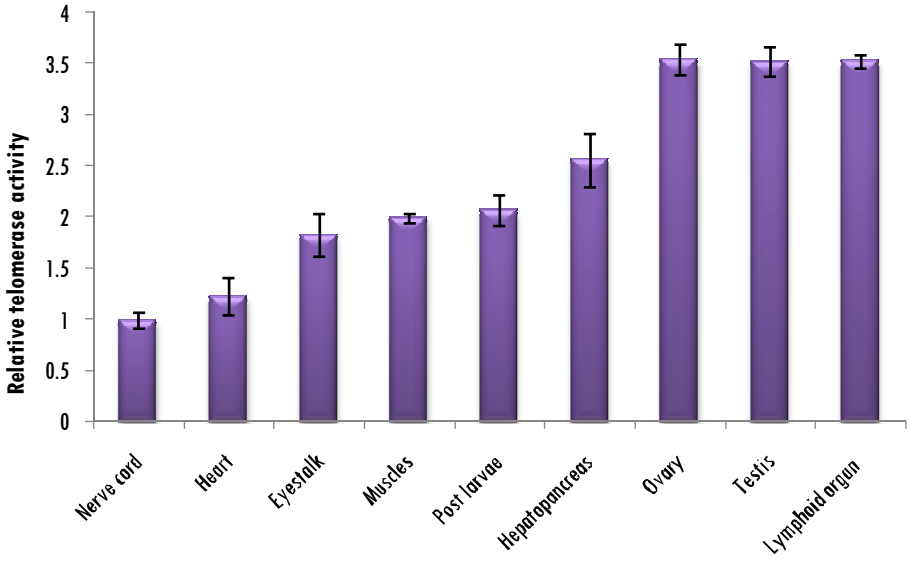
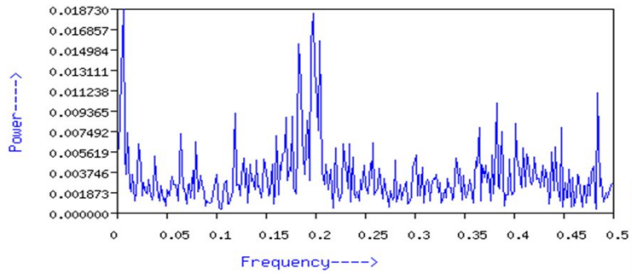


Fig.6. Relative telomerase activity in various tissues from *P. monodon* and its post larvae. The results represent the mean ± standard deviation (n= 3).

A



B

Pattern Searched	Region	Consensus	Copy No	Score
View Window Spectrum for 2 mer				
AT	1::525	AT	55	1.000
View Window Spectrum for 4 mer				
TAAT	1::469	TAAT	42	0.756
View Window Spectrum for 5 mer				
GATTA	1::627	GGTTA	32	0.806
TTATG	1::627	TTAGG	32	0.806
ATGTT	1::627	AGGTT	32	0.806
View Window Spectrum for 6 mer				
TATGTT	1::627	TAGGTT	17	0.843
View Window Spectrum for 7 mer				
AACATAA	1::518	AACATAA	7	0.878

C

Pattern Searched :: TTATG
Consensus Pattern :: TTAGG
Number of Copies :: 32
Score :: 0.806

```
100 TTCTGAATCG TGTATTTAAC GTATTGCGAC GCAAGATTAC AAGAATTATA TTCCCTGATA 159
160 AACGTTAATA CAGGAAAGAA ATGGAACAGC GATTTTATGT TCGAATGAAA ATCCTTGGAC 219
220 CGTGTATATG ATTTGGCTTA TTCATTTTTC TTTACAGATA AGTTTCGAAT ACTTCTAAGC 279
280 ACACAATATA AGTAGAAACT ACTATATGTA GAAGTTATTG ATTTCGACAA GGTATAACCC 339
340 TAGGGTGATT CTATCCTGAA ATCATGCCGG ACTCCCCTCA TGGCATAGCC CGGGCGAGGC 399
400 TGAGCTTCGC ATATCGGACT TATCCTTATC CTGAAATCCA TTGGCTTTGT CGACTCAATA 459
460 CTAAGTTCCA ACAAAGAAAA TAAAGCAAAA TGGAAATGTA TGCTAACTGA CATATTAACA 519
520 TTAGGTTTAG GTTAGGTTAG GTTAGGTTAG GTTAGGTTAG GTTAGGTTTA GGTTAGGTTA GGTTAGGCTG 579
580 GTTAGGTTAG GTTAGGTTAG GTTAGGTTAG GTTAGGTTAG GTTAGG 625

POSITIONS :

100  145  194  225  237  303  314  361  377  419  425  497  520  526  531

536  541  546  551  557  562  567  572  581  586  591  596  601  606  611

616  621
```

Fig. 7. Identification of the telomeric sequence using Spectral Repeat Finder (SRF). A: Fourier Spectrum of *P. monodon* telomeric sequence designed from SRF, B: Output from SRF, red box indicating that TTAGG are repeated 32 times in the given telomeric sequence, C: Position of TTAGG repeats in the telomeric sequence identified from SRF indicating contiguous TTAGG sequences

Table1 Primers used to amplify TERT gene from *P. monodon* (Custom made)

S.No	Species	Primers from *TERT* gene
1	*Bombyx mori*	F- 5′-TTTATCGAAATATGAATACCCCC-3′
		R-5′-CCATTCCATATATTCCAGTGAA-3′
		F-5′-GTGAAACGTCGATTTCTAGCTTAA-3′
		R-5′ GGTATTAAATTGGAACATTTCCATGTT-3′
2	*Tribolium castaneum*	F- 5′-ATGGTCCACTACTATCGC-3′
		R- 5′-AAATAACTCGCATCCACCTC-3′
3	*Danio rerio*	F-5'CCCCAAGCACGCGCACAGATGTC 3'
		R-5'ATGCTGTGTTTACGAGTGTGTGT 3'
4	*Apis mellifera*	F-5'TAGCTTAAGGGTTGTTGTTTT 3'
		R-5'ATGAAAAAAATGTGATAAAATA 3'
5	*Ciona intestinalis*	F-5'TTCGGTCGGTTTTGTATCTCCA 3'
		R-5'CACAGGGGAGGCAGGGATAGT 3'
6	*Homo sapiens*	F-5'GCCGAATTCTGCCGTTGCCCAAGAGG 3'
		R-5'GCGTGGATCCCAAGCAGCTCCAGA 3'

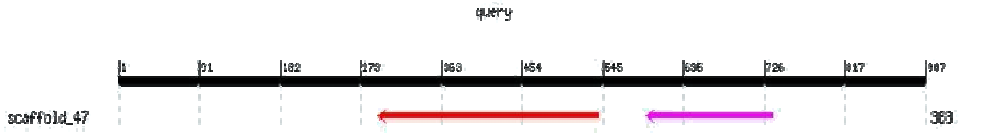


Fig. 8. TBLASTIN results suggesting that the *C. intestinalis* TERT sequence is complimentary with the sequence of *Daphnia pulex* genome scaffold 47, further confirming the complimentary region of *Daphnia pulex* genome containing putative *TERT* gene sequence.

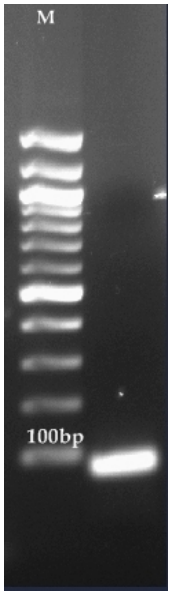


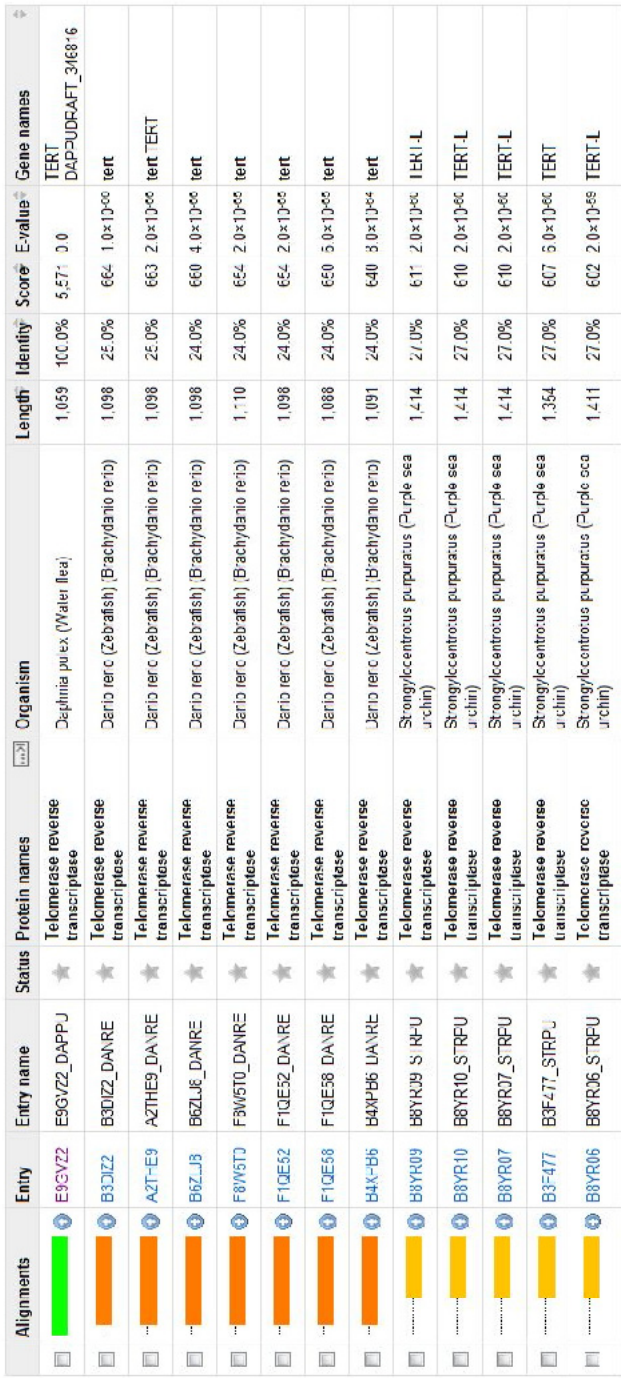
Fig. 9. Amplified *PmTERT* gene from *Penaeus monodon* using the *TERT* gene primer from *Daphnia pulex*

```
5'TTGAAGAAAATTCGTCTGGCATCCAGTGATGGCGAGCGTTTCCTTTATCTGACCACTAAT
   L  K  K  I  R  L  A  S  S  D  G  E  R  F  L  Y  L  T  T  N
GGCGAGCG 3'
 G  E
```

Fig.10. DNA sequence along with translated amino acid sequence (incomplete) of *PmTERT* gene. Translation was performed using ExPASy bioinformatics resource portal (http://web.expasy.org/translate/).

Alignment Hits										
Organism	Database	Scaffold	Protein	Start	End	Query Start	Query End	Strand	Score	E-Value
Daphnia pulex v1.0	allModels	scaffold_47	jgi|Dappu1|249148|SNAP_00017206	332059	358999	1	68	+	62	1.92E-20
Daphnia pulex v1.0	allModels	scaffold_47	jgi|Dappu1|107107|fgenesh1_pg.C_scaffold_47000049	331926	336625	1	32	+	32	1.56E-9
Daphnia pulex v1.0	allModels	scaffold_47	jgi|Dappu1|322437|NCBI_GNO_4700041	332505	335317	1	32	+	32	1.56E-9

Fig. 11. TBLASTN: blast nucleotide vs nucleotide using the programme *Daphnia pulex* v1.0 all gene models (transcripts) http://genome.jgi-psf.org (Altschul *et al.*, 1997)



Alignments	Entry	Entry name	Status	Protein names	Organism	Length	Identity	Score	E-value	Gene names
	E9GVZ2	E9GVZ2_DAPPU	★	Telomerase reverse transcriptase	Daphnia pulex (Water flea)	1,059	100.0%	5,671	0.0	TERT DAPPUDRAFT_346816
	B3DIZ2	B3DIZ2_DANRE	★	Telomerase reverse transcriptase	Danio rerio (Zebrafish) (Brachydanio rerio)	1,098	25.0%	664	1.0×10⁻⁶⁰	tert
	A2THE9	A2THE9_DANRE	★	Telomerase reverse transcriptase	Danio rerio (Zebrafish) (Brachydanio rerio)	1,098	25.0%	663	2.0×10⁻⁶⁰	tert TERT
	B6ZLJ8	B6ZLJ8_DANRE	★	Telomerase reverse transcriptase	Danio rerio (Zebrafish) (Brachydanio rerio)	1,098	24.0%	660	4.0×10⁻⁶⁰	tert
	F8WST0	F8WST0_DANRE	★	Telomerase reverse transcriptase	Danio rerio (Zebrafish) (Brachydanio rerio)	1,110	24.0%	654	2.0×10⁻⁵⁹	tert
	F1QE52	F1QE52_DANRE	★	Telomerase reverse transcriptase	Danio rerio (Zebrafish) (Brachydanio rerio)	1,098	24.0%	654	2.0×10⁻⁵⁹	tert
	F1QE58	F1QE58_DANRE	★	Telomerase reverse transcriptase	Danio rerio (Zebrafish) (Brachydanio rerio)	1,088	24.0%	650	5.0×10⁻⁵⁹	tert
	B4XHB6	B4XHB6_DANRE	★	Telomerase reverse transcriptase	Danio rerio (Zebrafish) (Brachydanio rerio)	1,091	24.0%	640	3.0×10⁻⁵⁴	tert
	B8YRJ9	B8YRJ9_STRPU	★	Telomerase reverse transcriptase	Strongylocentrotus purpuratus (Purple sea urchin)	1,414	27.0%	611	2.0×10⁻⁵⁰	TERT-L
	B8YR10	B8YR10_STRPU	★	Telomerase reverse transcriptase	Strongylocentrotus purpuratus (Purple sea urchin)	1,414	27.0%	610	2.0×10⁻⁶⁰	TERT-L
	B8YR07	B8YR07_STRPU	★	Telomerase reverse transcriptase	Strongylocentrotus purpuratus (Purple sea urchin)	1,414	27.0%	610	2.0×10⁻⁶⁰	TERT-L
	B3F477	B3F477_STRPU	★	Telomerase reverse transcriptase	Strongylocentrotus purpuratus (Purple sea urchin)	1,364	27.0%	607	5.0×10⁻⁶⁰	TERT
	B8YR06	B8YR06_STRPU	★	Telomerase reverse transcriptase	Strongylocentrotus purpuratus (Purple sea urchin)	1,411	27.0%	602	2.0×10⁻⁵⁹	TERT-L

Fig. 12. BLAST result of *PmTERT* partial gene sequence and its identity with water flea, zebra fish and sea urchin.

Chapter 5

Construction and evaluation of the versatile recombinant baculoviral vector systems with hybrid promoters designed for the expression of foreign gene in shrimp cells

5.1. Introduction

Studies in shrimp virology and functional genomics have been hampered by the lack of immortalized shrimp cell lines. Moreover, considering the mass mortality of the cultured animals being reported globally due to deadly viruses, establishment of permanent cell lines from shrimp turns out to be of paramount importance to undertake studies on viral morphogenesis, screening anti-viral molecules and for their doses specification. Several investigations have been conducted worldwide to overcome the WSSV havoc through prophylactic strategies (Sudheer *et al*., 2011; Syed Musthaq and Kwang, 2011). However, lack of a certified shrimp cell line remained all along a major impediment in the development of anti-WSSV drugs (Jose *et al*., 2012). In the previous chapters, a conclusion could be drawn based on the experience that shrimp cell line development would not be possible unless immortalization had been induced either with oncogenes or any other immortalizing gene. If it is so, a shrimp specific expression vector is the foremost requirement to achieve this target.

Gene transfer refers to the delivery of nucleic acid (gene) encoding a protein to the target cells. Such administration of foreign gene requires a transportation vehicle, called vectors, which carries the gene into the target cell. Various methods are being used to deliver vectors encoding foreign genes into eukaryotic cells, which include both physicochemical (electroporation, bombardment with gold or wolfram microparticles, etc.) and biological (lipid conjugates in the form of liposomes, recombinant viruses, etc.) techniques (Beljelarskaya, 2011). If the foreign DNA (transgene) is introduced into target cell via a viral vector through infection, the process is termed as transduction, which results in transformation or change of target cell genotype. The viral vectors have been proven as the most efficient tools for genetic modification of majority of somatic cells *in vitro* and *in vivo* (Sarkis *et al*., 2000). In this line, many viral vectors have been developed and widely used in gene transfer (transduction) and expression studies *in vivo* and *in vitro*. Such viral vectors are adenoviruses (Kozarsky and Wilson, 1993; Huard *et al*., 1995), retro and lentiviruses (Naldini *et al*., 1996; Felder and Sutton, 2009; Poluri *et al*., 2003), adeno associated viruses (Hermonat and Muzyczka, 1984; Carter, 2005) and baculoviruses (Zeng *et al*., 2009). Among them, the baculovirus has emerged as a promising gene delivery vector in recent years (Lo *et al*., 2009). However, in every such case, the efficiency of viral mediated transduction and transgene expression depends on the ability of viral particle to transmit their genome into the nucleus.

Baculoviruses, in particular *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) is a large enveloped virus with a double stranded, circular DNA genome of ~130-kb with 154 open reading frames (Ayres *et al*., 1994). This genome is condensed into a nucleoprotein structure known as a core and is located within a flexible rod shaped capsid, averaging 25-50 nm in diameter and 250-300 nm in length and can expand relatively freely to accommodate even very large recombinant molecules. The core and the capsid are known collectively

as the nucleocapsid. Membrane enveloped nucleocapsids are referred to as virus particles or virions (Mähönen, 2010a) (Fig. 1).

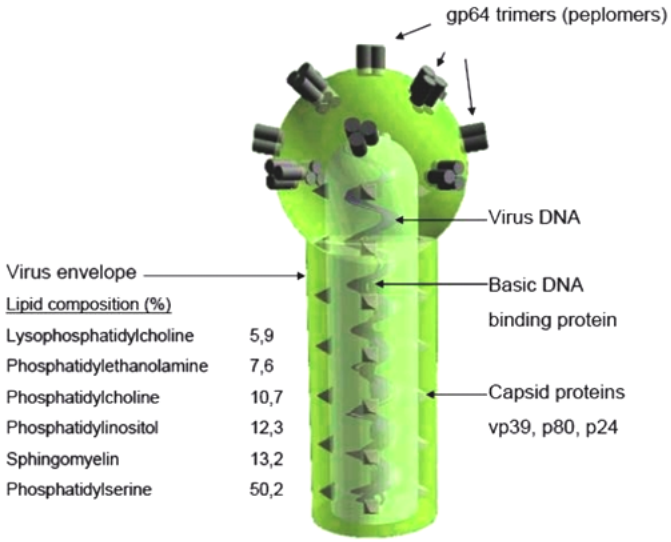


Fig. 1. Schematic structure of budded baculovirus (Airenne *et al.*, 2009).

Baculovirus mediated expression system has been widely used for high level gene expression in transduced cells and many such viral expression systems are available in the market in various brand names, an example being Bac-to-Bac baculovirus expression system (Invitrogen). In all such expression systems, the gene of interest is inserted in place of the AcMNPV polyhedrin gene, which is nonessential for viral replication in cell culture (O'Reilly and Miller, 1988). Even though the most studied baculovirus prototype, AcMNPV replicate only in insect cells and fails to replicate in vertebrate cells and it does express the foreign gene in a wide verity of eukaryotic cells including human cells (Condreay *et al.*, 1999). Its specialty is the presence of specific promoters (or hybrid promoters) that drive transcription in such category of variety of cell types (Fig. 2). In addition, Lewin *et al*. (2005) confirmed that baculovirus promoters carry structural features required by the bacterial RNA polymerase to initiate transcription and the gene expression in *Escherichia coli*.

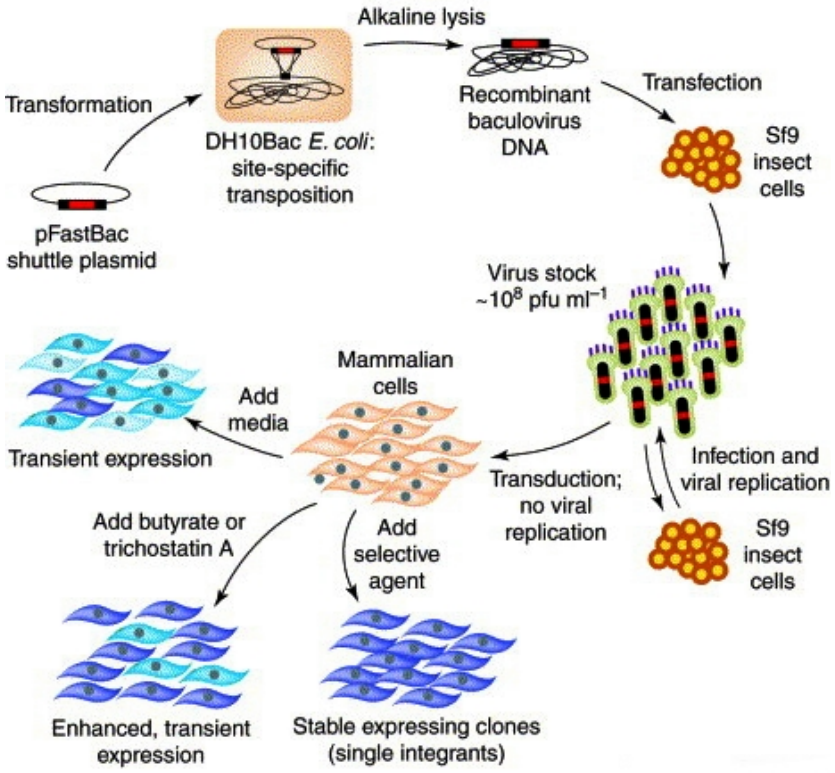


Fig. 2. Schematic diagram of baculovirus-mediated gene delivery in mammalian cells (Kost and Condreay, 2002)

The entry mechanism of baculovirus in to eukaryotic cells and the cell surface receptors for its docking have not yet been accurately understood. However, many reports published in this realm suggested that the cellular entry was dependent on electrostatic interaction (Duisit *et al*., 1999), pH trigger (Dong *et al*., 2010; Paul and Prakash, 2010), cell surface phospholipids (Tani *et al*., 2001) and heparin sulfate (Duisit *et al*., 1999), and the process of trans-membrane transport via adsorptive endocytosis (Volkman and goldsmith, 1985), clathrin-mediated endocytosis and macropinocytosis (Matilainen *et al*., 2005), and phagocytosis (Laakkonen *et al*., 2009; Abe *et al*., 2005). Inside the cell, the intracellular transport is initiated by acid-triggered gp64 fusion (baculovirus envelope protein) and endosomal escape (Kukkonen *et al*., 2003), followed by

actin (Salminen *et al*., 2005) and vimentin (Mahonen *et al*., 2010b) mediated nuclear transport (Chen *et al*., 2011). While the nucleocapsids enter into nucleus, the viral DNA is released, and the virus undergoes repeated rounds of transcription and replication, followed by the formation of virions embedded within the proteinaceous structures called occlusion bodies (Ghosh *et al*., 2002) (Fig 3).

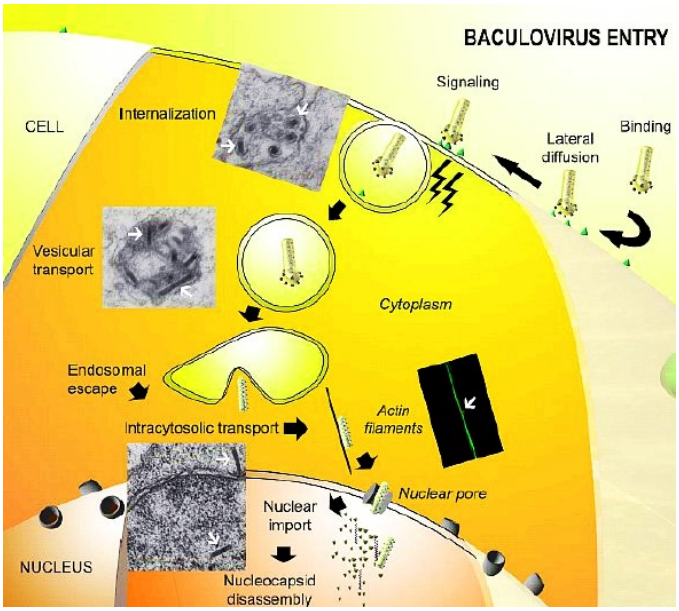


Fig. 3. Baculovirus entry mechanism in eukaryotic cells (Airenne *et al.*, 2009).

Baculoviruses have been studied since 1920s as biopesticides (Black *et al*., 1997). After a long history, in 1985, first successful *in vitro* gene transfer by a recombinant baculovirus was accomplished by Carbonell *et al*. (1985) that made baculovirus a tool in gene transfer technology, especially over expression of cloned genes (O'Reilly *et al*., 1992). Since then, the recombinant baculoviruses have been successfully used for gene transfer in various eukaryotic animals/cells such as in fishes (Smith *et al*., 1989; Leisy *et al*., 2003; Wagle and Jesuthasan, 2003; Wagle *et al*., 2004), in chicken and duck (Ping *et al*., 2006; Song *et al*., 2006), in fruit fly (Oppenheimer *et al*., 1999; Lee *et al*., 2000), in honey bees (Ando *et al*., 2007), in rabbit (Airenne *et al*., 2000), in monkey (Tani *et al*., 2001) and in human

(Hofmann *et al*., 1995; Kost and Condreay, 2002). Besides, recombinant baculovirus have been used for gene therapy (Luo *et al*., 2011; Zhao *et al*., 2012), and for vaccine production (van Oers, 2006; Treanor *et al*., 2007; Hu *et al*., 2008; Cox, 2012). More recently, Gamble and Barton (2011) expressed human telomerase reverse transcriptase in primary fibroblasts and extended its replicative lifespan *in vitro* using recombinant baculovirus successfully. Moreover, the recombinant baculovirus with WSSV Ie1 promoter has been successfully expressed in shrimp cells *in vivo* (Syed Musthaq *et al*., 2009; Syed Musthaq and Kwang, 2011) and *in vitro* (Lu *et al*., 2005).

As the primary shrimp cells were very sensitive to standard gene delivery systems especially liposome-based transfection and electroporation, for transgenic expression, we employed a transduction method mediated by recombinant baculovirus with shrimp viral promoters for the successful transduction of immortalizing gene (s) in shrimp cells *in vitro*. Accordingly, recombinant baculovirus AcMNPV constructs have been produced that carry expression cassettes consisting of gene coding GFP as a reporter linked either to WSSV Ie1 or IHHNV P2 promoter for crustacean specific transduction vectors. These versatile transduction systems were designed with a vision to develop cell lines from shrimp by immortalizing gene delivery, and can be applied for developing genetic vaccines to crustaceans against viruses and for developing specific pathogen resistant or multiple pathogen resistant animals.

5.2. Materials and methods

5.2.1. Plasmid vectors used for the experiment, extraction and its purification

P2 complete Fluc pGL3 basic vector containing IHHNV-P2 promoter (Dhar *et al*., 2007) was kindly received from Dr. Arun K. Dhar, Viracine Therapeutics Corporation, USA. pEGFP-N1 (Clontech) vector containing gene encoding green

fluorescent protein (GFP) was collected from National Centre for Cell Science (NCCS), Pune, India. pFastBac™ 1 containing polyhedrin (PH) promoter and DH10Bac™ (Invitrogen) containing bacmid and helper plasmids were kindly received from Dr. R.B Narayanan, Anna University, Chennai. All plasmids were transformed into respective *E. coli* host and maintained in glycerol stock at -80 ºC (New Brunswick Scientific, England)

5.2.1. 1. P2 complete Fluc pGL3 basic vector

P2 complete Fluc pGL3 basic vector (Dhar *et al*., 2007), is a modified pGL3-Basic vector (Promega, USA) containing a modified *luc* coding region of the firefly (*Photinus pyralis*) luciferase that has been optimized for evaluating transcriptional activity in transfected eukaryotic cells. The P2 promoter region of the infectious hypodermal and hematopoietic necrosis virus (IHHNV) was inserted between *Sac* I and *Xma* I sites of pGL3-basic upstream of the luciferase coding sequence (Fig. 4).

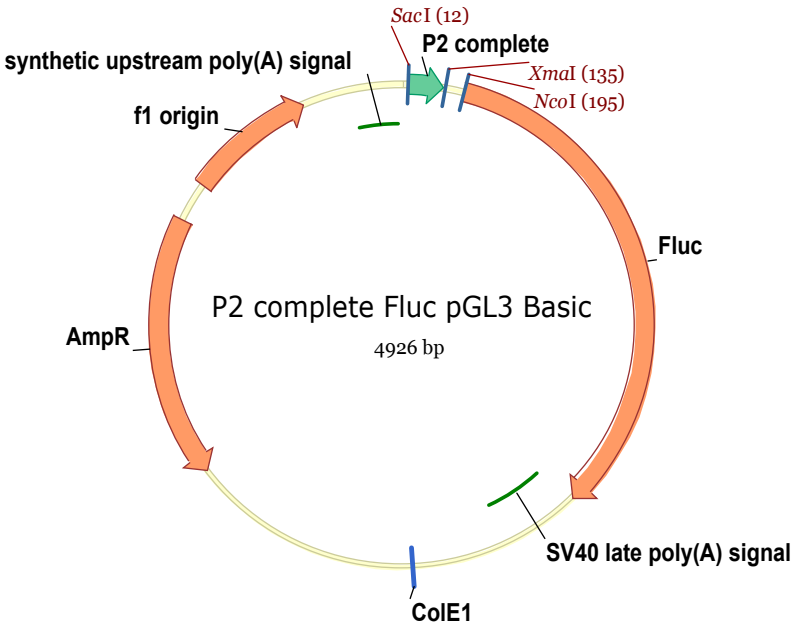


Fig.4. Vector map of P2complete Fluc pGL3 basic vector

5.2.1. 2. pFastBac™ 1 transfer vector

pFastBac™ 1 (Invitrogen) transfer vector into which the gene (s) of interest to be cloned to express in baculoviral expression system is under the control of *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) polyhedrin (PH) promoter for high-level expression in insect cells. This expression cassette is flanked by the left and right arms of Tn7 transposon, and also contains gentamicin resistance gene and SV40 polyadenylation signal to form a mini Tn7 for the site-specific transposition properties of the Tn7 transposon (Fig. 5).

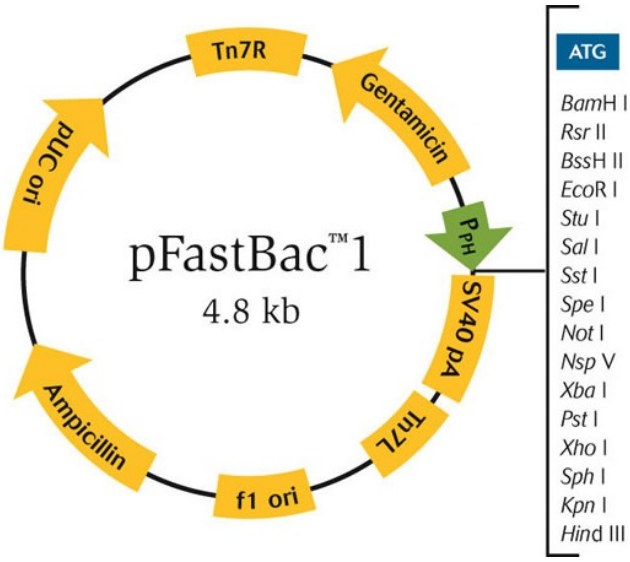


Fig.5. Vector map of pFastBac™ 1 transfer vector

5.2.1. 3. pEGFP-N1 vector

pEGFP-N1 (Clontech) encodes a red-shifted variant of wild-type green fluorescent protein (GFP) under the control of human cytomegalovirus (CMV) immediate early gene promoter for evaluating transcriptional activity in post-transfected eukaryotic cells.

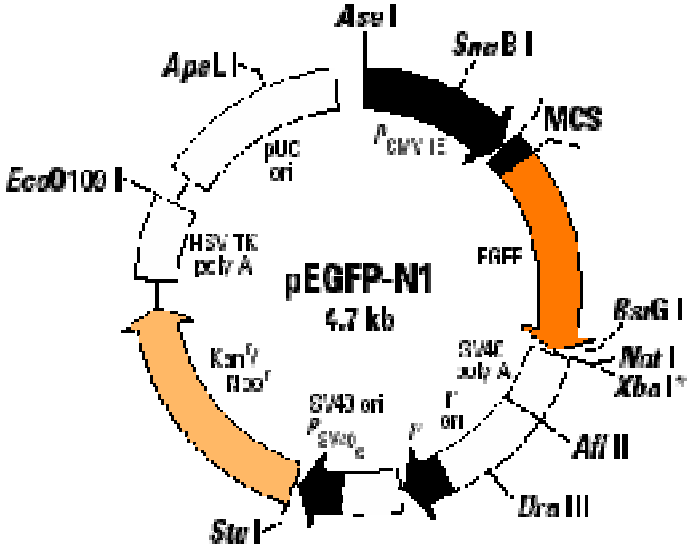


Fig.6. Vector map of pEGFP-N1 vector

The vector provides dominant selectable marker for resistance to neomycin (G-418) in mammalian or other eukaryotic cells and to kanamycin in *E. coli.* Fusions to the N terminus of EGFP retain the fluorescent properties of the native protein allowing the localization of the fusion protein *in vivo* (Fig. 6).

5.2.1. 4. Propagation of *E. coli* containing the plasmid vectors

E. coli JM109 with P2 complete Fluc pGL3 basic vector, *E. coli* DH5α with pFastBac™ 1 were propagated in LB ampicillin (100 µg μl^{-1}) and *E. coli.* DH5α with pEGFP-N1, in LB kanamycin (30 µg μl^{-1}), all cultures were incubated at 37 °C with shaking at 220 rpm.

5.2.1. 5. Plasmid extraction

Plasmid extraction was done using GenElute HP Plasmid Miniprep kit (Sigma Life Sciences) and by following manufacturer's instruction. Briefly, an aliquot of 2 ml culture after overnight incubation was pelletised at 12,000 x g for 1 min. The pellet was resuspended in 200 µl resuspension solution containing RNase A and lysed by adding 200 µl lysis buffer. An aliquot of 350 µl neutralization solution was added and

centrifuged at 12,000 x g for 10 min to remove the cell debris. Lysate was loaded into GenElute HP Miniprep binding column inserted into a microcentrifuge tube and centifuged at 12,000 x g for 1 min. Plasmid DNA bound to the column was washed twice with wash solution to remove the endotoxins, salt and other contaminants. To elute the plasmid DNA, the column was transferred to a fresh collection tube, added 100 µl 10 mM Tris-Cl and centrifuged at 12,000 x g for 1 min and stored at -20 ºC. Purity of the plasmid DNA obtained was analysed by agarose gel electrophoresis and by determining the ratio of the absorbance at 260/280 nm in a UV-VIS spectrophotometer (U2800, Hitachi, Japan) and fluorometrically using Qubit® fluorometer (invitrogen™, USA).

5.2.2. DH10Bac™ *E. coli* with baculovirus shuttle vector (Bacmid) and helper plasmid, pMON7124

The baculovirus shuttle vector (bacmid), bMON14272 (136-kb), present in DH10Bac™ *E. coli* contains a low-copy number mini-F replicon, kanamycin resistance marker and a segment of DNA encoding the LacZ peptide from a pUC-based cloning vector into which the attachment site for the bacterial transposon, Tn7 (mini-*att*-Tn7) has been inserted. The bacmid propagates in *E. coli* DH10Bac™ as a large plasmid that confers resistance to kanamycin and can complement a *lacZ* deletion present on the chromosome to form colonies that are blue (Lac+) in the presence of a chromogenic substrate X-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) and the inducer, IPTG (Isopropyl-beta-D-thiogalactopyranoside). Recombinant bacmids (composite bacmids) are generated by transposing a mini- Tn7 element from a pFastBac™ donor (transfer) plasmid to the mini-*att*-Tn7 attachment site on the bacmid. DH10Bac™ *E. coli* also contain the helper plasmid, pMON7124 (13.2-kb), which encodes the transposase and confers resistance to tetracycline.

5.2.2.1. Preparation of DH10Bac™ *E. coli* competent cells

DH10Bac™ *E. coli* was recovered from the glycerol stock at -80 ºC, an aliquot of 10 µl was inoculated into 100 ml LB broth containing kanamycin (50 µg ml^{-1}) and tetracycline (10 µg ml^{-1}), incubated at 37 °C for overnight with shaking (150rpm). An aliquot of 5 ml from overnight grown DH10Bac™ *E. coli* was further inoculated into 50 ml LB broth containing kanamycin (50 µg ml^{-1}) and tetracycline (10 µg ml^{-1}) and incubated at 37 °C for 2 h at 150 rpm. Transferred the cells to another 50 ml centrifuge tube, centrifuged at 6000 rpm for 20 min at 4 °C. The medium was removed from the pellet, drained off the traces of medium by keeping the tubes in an inverted position on a pad of tissue paper. The pellet was re-suspended by gentle vortexing with 50 ml ice-cold 0.1 M $CaCl_2$ solution, centrifuged at 6000 rpm for 20 min at 4 °C. The medium was removed from the pellet, drained off the traces of medium by keeping the tubes in an inverted position on a pad of tissue paper. The pellet was re-suspended in 50 ml ice-cold 0.1 M $CaCl_2$ solution by gentle vortexing. An aliquot of 80 µl above competent cells were mixed with 20 µl 60% glycerol in a 0.5 ml micro centrifuge tube (MCT) and stored at -80 °C. The above competent DH10Bac™ *E. coli* host was used for constructing recombinant baculovirus shuttle vector (Bacmid).

5.2.3. Crustacean specific putative promoter from WSSV and IHHNV

The WSSV-Ie1 (hereafter Ie1) basic promoter (Liu *et al*., 2005; Lu *et al*., 2005) region from -1 to -512 was PCR amplified from *P. monodon* challenged with WSSV through intramuscular injection (Sudheer *et al*., 2011). The putative P2 promoter of IHHNV (hereafter P2) was PCR amplified from the P2 complete Fluc pGL3 basic vector (Dhar *et al*., 2007) containing P2 promoter.

5.2.3.1. Genomic DNA extraction from WSSV infected animal for Ie1 promoter

Genomic DNA was extracted from infected animals using DNAzol® (Chomczynski *et al*., 1997; Dhar *et al*., 2001**)** and by following the method of

manufacturer (Molecular Research Center, Inc., Cincinnati, Ohio). Briefly, 50 mg gill tissue was collected from dead animal and macerated in 1000 µl DNAzol®, centrifuged at 10,000xg for 10 min. Supernatant was collected into a fresh micro centrifuge tube containing 500 µl ethanol (100%). Gently inverted the sample tube several times to mix and incubated at RT for 3 min, centrifuged at 4000xg at RT to palletize the DNA and supernatant was removed carefully. An aliquot of 1000 µl 70% ethanol was added to the DNA pellet and centrifuged at 4,000xg for 5 min at RT. The ethanol was tipped off and added fresh 1000 µl 70% ethanol and centrifuged at 4,000g for 5 min at RT, supernatant was removed and the DNA pellet was allowed to dry for 15 sec. The DNA was dissolved in 200 µl MilliQ and stored at -20 ^{o}C till use.

5.2.3.2. PCR amplification of Ie1 and P2 promoters

The Ie1 basic promoter region from -1 to -512 was PCR amplified using the primer set (enzyme site *Bam*H I is underlined) of F-5'- GGA TCC TCC CTA CGT ATC AAT TTT ATG TGG CTA ATG GAG A-3' and R- 5'- GGA TCC ACG CGT CGA CCT TGA GTG GAG AGA GAG CTA GTT ATA A-3' (Lu *et al*., 2005). P2 complete pGL3 vector (Dhar *et al*., 2007) was used for the PCR amplification of putative P2 promoter region using the primer set NP602F-5'GGA TCC CTG CGA GCG CTT CGC AG-3' and NP602R- 5'-GGA TCC TAG CAC TTG GAA TAG CCT CTT-3' (enzyme site *Bam*H I is underlined). The 25µl PCR reaction mixture containing 2.5 µl 10x buffer, 2.5 µl dNTP (2.5 mM), 1 µl Taq polymerase (0.5 U μl^{-1}), 1 µl genomic DNA for WSSV-Ie1 promoter and 0.2 µl plasmid DNA (P2 complete PGL3) for IHHNV-P2 promoter (~75 ng), 1 µl of each primer (10 pmol μl^{-1} and the mixture was made up to 25 µl with MilliQ. The hot start PCR programme used for the amplification was 95 °C for 5 min followed by hold at 80 °C, 32 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec, extension at 72 °C for 2 min, followed by final extension at 72 °C for 10 min. Ten µl of PCR products was analyzed by 1% agarose gel electrophoresis, stained with

ethidium bromide, visualized and documented using gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA).

5.2.3.3. Cloning with pGEM-T Easy vector

Amplified PCR products of Ie1 and P2 promoters were ligated with pGEM-T Easy vector (Promega, USA) by following the manufacture's instruction. Briefly, 10 µl ligation mixture containing 0.5 µl pGEM-T Easy vector, 3.5 µl PCR Product, 5 µl ligation buffer, 0.5 µl ligase enzyme and MilliQ was incubated at 4 °C, overnight. This allowed for the ligation of PCR products on pGEMT-Easy vector.

5.2.3.4. Transformation into *E. coli* DH5α

Thawed the competent cells (*E. coli* DH5α) by placing on ice for 5-10 min, added 10 µl of each ligation reaction to a sterile 15 ml culture tube already on ice, transferred 50-100 µl of competent cells into the 15 ml tubes (containing ligation mix) on ice, gently flicked the tubes to mix and placed them on ice for 20 min, heat shocked the cells for 90 sec in a water bath at exactly 42 °C, immediately returned the tubes to ice for 2 min, Added 600 µl super optimal broth with catabolite repression (SOC; Composition for 10 ml: 0.2 g tryptone ; 0.05 g yeast extract; 0.005 g NaCl, 100 µl 1M KCl; 50 µl 2 M $MgCl_2$; 200 µl 1 M glucose. $MgCl_2$ and glucose were added just before transformation) to the tubes containing cells transformed with ligation mixture, incubated for 2 h at 37 °C with shaking at 220-230 rpm, plated 100 µl of each transformation culture onto duplicate/triplicate on to LB/ampicillin/IPTG/X-gal plates and incubated the plates overnight (16-18 h) at 37 °C.

5.2.3.5. PCR confirmation of gene insert in the selected clones

The white colonies were selected and patched on ampicillin/IPTG/X-gal plates to reconfirm the transformation. All individually streaked colonies were subjected for colony PCR using vector primers designed from either side of the multiple cloning site of the vector so that whatever be the product formed, primer

could amplify it from either side. The 25 µl reaction PCR reaction mixture containing 2.5 µl 10x buffer, 2.5 µl dNTP (2.5 mM), 1µl Taq polymerase (0.5 U μl^{-1}), pinch of colony, 1 µl of T7 and SP6 primers each, and the mixture was made up to 25 µl with MilliQ. The hot start PCR programme used for the amplification of complete genes was 95 °C for 5 min followed by holding at 80 °C, 35 cycles of denaturation at 94 °C for 15 sec, annealing at 57 °C for 20 sec, extension at 72 °C for 1 min, followed by final extension at 72 °C for 10 min. Ten µl of PCR products was analyzed by 1% Agarose gel electrophoresis, stained with ethidium bromide, visualized and documented using gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA).

5.2.3.6. Propagation of confirmed colony and plasmid extraction

After confirmation, the transformed *E. coli* DH5α containing cloned vectors were propagated in 10 ml LB ampicillin (100 µg μl^{-1}) medium at 37 °C at 230 rpm. The methodology followed for plasmid exaction was explained elsewhere in this chapter.

5.2.3.7. Restriction digestion of cloned pGEM-T vector with *Bam*H I to release Ie1 and P2 promoters and its purification

The purified pGEM-T plasmid vectors containing Ie1 (pGEMT-Ie1) and P2 (pGEMT-P2) promoter were restriction digested with *Bam*H I enzyme (New England Biolabs, UK) to release the corresponding promoter sequences (Ie1 and P2). An aliquot of 20 µl PCR reaction mixture containing 5 µl plasmid, 0.5 µl enzyme (*Bam*H I -10,000 U ml^{-1}) 2 µl reaction buffer and 12.5 µl MilliQ water was incubated for 1 h at 37 °C. The restriction digestion was confirmed by 1% Agarose gel electrophoresis.

The restricted, released Ie1 (WSSV) and P2 (IHHNV) promoters were gel purified using GenElute™ Gel Extraction kit (Sigma, USA) by following manufacturer's instruction. Briefly, the agarose gel that contain DNA fragment of appropriate size was excised using X-tracta gel extraction tool (Sigma, USA). The

excised gel slice was taken in a 1.5 ml tube, weighed and added 3 gel volumes (~450 µl) of gel solubilization solution and incubated at 60 ºC for 10 min with repeated vortexing in every 2 min. After incubation, added 1 gel volume (~150 µl) of 100% isopropanol, mixed gently until it become homogenous. This solubilized gel solution was loaded into the binding column that was pre treated with column preparation solution, centrifuged at 12,000 x g for 1 min. Added 700 µl wash solution and centrifuged for 1 min at 12,000 x g , repeated the centrifugation and residual wash solution was removed. The binding column was transferred to a fresh collection tube (2 ml MCT) and added 50 µl of preheated (at 65 ºC) 10 mM Tris-HCl (pH 9.0), centrifuged at 12,000 x g for 1 min, stored at -20 ºC. The concentration of DNA was measured spectrometrically at 260/280 nm in a UV-VIS spectrophotometre (U2800, Hitachi, Japan) and fluorometrically using Qubit® flourometer (invitrogen™, USA).

5.2.4. Construction of the versatile recombinant baculoviral vector systems with hybrid promoters

5.2.4.1. Restriction digestion, CIP treatment and purification of pFASTBac™ 1 transfer vector

The pFASTBac™ 1 transfer vector was restriction digested with *Bam*H I enzyme and the methodology followed was same as explained in the previous section (5.2.3.7.). Restriction digested plasmid was CIP (Calf Intestinal Phosphatase) treated to remove the phosphate groups to prevent self ligation. The reaction mixture containing 20 µl plasmid, 0.1 µl CIP enzyme and 5 µl buffer was incubated at 37 ºC for 1 h followed by heat inactivation at 65 ºC for 20 min. Restriction digested, CIP treated vector was gel purified using GenElute™ Gel Extraction kit (Sigma, USA) and the methodology followed was well explained elsewhere in this chapter.

5.2.4.2. Insertion of crustacean specific viral promoters (Ie1 and P2) into pFASTBac™ 1 vector

The purified Ie1 and P2 promoters were ligated with the restriction digested, CIP treated pFASTBac™ 1 plasmid vector at *Bam*H I restriction region in the multiple cloning site (MCS) downstream to PH promoter to construct two hybrid transfer vector systems such as pBacIe1 and pBacP2 respectively. The 10 µl ligation mixture containing 2 µl CIP treated pFASTBac™ 1 vector, 3 µl gel purified Ie1 or P2 promoter, 1 µl 10X buffer, 0.5 µl T_4DNA ligase enzyme and 3.5 µl MilliQ water was incubated at 16 °C, overnight.

5.2.4.3. Transformation of vectors with hybrid viral promoters into *E. coli* DH5α and its propagation; extraction and purification of the vector systems

To the 10 µl ligation mixture in a 5 ml ice cold screw cap tube, added 50 µl competent *E. coli* DH5α cells, mixed gently and incubated on ice for 20 min. Heat shock was given for 90 sec at exactly 42 °C. The tubes were returned to ice for 2 min. Added 600 µl super optimal broth with catabolite repression (SOC; Composition for 10 ml: 0.2 g tryptone ; 0.05 g yeast extract; 0.005 g NaCl; 100 µl 1M KCl; 50 µl 2 M $MgCl_2$; 200 µl 1 M glucose. $MgCl_2$ and glucose were added just before transformation) and incubated for 2 h at 37 °C with shaking at 230 rpm. After incubation, 200 µl each was plated onto LB ampicillin (100 µg ml^{-1}) plate containing IPTG and X-gal (LB medium-2 g 100 ml^{-1}; Agar- 2g 100 ml^{-1}) and incubated for 24 h at 37 °C. After PCR confirmation, individual colonies developed (white) were inoculated into 100 ml LB ampicillin broth and incubated at 37 °C with shaking for plasmid extraction. Plasmid extraction was done using GenElute HP Plasmid Miniprep kit (Sigma Life Sciences, USA) and the methodology followed was explained in the previous section.

5.2.5. Insertion of of green fluourescent protein (GFP) into the vectors for analysing transcriptional activity of hybrid viral promoter system

Transcriptional activity of hybrid viral promoters in the versatile vectors was analyzed by expressing the green fluorescent protein (GFP) reporter gene. The GFP was inserted downstream to hybrid promoter (PH-Ie1 or PH-P2) in the transfer vector system pBacIe1 and pBaP2 respectively.

5.2.5.1. Gene encoding green fluorescent protein (GFP) and its purification

The gene encoding green fluorescent protein (GFP) was restriction digested from pEGFP-N1 vector with *Sal* I and *Not* I enzymes (New England Biolabs, UK) following double digestion. An aliquot of 50 µl reaction mixture containing 5 µl plasmid, 2 µl *Sal* I enzyme (20,000 U ml^{-1}), 2 µl *Not* I enzyme (2,500 U ml^{-1}), 4 µl reaction buffer, 0.4 µl bovine serum albumin (BSA) and 36.6 µl MilliQ water, incubated at 37 ºC for 2 h followed by heat inactivation at 65 ºC for 20 min. The restriction digested vector was subjected for gel purification to extract GFP using GenElute™ Gel Extraction kit (Sigma, USA) and the methodology followed was well explained in the previous section.

5.2.5.2. Insertion of of green fluourescent protein (GFP) downstream to hybrid viral promoter and its purification

The vectors pBacIe1 and pBacP2 were restriction digested with *Sal* I and *Not* I enzymes (New England Biolabs) and purified using GenElute™ Gel Extraction kit. The restriction digested, gel purified GFP gene was inserted to the corresponding restriction sites (*Sal* I and *Not* I) (Fig. 7). Ligated products were transfected into *E. coli* DH5α and the confirmed colonies were propagated for plasmid extraction. The methodology followed was well explained in the previous sections (5.2.3.7 and 5.2.5.1.).

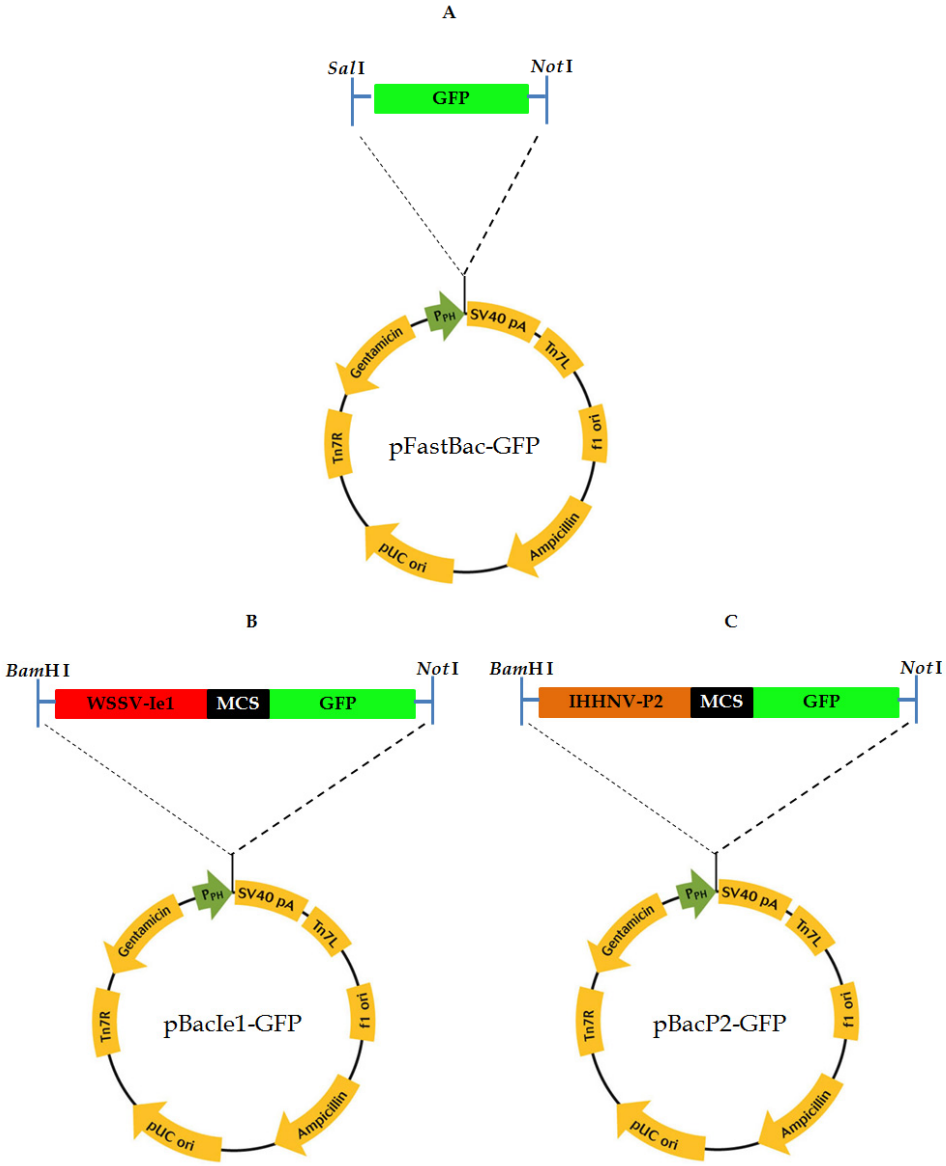


Fig.7. Construction of transfer vector containing hybrid promoter and GFP. A: GFP cassettes inserted between *Sal* I and *Not* I site in the transfer vector to construct wild-type baculovirus (control) expressing GFP; B: constructed transfer vector (pBacIe1-GFP) for generating recombinant baculovirus with PH-Ie1 hybrid promoter and GFP reporter; and C: transfer vector (pBacP2-GFP) for generating recombinant baculovirus with PH-P2 hybrid promoter and GFP reporter gene.

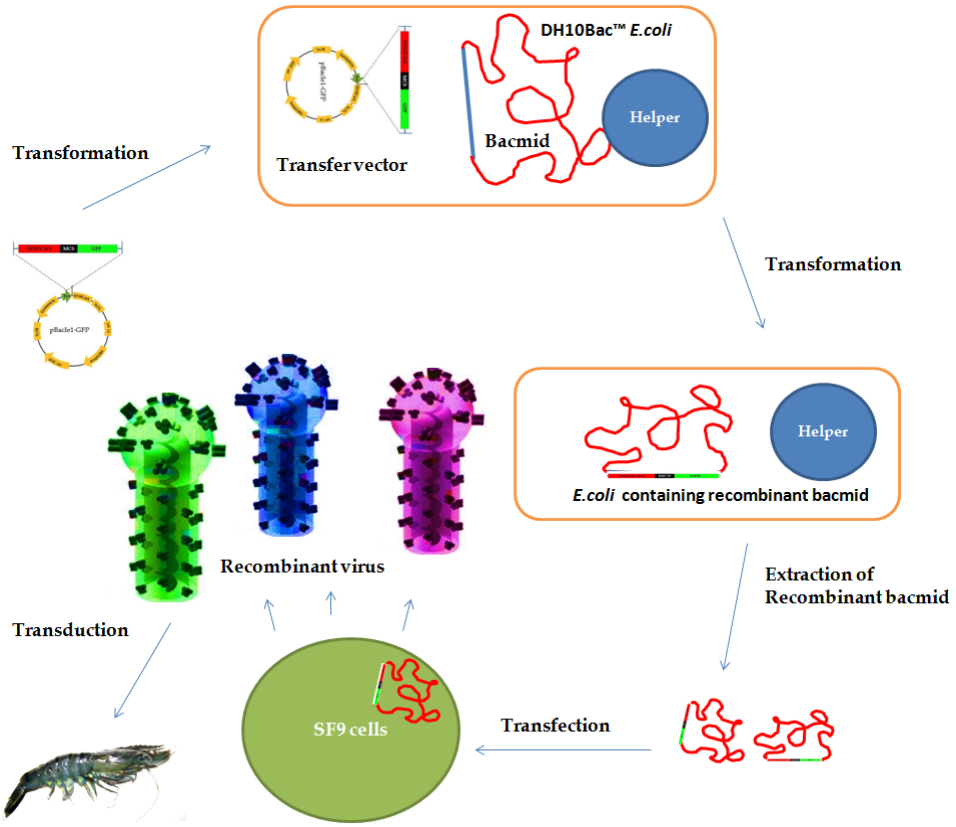


Fig.8. Schematic structure for the generation of recombinant baculovirus transduction vector from transfer vector

5.2.6. Generation of recombinant virus containing hybrid viral promoters and GFP

Bac-to-Bac™ baculovirus expression system (Invitrogen) based on site-specific transposition with Tn7 was used for generating the recombinant baculovirus. Here, the PH-Ie1-GFP and PH-P2-GFP cassettes in the pFastBac™ 1 transfer vector along with mini-Tn7 transposome element could transpose to the mini-*att*-Tn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid present in DH10Bac™ *E. coli*. Colonies containing recombinant bacmids were identified by antibiotic selection and blue/white screening. Since the transposition resulted in disruption of the *lacZα* gene

DH10Bac™ transformants produce white colonies. High molecular weight mini-prep DNA was prepared from selected *E. coli* clones containing the recombinant bacmid, and this DNA was then used to transfect insect cells to generate recombinant viral particles (Fig. 8)

5.2.6.1. Transformation of pBacIel-GFP and pBacP2-GFP transfer vectors containing hybrid promoter system into DH10Bac™ *E. coli.*

To the 10 µl ligation mixture containing pBacIel-GFP and pBacP2-GFP transfer vectors in a 5 ml ice cold screw cap tube, added 50 µl competent DH10Bac™ *E. coli* cells, mixed gently and incubated on ice for 20 min. Heat shock was given for 90 sec at exactly 42 °C. The tube was returned to ice for 2 min. Added 600 µl super optimal broth with catabolite repression (SOC; composition for 10 ml: Tryptone-0.2 g; yeast exytract-0.05 g; NaCl-0.005 g; 1 M KCl- 100 µl; 2 M $MgCl_2$-50µl; 1 M glucose-200 µl. $MgCl_2$ and glucose were added just before transformation) and incubated for 2 h at 37 °C with shaking at 230 rpm. After incubation, 200 µl each was plated onto LB plate containing (LB medium-2 g 100 ml^{-1}; Agar- 2 g 100 ml^{-1}) kanamycin (50 µg ml^{-1}), gentamicin (7 µg ml^{-1}), tetracycline (10 µg ml^{-1}), IPTG (40 µg ml^{-1}), and X-gal (100 µg ml^{-1}), and incubated for 24 to 48 h at 37 °C to select for DH10Bac™ transformants.

5.2.6.2. Propagation of recombinant bacmid DNA in DH10Bac *E. coli*

The individual colonies developed (white) were propagated into 100 ml LB broth containing kanamycin (50 µg ml^{-1}), gentamicin (7 µg ml^{-1}), tetracycline (10 µg ml^{-1}), and incubated at 37 °C with shaking (225 rpm) for plasmid extraction.

5.2.6.3. Isolation of recombinant bacmid DNA from DH10Bac *E.coli*

The recombinant bacmid DNA containing crustacean specific promoter and GFP reporter was isolated and purified using PureLink, HiPure Plasmid Miniprep kit (Invitrogen, Germany) by following manufacturer's instruction. Briefly,

centrifuged an aliquot of 8 ml overnight culture of DH10Bac containing recombinant bacmid at 15,000xg at RT for 15 min, removed the entire medium. Resuspended the pellet in 0.4 ml of cell suspension buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.2 mg ml^{-1} RNase A. Added 0.4 ml of cell lysis solution containing 200 mM NaOH and 1% SDS, mixed gently by inverting the capped tube five times and incubated at RT for 5 min. After incubation, added 0.4 ml of neutralization buffer containing 3.1 M potassium acetate (pH 5.5) and mixed immediately by inverting the tube five times. Centrifuged at 15,000xg at RT for 10 min and transferred the supernatant onto the equilibrated column and allowed the solution in the column to drain by gravity flow. The column equilibration was performed by adding 2 ml equilibration buffer containing 600 mM NaCl, 100 mM sodium acetate (pH 5.0) and 0.15% Triton X-100, and the buffer was drained off by gravity. After complete removal of supernatant from the equilibrated column, washed the column two times with 2.5 ml wash buffer containing 800 mM NaCl, 100 mM Sodium acetate (pH 5.0) and allowed the solution in the column to drain off. The recombinant bacmid DNA attached on the column membrane was eluted by adding 0.9 ml of elution buffer containing 1.25 M NaCl, 100 mM Tris-HCl (pH 8.5) and allowed the solution in the column to drain to a 2 ml sterile micro centrifuge tube (MCT). The recombinant bacmid DNA was precipitated by adding 0.63 ml isopropanol, mixed and placed on ice for 10 min, centrifuged at 15,000xg at RT for 30 min. Carefully discarded the supernatant and washed the bacmid DNA pellet with 1 ml of ice cold 70% ethanol and centrifuged at 15,000xg at RT for 5 min. Carefully drained off the ethanol and air dried the pellet for 10 min at RT, dissolved in 50 µl of TE buffer containing 10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA. The concentration of recombinant bacmid DNA was calculated fluorometrically using Qubit Fluorometer (Invitrogen) and stored at -20 °C.

5.2.6.4. PCR confirmation of insert orientation in bacmid DNA

The orientation of bacmid DNA-carrying PH-Ie1-GFP and PH-P2-GFP fragments (cassettes) were confirmed by PCR amplification using the M13F (5' CCC AGT CAC GAC GTT GTA AA ACG 3') bacmid primer and GFP specific primer (NP266R-5' CAC GAA CTC CAG CAG GAC CAT G 3'). The 25 µl PCR reaction mixture containing 2.5 µl 10x buffer, 2.5 µl dNTP (2.5 mM), 1 µl Taq polymerase (0.5 U μl^{-1}), 0.2 µl bacmid , 1 µl of each primer (10 pmol μl^{-1}) and the mixture was made up to 25 µl with MilliQ. The hot start PCR programme used for the amplification was 95 °C for 5 min followed by hold at 80 °C, 32 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec, extension at 72 °C for 2 min, followed by final extension at 72 °C for 10 min. An aliquot of 10 µl of PCR products was analyzed by 1% Agarose gel electrophoresis, stained with ethidium bromide, visualized and documented using gel documentation system (Gel Doc™XR+ imaging system, Bio-Rad, USA).

5.2.6.5. Transfection of recombinant bacmid shuttle vector into Sf9 cells to generate recombinant baculovirus

The Sf9 cells at a cell density 1.5 x 10^6 cells ml^{-1} grown in TNM-FH medium (Sigma, USA) without antibiotics and serum in 35 mm culture dish was selected for transfection.

To generate the recombinant virus, Sf9 cells were transfected with the confirmed bacmid DNA using Cellfectin® II reagent (Invitrogen) by following manufacturer's instruction. Briefly, an aliquot of 1 µl (500 ng ml^{-1}) recombinant bacmid DNA was diluted with 100 µl antibiotic and serum free TNM-FH medium and mixed with Cellfectin® II which was previously diluted by adding 8 µl into 100 µl TNM-FH medium (antibiotics and serum free). The lipid-bacmid mixture (transfection mixture) was mixed gently and incubated at RT for 45 min, added drop wise onto the cells and incubated at 28 °C for 6 h. After incubation, the

transfection mixture was replaced with TNM-FH medium containing 15% fetal bovine serum (FBS) and antibiotics. The cells were further incubated at 28 °C until the sign of viral infection (occlusion bodies) and fluorescent signals from green fluorescent protein could be visualized.

5.2.6.6. Isolation, amplification and storage of recombinant baculovirus containing hybrid viral promoters

Recombinant baculovirus released in the TNM-FH medium was collected from each culture dish and re-infected on to another sets of Sf9 cells (1.5×10^6 cells ml^{-1}) to reamplify the viral stock. After reamplification, the medium containing concentrated virus was collected into sterile centrifuge tube and centrifuged at 500xg for 5 min to remove the cell debris. The clear supernatant was transferred to fresh cryovials, covered with aluminum foil to protect from light and stored at -80 °C until transduction experiment was carried out in lymphoid cell culture from *P. monodon*.

5.2.7. Analysis of hybrid viral promoters mediated transcriptional activity in Sf9 cells

Transcriptional activity of the recombinant baculovirus constructs that carry expression cassettes consisting of gene encoding GFP as a reporter linked either to PH-Ie1 or PH-P2 hybrid viral promoter was carried out in Sf9 cells. The hybrid promoter activity of PH-Ie1 or PH-P2 cassettes in the recombinant baculovirus BacIe1-GFP and BacP2-GFP respectively were determined by fluorescence microscopic examination of GFP (reporter gene) signals from the transduced Sf9 cells and by the analysis of the SDS-PAGE separated green fluorescent protein. Activity was compared with that of single promoter (PH) linked GFP cassette (PH-GFP), in recombinant baculoviral vector Bac-GFP as control. Human cell lines such as HeLa and HEp2 were selected to check whether the virus caused any infection in human.

5.2.7.1. Analysis of GFP signals from transduced Sf9 cells

Promoter activity in Sf9 cells transduced with recombinant baculovirus (BacIe1-GFP or BacP2-GFP) consisting of either PH-Ie1-GFP or PH-P2-GFP hybrid viral promoter cassettes with GFP were carried out. Baculovirus with PH-GFP cassettes, in the Bac-GFP was used as the control. Expression of green fluorescent protein under the control of either of these promoter cassettes was evaluated by microscopic examination using Fluorescence-Inverted phase contrast microscope (Leica DMIL, Germany) with GFP filter, and controlled by image acquisition software (LAS, Leica). Transduced cells were observed in every 3 h for 24 h, subsequently in every 24 h for 3 days for the phenotypic changes and the GFP signals. Once the virus was amplified in Sf9 cells, GFP expression was observed, demonstrating the hybrid promoter activity in these cells.

5.2.7.2. Analysis of hybrid promoter mediated protein expression in transduced Sf9 cells

Transduced Sf9 cell after 72 h was harvested from 35 mm culture dish and protein was extracted using 400 µl of 1x SDS-PAGE lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS) and boiled for 5 min. The extracted protein was subjected to reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) following the method of Laemmli (1970). Briefly, protein extract was (10 µl) mixed with 10 µl of gel loading dye, boiled for 5 min. The protein was separated and analyzed using 4% stacking gel and 15% resolving gel prepared into 10 x 10.5 cm vertical gel plate of miniVE™ mini vertical electrophoresis unit (Hoefer-Amersham, India). Electrophoresis was performed in 1x Tris-glycine SDS (pH 8.3) buffer (tank buffer) at a voltage of 12 mA (EPS 301, Amersham, India). After electrophoretic separation, gel was stained in coomassie brilliant blue stain R-250 (0.025% coomassie brilliant blue R-250, 40% methanol and 7% acetic acid in distilled water), de-stained in de-staining solution I (40% methanol and 7% acetic acid in distilled water) and de-staining solution II (5% methanol and 7%

acetic acid in distilled water), and photographed using Gel Doc™ XR+ imaging system. Protein expression was determined by comparing with the protein profile of un-transduced Sf9 cells (control). Molecular weight of protein band was determined by comparing with that of standards (Genei, India).

5.2.8. Transduction of shrimp cells *in vitro* and *in vivo* with recombinant baculovirus encoding GFP under the control of hybrid viral promoters

Recombinant baculovirus constructs that carry expression cassettes specific to shrimp cells was transduced into primary cell cultures from *Penaeus monodon* and into the whole animal. These experiments were conducted to establish the understanding that the hybrid promoter mediated transcription could be carried out in the cells *in vitro* and *in vivo* (in shrimp).

5.2.8.1. Transduction of shrimp cells *in vitro*

Recombinant baculovirus constructs that carry expression cassettes consisting of gene encoding GFP as a reporter linked to the hybrid promoter either to PH-Ie1 or PH-P2 was transduced into primary cell cultures from *P. monodon.* Cell culture for transduction experiment from various cell types/ tissues of *P. monodon* were prepared in 35 mm culture dish in shrimp cell culture medium (SCCM) by following the methodology explained in the previous chapter (Chapter 1). After 24 h incubation of cell cultures at 25 °C, the medium was replaced with 1 ml SCCM and 1 ml supernatant containing recombinant baculovirus (1:1) and incubated for 6 h at 25 °C, replaced with growth medium (SCCM) containing 15% fetal bovine serum (FBS). Once the virus was amplified in cell culture, GFP expression was observed, demonstrating the hybrid promoter activity in shrimp cells *in vitro*. Expression of green fluorescent protein under the control of either of these promoter cassettes was evaluated by microscopic examination using Fluorescence-Inverted phase contrast microscope (Leica DMIL) with GFP filter,

and controlled by image acquisition software (LAS, Leica). Transduced cell cultures were observed in every 3 h for 24 h, subsequently in every 24 h for 3 days for the phenotypic changes and the GFP signals.

5.2.8.1. Transduction of shrimp cells *in vivo*

An aliquot of 10 µl viral preparation (~$1x10^{-4}$ pfu) was given intramuscularly to healthy *P. monodon* (Sudheer *et al.*, 2011). The recombinant baculovirus BacIe1-GFP and BacP2-GFP carrying expression cassettes PH-Ie1-GFP and PH-P2-GFP respectively were used for this experiment along with Bac-GFP as control vector (PH promoter only). After injection animals were maintained in the laboratory condition for 45 days in aerated seawater of 15 ‰ salinity and followed strict bio-security norms to prevent the entry of this virus to natural ecosystem. After 45 days, the animals were sacrificed and various tissue/ cells of the animals were observed for green fluorescent protein expression using Inverted phase contrast fluorescence microscope (Leica, Germany).

5.3. Results

5.3.1. Construction of the versatile vector systems with hybrid viral promoters

Two recombinant baculoviral transduction vectors, BacIe1-GFP and BacP2-GFP that carried expression cassettes consisting of gene encoding GFP as a reporter linked to the hybrid promoter either to PH-Ie1 or PH-P2 were successfully constructed. The transfer vector pFastBac™1 (Invitrogen) provided the strong polyhedrin (PH) promoter from *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV). Either Ie1 or P2 promoter was inserted at the position 4032 (4032^{th} base in vector map) in continuation with 128-bp sized PH promoter (3904 to 4032) in pFastBac™1 transfer vector to make the hybrid promoter system. The 4032^{th} site of the pFastBac™1 transfer vector was cleaved with *Bam*H I enzyme to insert the shrimp viral promoter Ie1 or P2 (Fig. 9) with a sequence size 116-bp and 502-bp respectively. Ie1 and P2 were the crustacean

specific promoters from white spot syndrome virus (WSSV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) respectively. Gene encoding green fluorescent protein (GFP), the reporter gene was inserted at the multiple cloning sites (4037 to 4142) between 4070 and 4090 position by restriction digestion with *Sal* I and *Not* I enzymes (Fig. 10), confirming that the GFP was in-frame with hybrid promoter. A total of 20 bases were removed while replacing GFP gene sequence at this position. The results from colony PCR using forward primer of either Ie1 or P2 and the reverse primer of GFP confirmed the alignment of insert in the transfer vector (Fig. 11). The expression cassettes containing hybrid promoter, multiple cloning sites, green fluorescent protein (GFP) were at the position between transposon elements Tn7R and Tn7L allowed the site specific transposition of the expression cassettes along with a gentamicin resistance gene in to the baculoviral genome. Transposon mediated transposition in bacmid was confirmed using M13 (forward) and GFP (reverse) primers, indicating that the inserts were transpositioned in correct orientation (Fig. 12).

5.3.2. Transduction of cell lines *in vitro* and evaluation of transcriptional activity of hybrid viral promoters in Sf9 cells

The efficiency of newly designed baculovirus-mediated transduction vector in gene transfer was successfully confirmed with Sf9 cells. Baculovirus-derived vector expressing the GFP reporter gene under the control of either PH-Ie1 or PH-P2 hybrid promoters were expressed, suggesting the transcriptional initiation and transduction in Sf9 cells. In the case of both the transduction vectors, expression of GFP was observed in Sf9 cells within 6 h of post transduction. After 6 h of infection with recombinant virus, 10% of the cells expressed green fluorescent protein and this value was increased to 20% within 12 h of post transduction followed by 80% within 24 h and 100% within 32 h (Fig. 13). Moreover, typical baculoviral cytopathic effects (CPE) including the occlusion bodies were observed in 12 h of post infected cells (Fig. 14). Furthermore, the hybrid promoter induced

expression of GFP was confirmed by SDS-PAGE analysis (Fig. 15), resulted in the presence of expressed protein under the control of PH-Ie1 and PH-P2 hybrid promoter cassettes in the cells transduced with recombinant virus BacIe1-GFP and BacP2-GFP respectively. Observed fluorescence intensity from transduced cells suggested that transcriptional activity in the Sf9 cells was more or less similar under the control of hybrid promoters, and high intensity of fluorescent signals was observed at 24 h post transduction. Further, the transduction efficiency and the infectivity of the recombinant virus were found to be 100% within 32 h of post transduction with ~$1x10^{-4}$ pfu (approximate value, suggested by the manufacturer). Sign of infection and transduction (no GFP signal) were not observed in human cell lines HeLa and HEp2, confirming that the recombinant viral particles would not be infecting human cells (data not shown).

5.3.2. Transduction of shrimp cells *in vivo* and *in vitro*, and evaluation of transcriptional activity of hybrid viral promoters

As expected, the recombinant baculovirus BacIe1-GFP and BacP2-GFP containing PH-Ie1 and PH-P2 hybrid promoter cassettes drove the expression of GFP reporter gene in shrimp cells *in vitro* and *in vivo*, demonstrated by fluorescent microscopy. However, PH-GFP (Control) expression cassettes under the control of PH promoter in the Bac-GFP virus didn't express GFP to a detectable level either in *in vitro* or *in vivo* experiments. Very late and a feeble transduction efficiency was observed in shrimp primary cells in comparison with Sf9 cells. Transduction efficiency in shrimp cells with both recombinant virus BacIe1-GFP and BacP2-GFP was found to be lower (10-20%) in comparison to the efficiency in Sf9 cells, which was 100%. *In vivo* experiments with recombinant viral vector proved that the viruses were infective to most of the cell/tissue types tested. The recombinant baculovirus BacIe1-GFP with PH-Ie1 hybrid promoter was infective to gills, nerve ganglion, intestine, muscles, haemocytes and lymphoid organ (Fig. 16 & 17). Whilst, recombinant baculovirus BacP2-GFP with PH-P2 hybrid promoter was

more infective to hepatopancreas, haemocytes, gills and lymphoid organ (Fig. 18 & 19). In the *in vitro* experiments, haemocytes, hepatopancreas, lymphoid and heart tissues were used to confirm their susceptibility to the recombinant virus particle. The PH-Ie1 promoter system in BacIe1-GFP virus initiated transcription and expressed GFP in all cell types tested *in vitro*. Whilst, with PH-P2 promoter system in BacP2-GFP, the virus showed more infectivity to lymphoid cells and found more transduced cells with GFP expression than BacIe1-GFP transduced cells. In both the cases, the expression was observed after 48 h of infection. Control animals were subjected for imaging under fluorescence microscope to avoid misinterpretation from the auto-fluorescence from its exoskeleton and eye stalk (Fig. 20). The shrimp injected with the above recombinant viruses (BacIe1-GFP and BacP2-GFP) survived for 45 days without any mortality (Fig. 21), suggesting the vector system nontoxic to animal which could also be used for immunization (DNA vaccine) against pathogenic bacteria and viruses.

5.4. Discussion

Studies on the development and establishment of shrimp cell lines have been hampered by the lack of effective molecular tools for gene transfer into primary shrimp cell cultures. Because the spontaneous transformation of shrimp cells *in vitro* and their establishment as permanent cell lines were found impossible to achieve, the induced immortalization was hypothesized to be the only option left to attempt develop shrimp cell lines (Jayesh *et al.*, 2012). Under such a situation, vectors capable of enhanced and long term delivery of immortalizing gene to the primary shrimp cell culture are required to evade the molecular blocks that prevent *in vitro* transformation. Moreover, primary shrimp cells were found to be very sensitive to standard gene delivery systems especially liposome-based transfection and electroporation. Thus, for the transgenic expression, viral mediated transduction was the better choice amongst all such methods. In this context, this study describes the construction of two recombinant baculovirus vectors with shrimp virus promoters designed to transfer foreign genes in to shrimp cells.

The putative promoters from shrimp viruses (WSSV-Ie1 and IHHNV-P2) such as white spot syndrome virus and infectious hypodermal and hematopoietic necrosis virus have been considered for constructing recombinant baculovirus vectors (BacIe1-GFP and BacP2-GFP). Immediately early (IE) gene *Ie1* of WSSV along with *ie2*, and *ie3* were identified in infected shrimps (Liu *et al*., 2005), wherein *Ie1* gene promoter has been considered as an efficient viral promoter to construct expression vectors. Moreover, WSSV Ie1 promoter was found active and control transcription in insect, shrimp, avian and mammalian cells (Prabakaran *et al*., 2010; Syed Musthaq *et al*., 2009; He *et al*., 2008; Gao *et al*., 2007). Syed Musthaq *et al*. (2009) constructed a recombinant baculovirus encoding VP28 envelop protein under the control of WSSV Ie1 protein and expressed this vector in shrimp tissue. He *et al.* (2008) suggested that recombinant baculovirus with WSSV Ie1 promoter was more active than with CMV (cytomegalovirus) promoter for displaying expression of haemagglutination activity of H5N1 virus.

IHHNV P2 promoter was proved to control transcription in insect, fish and crustacean cells (Dhar *et al*., 2007), that possessed the canonical TATA box (TATATAA). Moreover, Dhar *et al.* (2007) suggested that, even though the results were highly variable, transient expression of luciferase could be achieved under the control of P2 promoter in the constructed vector P2 complete pGL3. Because, it is located near map unit 2, Shike *et al*. (2000b) named the promoter as P2. In the present study, the same P2 promoter was used for constructing the recombinant baculovirus; the vector P2 complete pGL3 was kindly given by Dr. Arun K. Dhar, Viracine Therapeutics Corporation, USA.

As the recombinant baculovirus have emerged as a potent tool for protein production (Liu *et al*., 2010), virus production (Zheng *et al*., 2010; Lesch *et al*., 2011), vaccine development (Madhan *et al*., 2010), cancer therapy (Wang and Balasundaram, 2010), tissue engineering (Lin *et al*., 2010) and especially used for expression in shrimp (Syed Musthaq *et al*., 2009). In the present study, baculovirus vectors were used as the backbone to construct recombinant vector with shrimp viral promoters. Additionally, Condreay *et al.* (1999) described the use of a recombinant baculovirus vector carrying a mammalian expression cassette

comprising the cytomegalovirus immediate early (CMV-IE) promoter and the gene for green fluorescent protein (GFP) to direct gene expression in a wide variety of mammalian cell lines as well as primary human cells derived from different tissues. Likewise, studies suggested that recombinant baculovirus vectors carrying GFP reporter gene under the control of WSSV Ie1 promoter were capable of transducing shrimp cells *in vivo* (Syed Musthaq *et al*., 2009; Syed Musthaq and Kwang, 2011) and *in vitro* (Lu *et al*., 2005).

The hybrid promoter PH-Ie1 and PH-P2 in the recombinant baculovirus vector BacIe1-GFP and BacP2-GFP respectively could control transcriptional initiation in shrimp cells *in vitro* and *in vivo*. However, we could transduce only 10-20% shrimp cells with the hybrid promoter system. Further improvisation in transduction has to be carried out in the presence of histone deacetylase inhibitors such as sodium butyrate. Guo *et al*. (2010) supporting this hypothesis proved that sodium butyrate enhanced the expression of baculovirus-mediated sodium/iodide symporter gene in A549 lung adenocarcinoma cells. Many other earlier reports have also stated that sodium butyrate could significantly enhance baculovirus mediated gene expression in vertebrate cells (Condreay *et al*., 1999; Airenne *et al*., 2000). On the contrary, Lu *et al*. (2005) suggested that the low transduction efficiency might be due to the possible inhibition effect, especially the pH of the medium, on the attachment of baculovirus to the cell membrane. Lu *et al*. (2005) used L-15 medium for this experiment with a pH above 7.0, however, we used newly designed shrimp cell culture medium (SCCM) with a pH 6.8, a condition well sufficient to amplify the virus.

In conclusion, two recombinant baculoviral transduction vectors (BacIe1-GFP and BacP2-GFP) that carried expression cassettes consisting of gene encoding GFP as a reporter linked to the hybrid promoter either to PH-Ie1 or PH-P2 were successfully constructed and expressed in insect cell line and shrimp cells *in vivo* and *in vitro*. Because of its successful expression in shrimp cells without any toxicity, these versatile transduction systems could be used for expression of oncogenes or an immortalizing gene like telomerase reverse transcriptase (*TERT*) to effect

immortalization of shrimp cells. Moreover, these virus vectors can find application in the development of DNA vaccination and generation of transgenic animals.

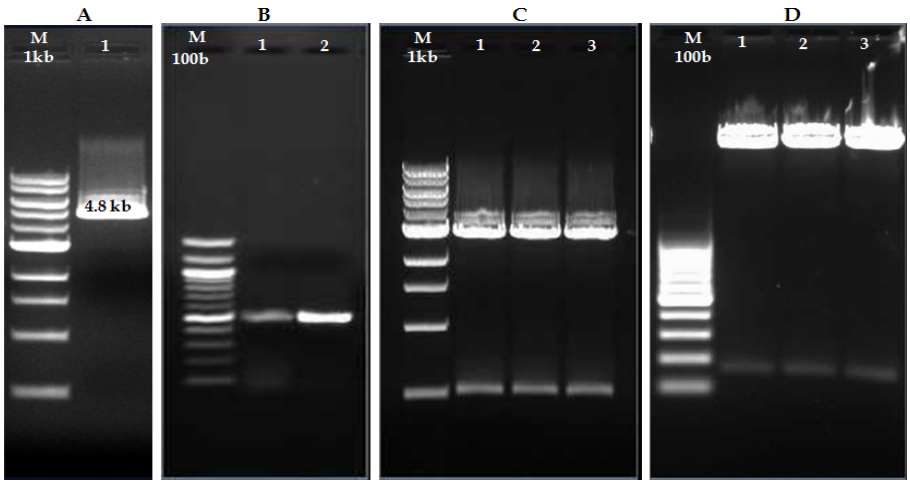


Fig.9. Agarose gel showing –A: Linearized plasmid pFastBac™ 1 digested with *Bam*H I, B: PCR amplified WSSV immediate early gene (Ie1) product of 502-bp size from infected animal, C: WSSV Ie1 promoter (502-bp) released from pGEM-T vector after restriction digestion with *Bam*H I enzyme, D: IHHNV P2 promoter (116-bp) released from PGEMT-T vector after restriction digestion with *Bam*H I enzyme.

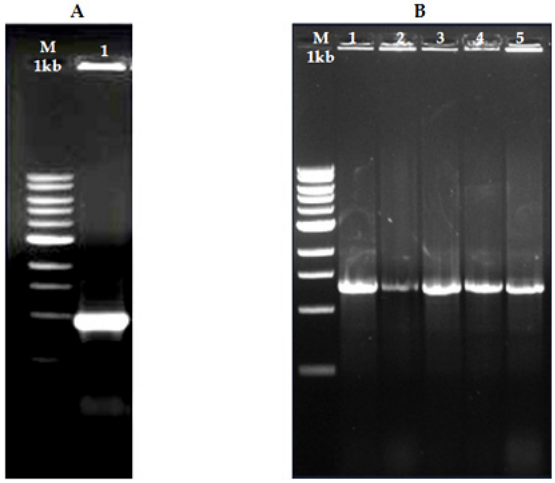


Fig.10. Agarose gel showing – A: Green fluorescent protein (GFP) gene restriction digested from pEGFP N1 with *Sal* I and *Not* I enzyme, B: Colony PCR performed for confirming the alignment of inserted GFP gene in pFastBac™ 1 vector between *Sal*I and *Not*I enzyme sites.

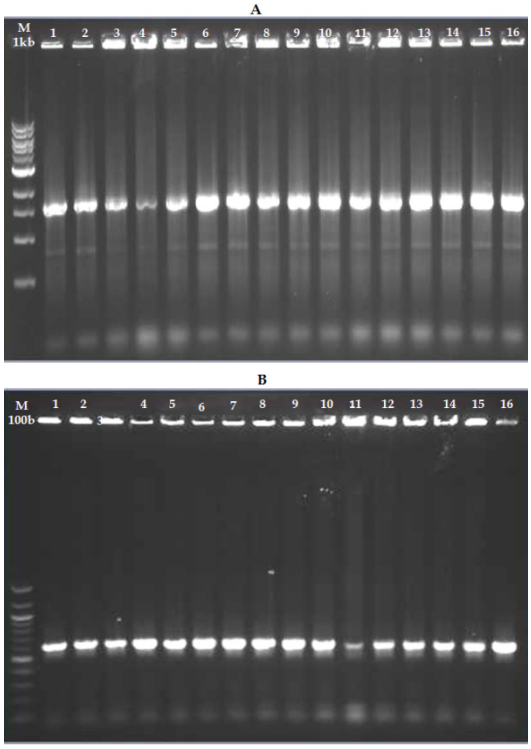


Fig.11. Agarose gel picture from colony PCR for confirming the insert orientation in pFastBac™1 before generating recombinant baculovirus. A: Ie1-GFP alignment confirmation using Ie1 forward primer and GFP reverse primer, B: P2-GFP alignment confirmation using P2 forward primer and GFP reverse primer.

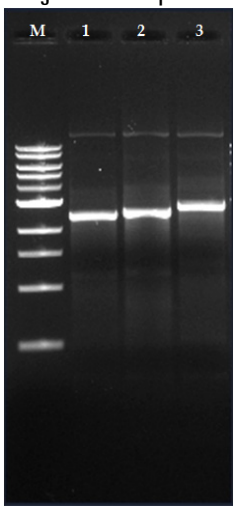


Fig.12. PCR confirmation of the recombinant bacmid using M13 forward and GFP reverse primers. M: molecular marker of 1-kb size, 1: wild type baculovirus tagged with GFP, 2: recombinant bacmid with P2 promoter, 3: recombinant bacmid with Ie1 promoter

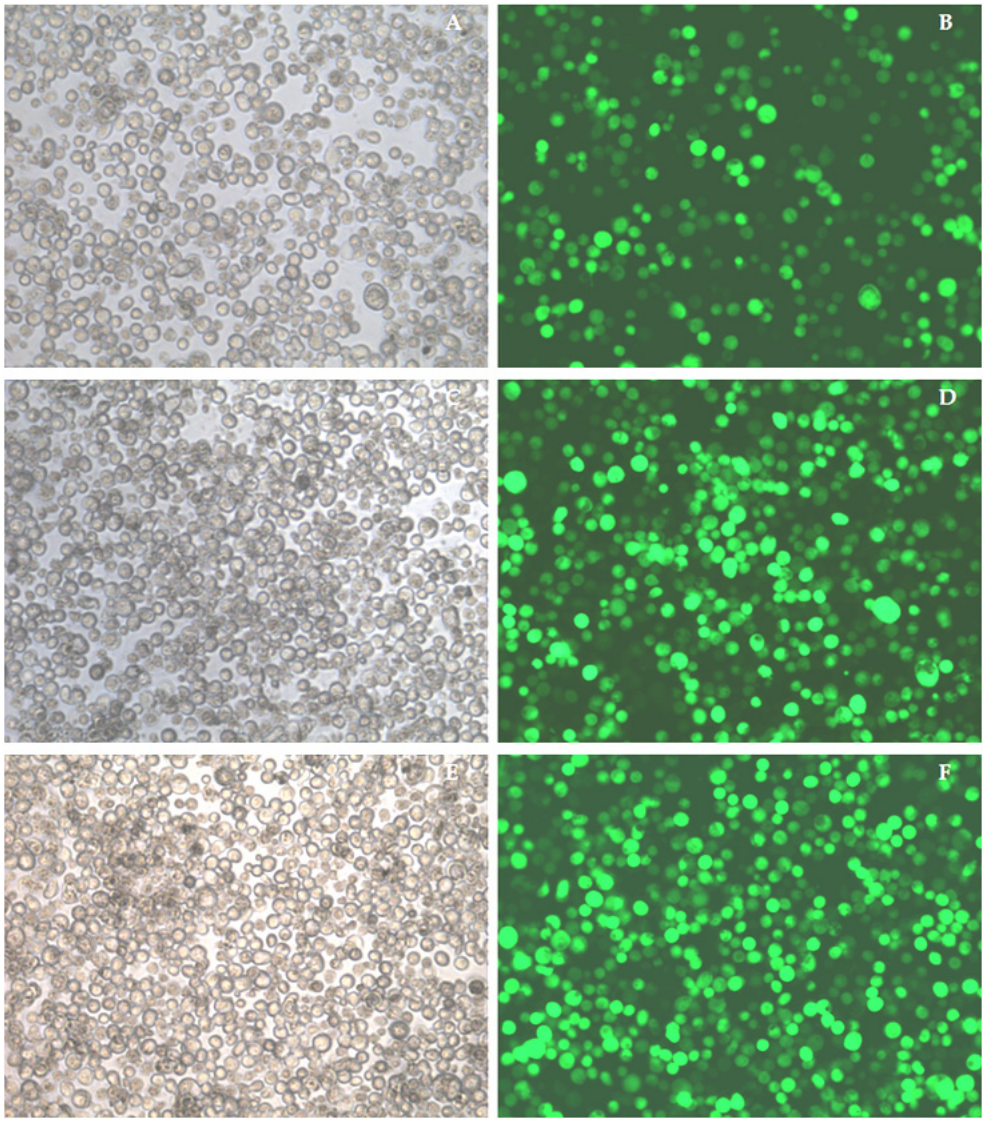


Fig.13. Recombinant baculovirus mediated tranduction in insect cells (Sf9). A, C, E: phase contrast image of the transduced Sf9 cells with Bac-GFP, Bacle1-GFP and BacP2-GFP respectively; B, D, and F: corresponding image under fluorescence microscope. The green fluorescent signals indicate the active viral transcription inside the cells.

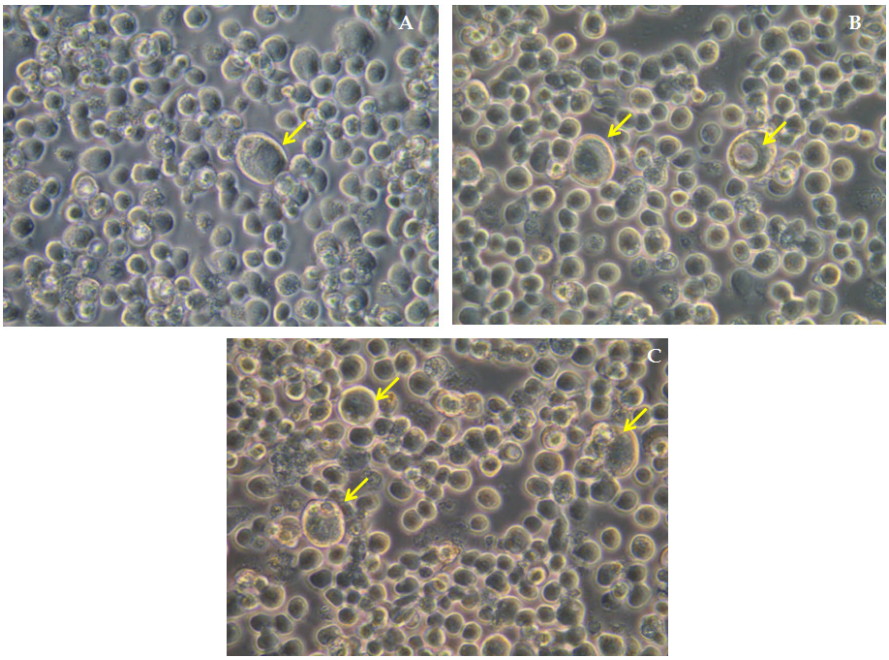


Fig.14. Sf9 cells showing cytopathic effect (arrow) after 12h of post infection with recombinant baculovirus. A: infected with Bac-GFP; B: Bacle1-GFP and C: BacP2-GFP virus.

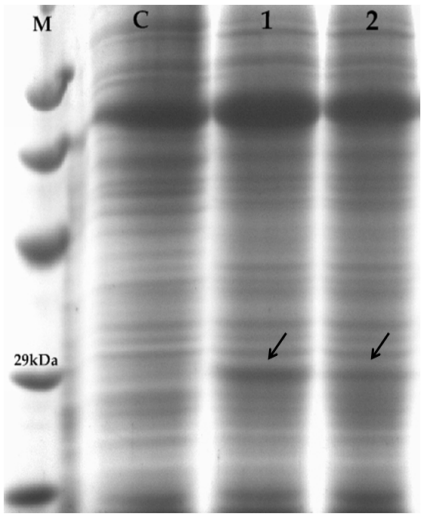


Fig.15. SDS-PAGE analysis of reporter protein (GFP) synthesis in Sf9 cells infected with recombinant baculovirus (vector): M: molecular marker, C: control cells without infection, 1: protein expression under the control of PH-Ie1 promoter (Bacle1-GFP vector), and 2: protein expression under the control of PH-P2 promoter (BacP2-GFP vector). Arrow indicates green fluorescent protein.

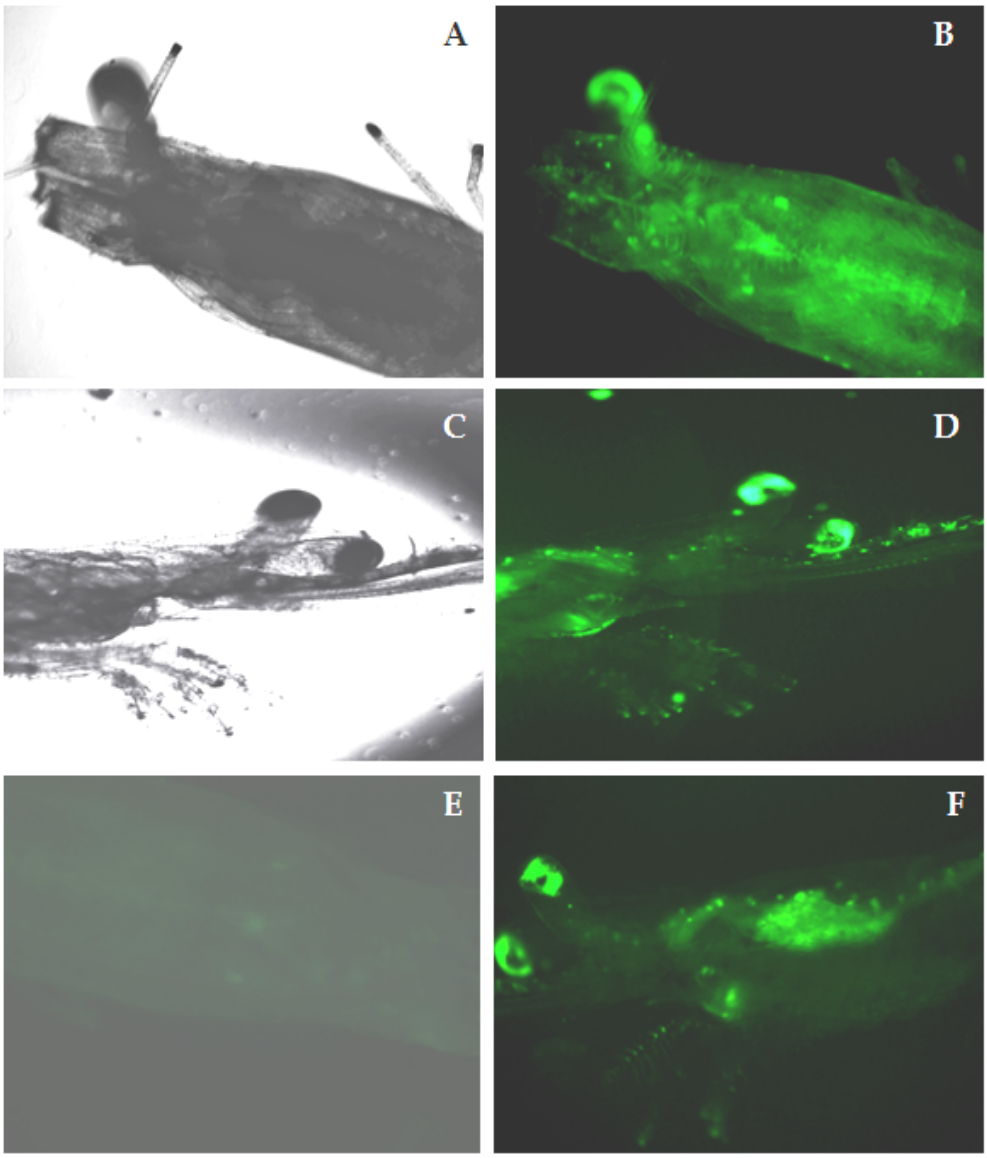


Fig.16. Transduction of GFP expressing recombinant baculovirus *in vivo* in susceptible larvae of *P. monodon* 3 days post infection obtained under microscope (10 x magnifications). A, C: bright field image of the animal infected with recombinant baculovirus containing PH-Ie1 hybrid promoter (BacIe1-GFP); B, D: corresponding animals expressing GFP; E: uninfected animal, and F: GFP expression from animal infected with recombinant baculovirus containing PH -P2 hybrid promoter (BacP2-GFP).

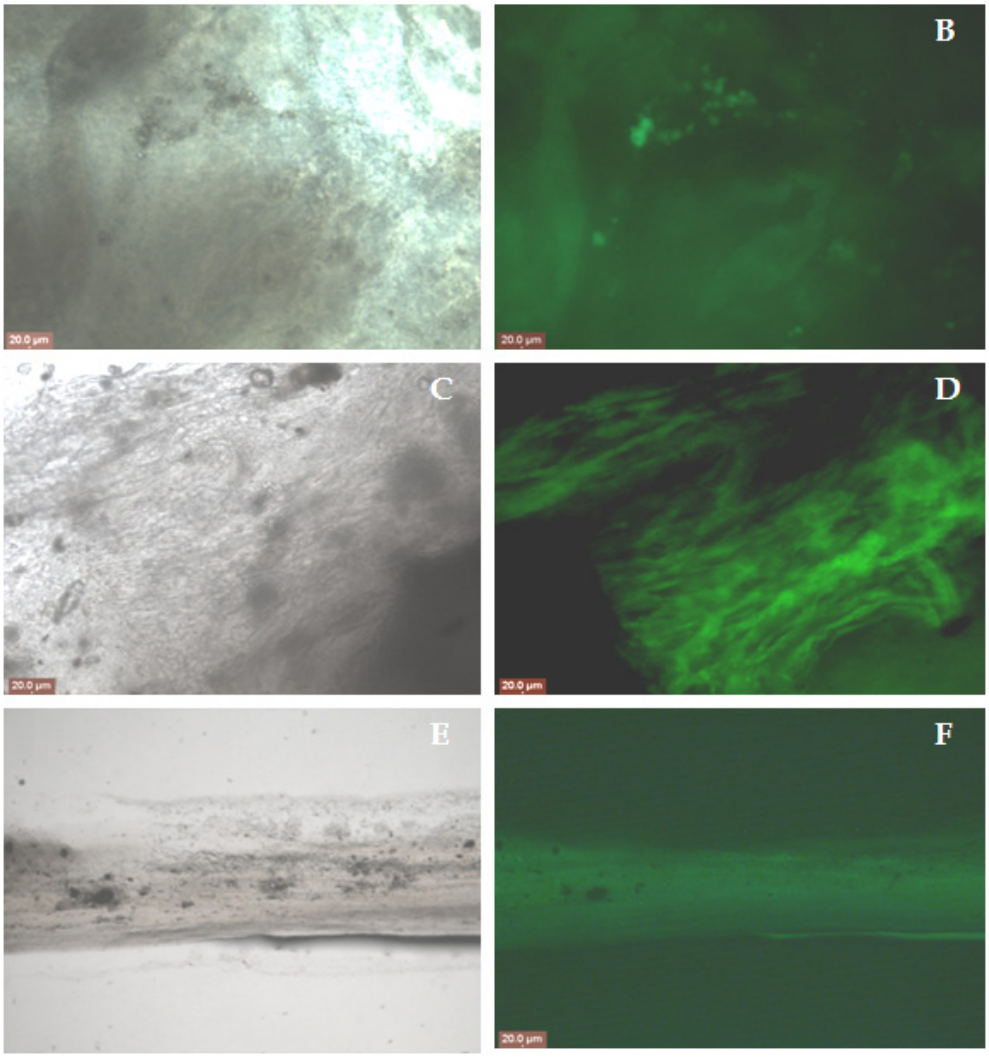


Fig.17. Expression of GFP from various organ/tissue of *P. monodon* transduced with recombinant baculovirus containing PH -Ie1 hybrid promoter (Bac-Ie1GFP). A, C, E: phase contrast image of heart, muscle and intestine, and B: expression of GFP from heart, D: muscle and F: intestine. Images were taken 3 days post infection under microscope (20 x magnifications) .

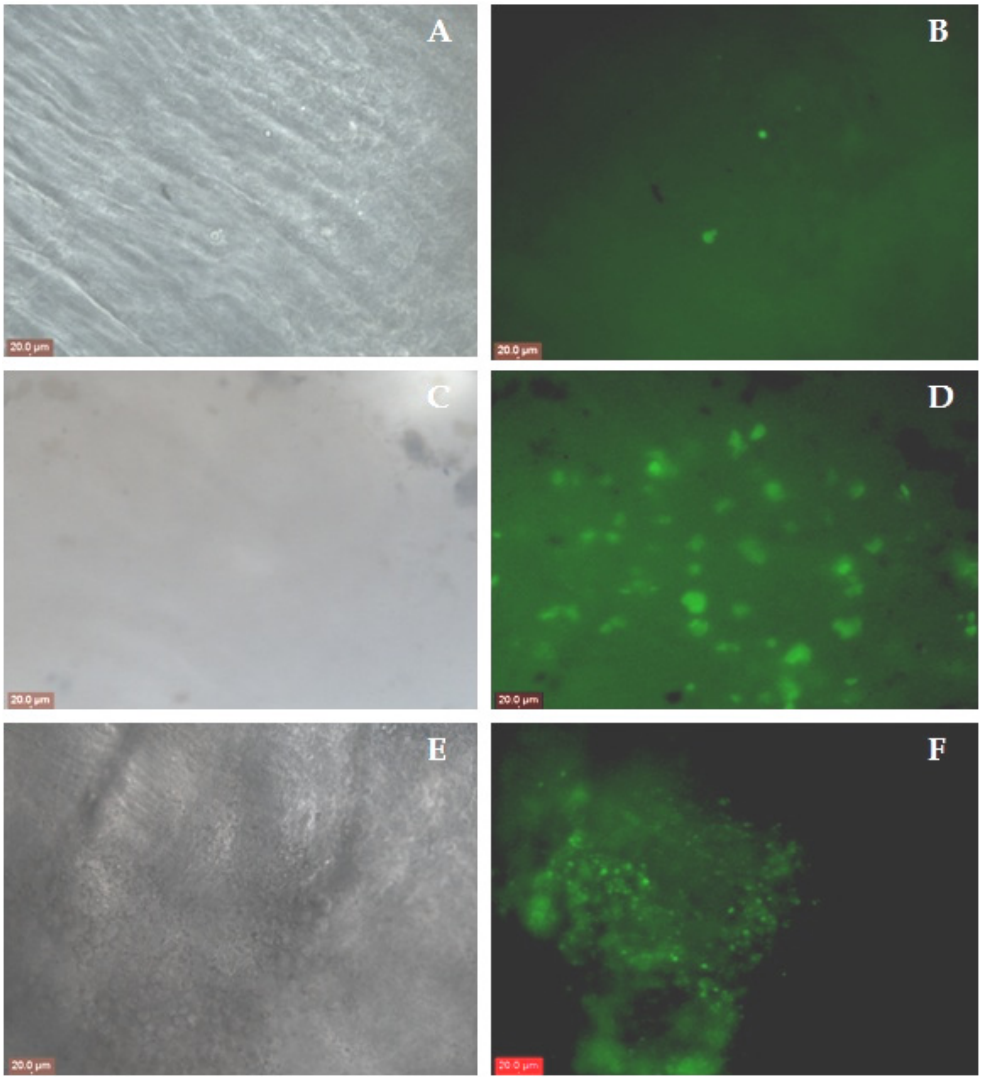


Fig.18. Expression of GFP from various organ/tissue of *P. monodon* transduced with recombinant baculovirus containing PH –P2 hybrid promoter (Bac-P2GFP). A, C, E: phase contrast image of heart, lymphoid organ and hepatopancreas, and B: the expression of GFP from heart, D: lymphoid organ and F: hepatopancreas. Images were taken 3 days post infection under microscope (20 x magnifications) .

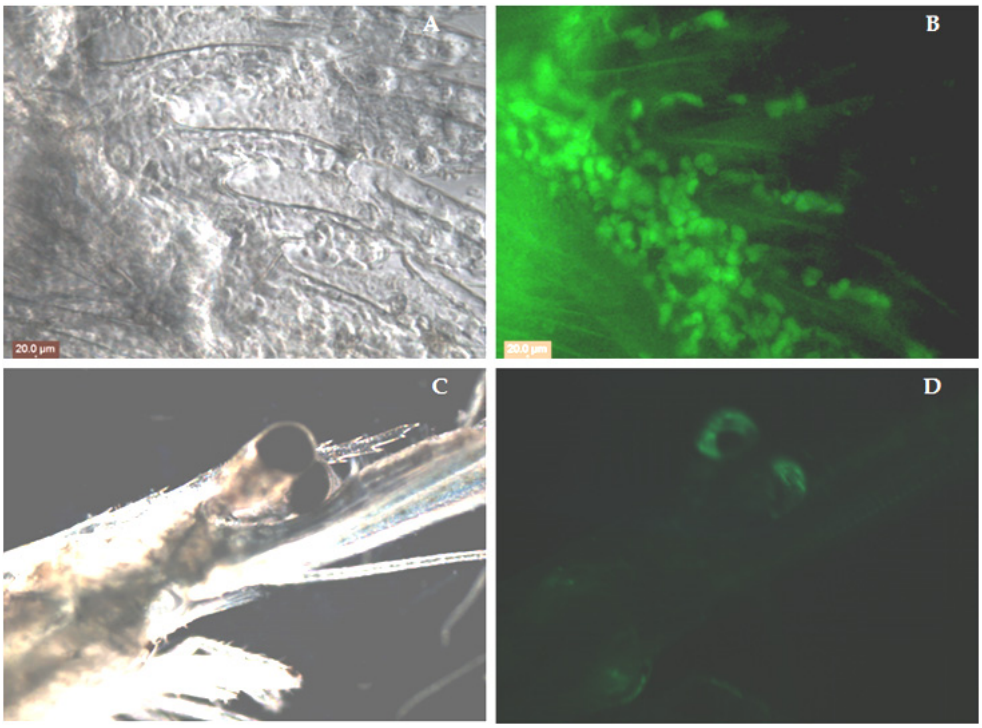


Fig.19. Expression of GFP from various gill tissue of *P. monodon* transduced with recombinant baculovirus (BacP2-GFP) containing PH –P2 hybrid promoter. A: phase contrast image of gill tissue; B: expression of GFP from same under fluorescence microscope indicating the viral transcription and successful transduction; C and D: control animal under bright field and fluorescent microscope; low level of auto fluorescence was observed in eyestalk. The Images were taken 3 days post infection under microscope (20 x magnifications) .

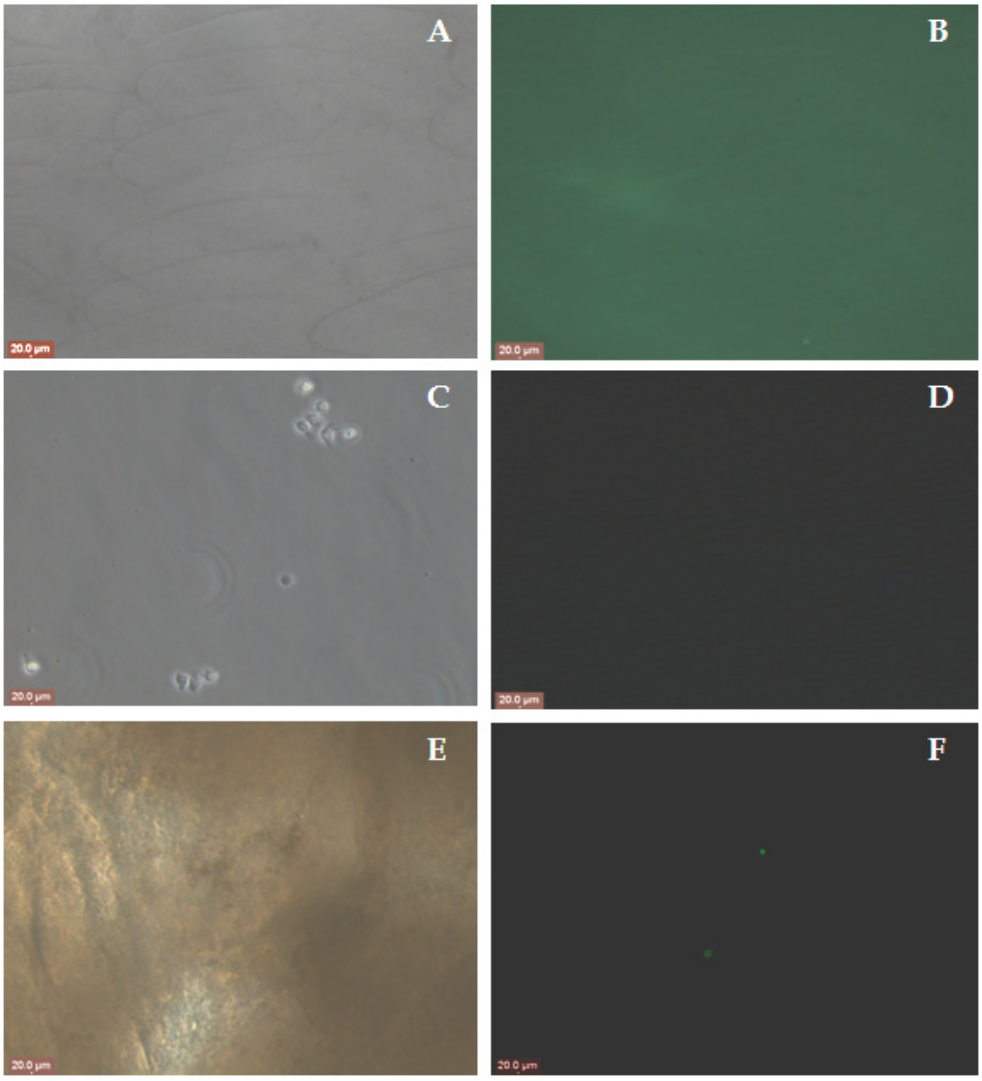


Fig.20. Transduced *P. monodon* with wild-type baculovirus containing GFP (Control). A, C, E: phase contrast image of gills, haemocytes and hepatopancreas, and B, D, F: same tissue/cells under fluorescence microscope. Images were taken 3 days post infection under microscope (20 x magnifications).

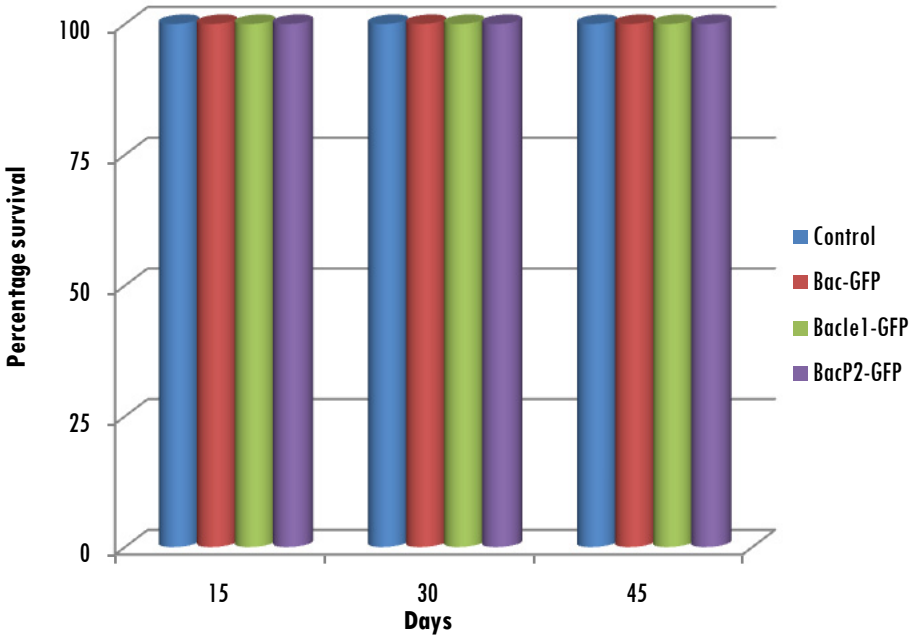


Fig. 21. Survival of animals injected with recombinant and wild-type baculovirus

Chapter 6

Transfection and transduction mediated oncogene expression in lymphoid cell cultures from *Penaeus monodon* for its *in vitro* transformation

6.1. Introduction

In general, when animal cells isolated from tissue are cultured *in vitro*, they stop dividing after a finite number of divisions due to senescence (Hayflick and Moorhead, 1961). If a cell overcomes senescence, it is said to have become immortal since it has acquired an infinite life span (Powell *et al*., 1999). Such genetic modification in cells, called *in vitro* transformation, may lead to immortalization. If the cells are unable to produce cell lines by spontaneous transformation, they are subjected to transformation with foreign gene expression, especially oncogenes, using various methods such as calcium phosphate precipitation, lipid-mediated transfections, electroporation, and viral mediated transduction. Moreover, it has been proven that the introduction of a cellular or viral oncogene could induce *in vitro* transformation in cells through oncogenesis, a process whereby a cell escapes from the normal cellular proliferative controls (Weigel *et al*., 1990), and achieves indefinite cell division and thus immortalization. Oncogene mediated immortalization in cells has been experimented and proven with various oncogenes, such as the simian virus 40 (SV40) large tumour (T) antigens (Fitzgerald *et al*., 1994; Jha *et al*., 1998;

Kirchhoff *et al*., 2004), adenoviral E1A gene (Weigel *et al*., 1990), C-MYC (De Filippis *et al*., 2008), E6 and E7 encoded by the human papilloma virus (Wazer *et al*., 1995; Jansen-Durr, 1998); virus mediated immortalization using Epstein-Barr virus (Gao *et al*., 2002) and hTERT mediated immortalization (Bodnar *et al*., 1998).

SV40-T antigen is a multifunctional nuclear phospho- protein of 708 amino acids (Soule and Butel, 1979) that has been shown to be the simplest and most reliable agent for the immortalization of many different cell types and the mechanism of SV40 T antigen in cell immortalization is relatively well understood (Lundberg *et al*., 2000). Moreover, studies suggested that SV40 -T antigen induced *in vitro* transformation resulted in phenotypic variation in diverse species (Ozer *et al*., 1996). In addition, recent studies have also shown that SV40 infection can induce telomerase activity in human cells (Foddis *et al*., 2002). Claydon and Owens (2008) suggested that human papilloma virus expressed E6 and E7 proteins that function concomitantly to disrupt the p53 and retinoblastoma (Rb) tumor suppressor genes, regulators of the cell-cycle checkpoints at the first gap (G1) phase. Adenovirus type 12 early region 1A (12S E1A), oncoprotein from 12S mRNA of 243 amino acids has also been shown to affect a variety of cellular functions, most notably the immortalization of primary cells and the promotion of quiescent cells into S phase (Mal *et al*., 1996). Furthermore, the 12S ElA oncogene alters the differentiation process in malignant cells and can induce cell proliferation, resulting in the establishment of permanent cell lines (Weigel *et al*., 1990).

The transforming effect of oncogenes of DNA viruses (e.g., 12S E1A, HPV E6, E7, and SV40-T) is due to their interference with negative regulators of cell progression through the cell cycle (Bulavin *et al*., 2002). In most of the cases, oncogenes achieve *in vitro* transformation and immortalization by inactivating the tumor suppressor genes such as p53, p16, pRb, the negative regulators of G1/S

transition, and other regulating genes including cyclins (Weigel *et al.*, 1990; Zerfass *et al.*, 1996; Jansen-Durr, 1998; Bulavin *et al.*, 2002), pro-apoptotic Bax (Nowak *et al.*, 2004) or by activating the telomerase genes, that all can induce a replicative senescence in cells (Lundberg *et al.*, 2000). However, in certain cases, both telomerase activation and tumor suppressor inactivation (eg. $p16^{INK4A}$ gene) by genetic or epigenetic mechanisms is required to bypass senescence and render the cells immortality (Kiyono *et al.*, 1998; Vaziri and Benchimol, 1999; Yamamoto *et al.*, 2003).

Transformation studies in shrimp primary cultures were first reported by Tapay *et al.* (1995). Since then, various methods of transformation such as lipofection, (Tapay *et al.*, 1995), retroviral infection (Shike *et al.*, 2000a; Hu *et al.*, 2008, 2010) and recombinant baculoviral mediated transduction (Lu *et al.*, 2005) were employed in shrimps. However, inspite the variations in the methods of transformation, the choice of transgene in most of the studies was SV40-T antigen (Tapay *et al.*, 1995; Hu *et al.*, 2008, 2010). However, despite all these transformation studies, shrimp cells with constitutive expression of exogenous gene (oncogene) via stable integration into the genome of dividing cells have not yet been attained. This may be due to the selection of inappropriate method of transformation and wrong choice of oncogenes. Conversely, the technique which would cause minimum toxicity to the cell would be a better choice for the transformation, besides the appropriate selection of oncogene or immortalizing gene.

In this context, the present study was designed with a focus on transfection and transduction mediated oncogene expression in lymphoid cell cultures. Lipofection and electroporation mediated transfection of cells with SV40-T, and recombinant baculovirus mediated transduction of adenoviral oncogene 12S E1A were tested for *in vitro* transformation of lymphoid cell cultures generated from *P. monodon*.

6.2. Materials and methods

6.2.1. Cells and cell culture

Primary lymphoid cell culture from *P. monodon* was used as the platform for studying transfection and transduction mediated oncogene expression in shrimp cells *in vitro*. Lymphoid cell culture was developed in shrimp cell culture medium (SCCM) by following the methodology explained in Chapter 2.

The insect cell line, Sf9 was used for generating baculovirus from the recombinant bacmid containing PH-P2-12S E1A-GFP expression cassettes. Insect cell line originated from *Spodoptera frugiperda* pupal ovarian tissue was maintained at 28 °C in Grace's insect medium with 2 mM L-glutamine, 500 mg l^{-1} calcium chloride, 2.8 g l^{-1} potassium chloride, 3.33 g l^{-1} lactalbumin hydrolysate supplemented with 10% fetal bovine serum and antibiotic mixture containing 100 µg ml^{-1} streptomycin and 100 IU ml^{-1} penicillin. The growth medium was changed once in every 2-3 days.

Human cervical carcinoma cell, HeLa (Henrietta Lacks) was used as the control for transfection experiment which was maintained at 37 °C in Dulbecco's modification of minimum essential medium (DMEM) with 2 mM L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g l^{-1} sodium bicarbonate, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate supplemented with 10% fetal bovine serum and antibiotic mixture containing 100 µg ml^{-1} streptomycin and 100 IU ml^{-1} penicillin. The growth medium was changed three times in a week.

6.2.2. Oncogene (s) and vectors used for the experiment

pSV3-neo vector encoding SV40-T oncogene and recombinant baculovirus BacP2-12S E1A-GFP encoding 12S E1A oncogene were used for the transfection and transduction experiment respectively in lymphoid cells. pEGFP-C1 vector

containing green fluorescent protein reporter gene was used for co-transfection experiment in HeLa cells (control) to detect the transcriptional activity in post-transfected cells as transfection marker, and pEGFP-N1 for GFP tagging.

6.2.2.1. pSV3-neo vector encoding simian virus 40-T (SV40-T) oncogene

pSV3-neo vector encoding simian virus 40 large T (SV40-T) oncogene was purchased from ATCC (# 37150). Vector provides dominant selectable marker for resistance to neomycin (G-418) in mammalian or other eukaryotic cells and to ampicillin in *E. coli* (Fig. 1).

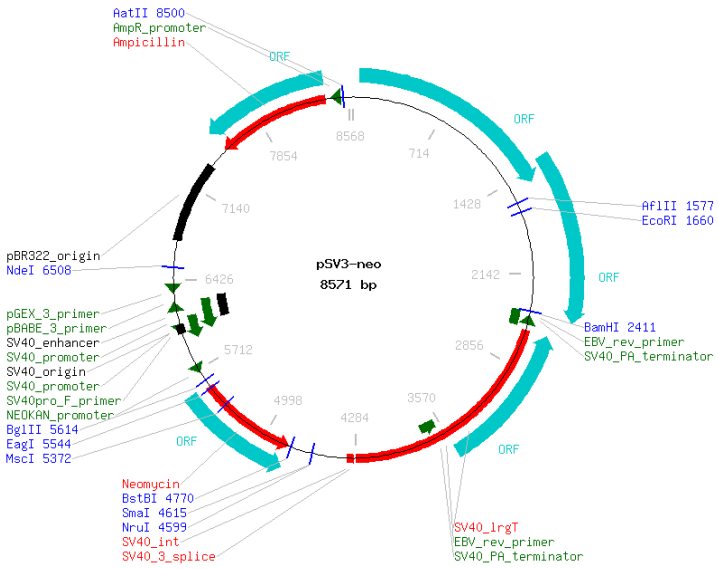


Fig.1. Vector map of pSV3-neo vector encoding SV40-T oncogene

6.2.2.2. pWZL hygro 12S E1A viral vector encoding adenoviral 12S E1A oncogene

Adenoviral 12S E1A oncogene encoding retroviral vector, pWZL hygro 12S E1A (addgene #18748, GenBank ID AAQ72378) constructed by Serrano *et al.* (1997) was collected through addgene, USA. The 1501-bp sized 12S E1A oncogene was restriction digested with *Bam*H I enzyme, released from the vector backbone and used for viral mediated transduction studies in lymphoid cells from *P. monodon* (Fig. 2).

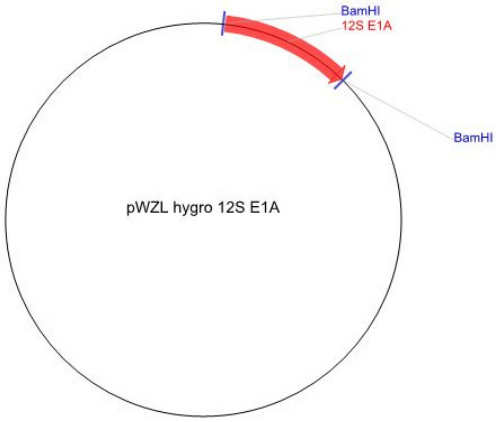


Fig.2. Vector map of pWZL hygro 12S E1A vector encoding adenoviral oncogene 12S E1A (Addgene # 18748)

6.2.2.3. pBacP2 transfer vector with PH-P2 hybrid promoter

pBacP2 transfer vector was designed by inserting P2 promoter from infectious hypodermal and hematopoietic necrosis virus (IHHNV) in-frame with PH promoter of *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) at *Bam*H I site in the pFastBac™1 transfer vector. This hybrid promoter system (PH-P2) is flanked by the left and right arms of Tn7 transposon, and also contains gentamicin resistance gene and SV40 polyadenylation signal for the site-specific transposition properties of the Tn7 transposon. Construction of pBacP2 transfer vector (Fig 3) was explained in the previous chapter (Chapter 5).

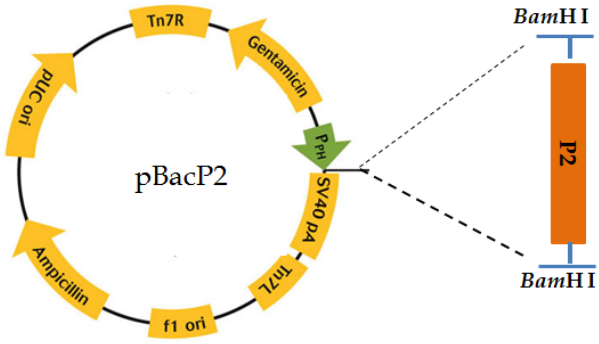


Fig.3. Vector map of pBacP2 vector with PH-P2 promoter

6.2.2.4. Green fluorescent protein encoding pEGFP-C1 and pEGFP-N1 vector

pEGFP-C1 and pEGFP-N1 vectors (Clontech) encoding red-shifted variant of wild-type green fluorescent protein (GFP) under the control of human cytomegalovirus (CMV) immediate early promoter for evaluating transcriptional activity in transfected eukaryotic cells were used. The vectors provided dominant selectable markers for resistance to neomycin (G-418) in mammalian or other eukaryotic cells and to kanamycin in *E. coli*. Fusion to the C terminus or N terminus of EGFP retained the fluorescent properties of the native protein allowing the localization of the fusion protein *in vivo* (Fig. 4).

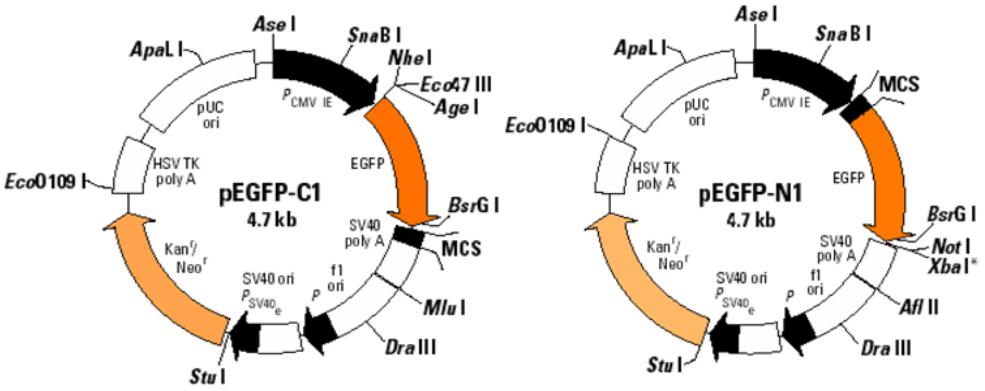


Fig.4. Vector map of pEGFP-C1 and pEGFP-N1 vectors encoding red-shifted variant of wild-type GFP

6.2.3. Propagation of *E. coli* containing the plasmid vectors pSV3-neo, pWZL hygro 12S E1A, pBacP2, pEGFP-C1 and pEGFP-N1 and plasmid extraction

E. coli HB101 with pSV3-neo vector, *E. coli* DH5α with pWZL hygro 12S E1A and pBacP2 were inoculated into 10 ml LB broth containing 100 µg ml^{-1} ampicillin. *E. coli* DH5α with pEGFP-C1 and pEGFP-N1 vectors were inoculated into 10 ml LB broth containing 30 µg ml^{-1} kanamycin. The cultures were incubated at 37 °C on a rotary shaker at 220 rpm for plasmid extraction.

Plasmid extraction was done using GenElute HP Plasmid Miniprep kit (Sigma Life Sciences) following manufacturer's instruction . Briefly, an aliquot of 2 ml culture after overnight incubation was pelletised at 12,000 x g for 1 min. The pellet was resuspended in 200 µl resuspension solution containing RNase A and lysed by adding 200 µl lysis buffer. An aliquot of 350 µl neutralization solution was added and centrifuged at 12,000 x g for 10 min to remove the cell debris. Lysate was loaded into GenElute HP Miniprep binding column inserted into a microcentrifuge tube and centifuged at 12,000 x g for 1 min . Plasmid DNA bound to the column was washed twice with wash solution to remove the endotoxins, salt and other contaminants. To elute the plasmid DNA, the column was transferred to a fresh collection tube, added 100 µl 10 mM Tris-Cl and centrifuged at 12,000 x g for 1 min and stored at -20 ºC. Purity of the plasmid DNA obtained was analysed by agarose gel electrophoresis and by determining the ratio of the absorbance reading at 260 nm/280 nm in a UV-VIS spectrophotometre (U2800, Hitachi, Japan) and fluorometrically using Qubit® flourometer (invitrogen™, USA).

6.2.4. Transfection mediated SV40-T oncogene expression in lymphoid cell culture and analysis of post transfected cells

Electroporation and lipofection, of lymphoid cells were performed as the methods of transfection.

6.2.4.1. Electroporation of lymphoid cell culture with pSV3-neo vector encoding SV40-T oncogene

Electroporation of lymphoid cells was performed in accordance with the study of Seena Jose explained in her thesis (Jose, 2009) with slight modifications. Briefly, a mixture of 400 µl lymphoid organ cells suspended in 600 mOsm kg^{-1} electroporation buffer (Eppendorf, Germany) and 5 µg ml^{-1} pSV3-neo plasmid vector encoding SV40-T oncogene was prepared, transferred to electroporation cuvette of 2 mm gap width (Eppendorf, Germany). Control (negative) consisted of

lymphoid cells without the addition of the plasmid, and HeLa cells were used as positive control. In HeLa cells, co-transfection experiment was performed with 3 µg ml^{-1} pSV3-neo vector and 3 µg ml^{-1} pEGFP-C1 vector as transcription marker to confirm the successful transformation by GFP expression. After two electric pulses at 500 v voltage for 100 µs in eukaryotic mode of the multiporator (Eppendorf, Germany), the cells were allowed to remain in the cuvett for 20 min at RT. Carefully added 200 µl of heat inactivated FBS to the cuvett, transferred this mixture to 25 ml tissue culture flask containing 500 µl shrimp cell culture medium (SCCM) and kept at 25 ºC for 12 h. After incubation complete medium was drained off and added fresh SCCM containing 400 µg ml^{-1} Geneticin 418 (sigma). In the case of HeLa cells, minimal essential medium (MEM) was used. All cells were incubated for 24 h and observed under fluorescent-inverted-phase contrast microscope.

6.2.4.2. Lipofection of lymphoid cell culture with pSV3-neo vector encoding SV40-T oncogene

Lymphoid cells grown in shrimp cell culture medium (SCCM) in 24 well plate (60-80% confluent) was selected for transfection studies. The cells were washed with growth medium (SCCM) without serum and antibiotics and subjected to transfection with pSV3-neo vector and both pSV3-neo vector and pEGFP-C1 vector (as transcription marker) was used for co-transfection experiment. An aliquot of 8 µg pSV3-neo vector was diluted with 200 µl SCCM without antibiotics and serum and mixed with Cellfectin (Invitrogen) which was previously diluted by adding 60 µl into 200 µl SCCM (antibiotics and serum free). In the case of co-transfection experiment 4 µg pSV3-neo vector and pEGFP-C1 vector were used. The lipid-DNA mixture was mixed gently and incubated at RT for 45 min and diluted to 2 ml with serum free, antibiotic free medium and subsequently 0.25 ml of the transfection suspension was added to each well. After 24 h incubation in transfection medium, the medium was removed from each well without disturbing

the cells and normal growth medium was added. pSV3-neo transfected cells along with co-transfected cells were subjected for microscopic observation, and analysis of transcriptional activity and for immunefluorescence assay with anti-SV40-T antigen.

6.2.4.3. Analysis of lymphoid cells transfected with pSV3-neo vector encoding SV40-T oncogene

Post-transfected mitotic events in the lymphoid cells were evaluated by microscopic examination using Inverted phase contrast microscope (Leica) connected with time lapse imaging facility controlled by image acquisition software (LAS, Leica). This was mainly performed to observe oncogene induced mitotic events and phenotypic variation in cells *in vitro*.

The cultured cells were observed daily, and the 48 h post transfected lymphoid cells were subjected for immunofluorescence assay with anti-SV40-T antigen. Briefly, the cells were pelletized by centrifugation at 400 g for 5 min, washed with PBS (720 mOsm) and fixed in 10% paraformaldehyde, and subsequently with 70% ethanol. Cells were attached on to glass slides, pre coated with poly-L-lysine (0.01%) using a cyto-centrifuge (Wescor, USA). Subsequently the free sites were blocked using 3% BSA in PBS and incubated in a humidified chamber for 1 h. The slides were washed in PBS/Tween-20 (0.01%) and added 100 µl anti-SV40-T antigen (5 µg ml^{-1}) (Calbiochem) on the slide, incubated for 1 h and washed three times in wash buffer. It was again incubated for an hour after addition of 100 µl rabbit anti-mouse FITC conjugate, 1: 50 dilution (Sigma) and subjected for washing. After incubation with general nuclear stain DAPI (10 µl, 0.2 µg ml^{-1}) for three minutes, the slides were rinsed with distilled water, air dried, mounted (Vectashield, USA) and observed under an Inverted fluorescence phase contrast microscope (Leica, Germany). DAPI and FITC were viewed under filters with excitation wavelength 360-370 nm and 470-490 nm respectively. The slides were compared with untransfected control cells.

The images were processed and merged using the image acquisition software (LAS, Leica).

The green fluorescent signals from pEGFP-C1 vector used in co-transfection experiment was observed using Inverted fluorescence phase contrast microscope with GFP filter. Both lymphoid and HeLa (control) cells were subjected for transcription marker (GFP) analysis to confirm the successful transformation.

6.2.5. Recombinant baculovirus BacP2-12S E1A-GFP mediated transduction and expression of 12S E1A oncogene into lymphoid cells and its confirmation

6.2.5.1. Construction of transfer vector encoding 12S E1A tagged with GFP for generating recombinant baculovirus

The transfer vector pBacP2-12S E1A-GFP was constructed by inserting GFP tagged 12S E1A oncogene. The construction of pBacP2 transfer vector containing PH-P2 hybrid promoter system was explained in the previous chapter (Chapter 5). The recombinant baculovirus was generated by transfection of recombinant bacmid generated in DH10Bac *E. coli* into insect cell lines. The recombinant bacmid was developed by transformation of the transfer vector pBacP2-12S E1A-GFP into DH10Bac *E. coli.*

6.2.5.1.1. Green fluorescent protein (GFP) tagging of 12S E1A oncogene

Green fluorescent protein was tagged at the 3' end of the 12S E1A oncogene to visualize the oncogene expression in the cells *in vitro.*

6.2.5.1.1.1 Restriction digestion of pEGFP-N1 vector encoding GFP with Bam H I and its purification

pEGFP-N1 plasmid vector encoding GFP gene was restriction digested with *Bam* H I (New England Biolabs) enzyme. Briefly, an aliquot of 20 µl reaction mixture containing 5 µl plasmid, 0.5 µl enzyme (10,000 U ml^{-1}) 2 µl reaction buffer and 12.5 µl

MilliQ water was incubated for 1 h at 37 ºC. Restriction digested plasmid was CIP (Calf Intestinal Phosphatase) treated to remove the phosphate groups to prevent self ligation. The reaction mixture containing 20 µl plasmid, 0.1 µl CIP enzyme and 5µl buffer was incubated at 37 ºC for 1 h followed by heat inactivation at 65 ºC for 20 min. Restriction digested, CIP treated pEGFP-N1 vector was gel purified using GenElute™ Gel Extraction kit (Sigma, USA) by following manufacturesr's instruction. Briefly, the agarose gel that contain DNA fragment of appropriate size was excised using X-tracta gel extraction tool (Sigma, USA). The excised gel slice was taken in a 1.5 ml tube, weighed and added 3 gel volumes (~450 µl) of gel solubilization solution and incubated at 60 ºC for 10 min with repeated vortexing in every 2 min. After incubation, added 1 gel volume (~150 µl) of 100% isopropanol, mixed gently until it become homogenous. This solubilized gel solution was loaded into the binding column that was pre-treated with column preparation solution, centrifuged at 12,000 x g for 1 min. Added 700 µl wash solution and centrifuged for 1 min at 12,000 x g , repeated the centrifugation and residual wash solution was removed. The binding column was transferred to a fresh collection tume (2 ml MCT) and added 50 µl of pre-heated (at 65 ºC) 10 mM Tris-HCl (pH 9.0), centrifuged at 12,000 x g for 1 min, stored at -20 ºC. The concentration of DNA was measured spectrometrically reading at 260/280 nm in a UV-VIS spectrophotometer (U2800, Hitachi, Japan) and fluorometrically using Qubit® fluorometer (invitrogen™, USA).

6.2.5.1.1.2. Restriction digestion of pWZL hygro 12S E1A vector with Bam H I to release 12S E1A oncogene and its purification

The purified pWZL hygro 12S E1A plasmid vector was restriction digested with *Bam* H I enzyme (New England Biolabs) to release 12S E1A oncogene. An aliquot of 20 µl reaction mixture containing 5 µl plasmid, 0.5 µl enzyme (10,000 U ml^{-1}) 2 µl reaction buffer and 12.5 µl MilliQ water was incubated for 1 h at 37 ºC. The released 12S E1A gene (oncogene) product was confirmed by 1% agarose gel

electrophoresis. After electrophoresis, the agarose gel containing 12S E1A was purified by the methodology explained in the previous section (6.2.5.1.1.1).

6.2.5.1.1.3. Ligation of 12S E1A oncogene into pEGFP-N1 vector, transformation into E. coli DH5α and plasmid extraction

The purified 12S E1A oncogene was ligated with the restriction digested, CIP treated pEGFP-N1 plasmid vector at *Bam* H I restriction site upstream to GFP gene. This allows tagging of GFP at 3' end of 12S E1A oncogene. The 10 µl ligation mixture containing 2 µl CIP treated pEGFP-N1 vector, 3 µl gel purified 12S E1A oncogene, 1 µl 10x buffer, 0.5 µl T_4 DNA ligase enzyme and 3.5 µl MilliQ water was incubated at 16 °C, overnight. After incubation, added 10 µl of ligation mixture to a sterile 15 ml culture tube already on ice, transferred 50-100 µl of competent cells (thawed) into the 15 ml tubes (containing ligation mix) on ice, gently flicked the tubes to mix and placed them on ice for 20 min, heat shocked the cells for 90 sec in a water bath at exactly 42 °C. immediately returned the tubes to ice for 2 min., Added 600 µl super optimal broth with catabolite repression (SOC; Composition for 10 ml: 0.2 g tryptone; 0.05 g yeast extract; 0.005 g NaCl; 100 µl 1 M KCl; 50 µl 2 M $MgCl_2$; 200 µl 1 M glucose. $MgCl_2$ and glucose were added just before transformation) to the tubes containing cells transformed with ligation mixture, incubated for 2 h at 37 °C with shaking at 220-230 rpm, plated 100 µl of each transformation culture (duplicate/triplicate) onto LB/ampicillin/ IPTG/X-gal plates and incubated the plates overnight (16-18 h) at 37 °C. After PCR confirmation of the white colonies, propagation and plasmid extraction were performed by following the methodology explained elsewhere in this chapter.

6.2.5.1.1.4. Restriction digestion and release of GFP tagged 12S E1A oncogene (12S E1A-GFP), and its purification

Green fluorescent protein (GFP) tagged 12S E1A oncogene was restriction digested from pEGFP-12S E1A vector with *Sal* I and *Not* I (New England Biolabs)

enzymes following double digestion protocol (New England Biolabs). Briefly, an aliquot of 50 µl reaction mixture containing 5 µl plasmid, 2 µl *Sal* I enzyme (20,000 U ml^{-1}), 2 µl *Not* I enzyme (2,500 U ml^{-1}), 4 µl reaction buffer, 0.4 µl bovine serum albumin (BSA) and 36.6 µl MilliQ water, incubated at 37 ºC for 2 h followed by heat inactivation at 65 ºC for 20 min. Restriction digested vector was subjected for gel purification to extract GFP tagged 12S E1A oncogene using GenElute™ Gel Extraction kit (Sigma, USA) and the methodology followed was well explained in the previous section (6.2.5.1.1.1) in this chapter.

6.2.5.1.2. Construction of pBacP2-12SE1A-GFP transfer vector encoding GFP tagged 12S E1A oncogene, extraction and purification

6.2.5.1.2.1 Restriction digestion pBacP2 transfer vector, and its purification

pBacP2 transfer vector was restriction digested with *Sal* I and *Not* I enzymes (New England Biolabs) following double digestion protocol (New England Biolabs). An aliquot of 50 µl reaction mixture containing 5 µl plasmid, 2 µl *Sal* I enzyme (20,000 U ml^{-1}), 2 µl *Not* I enzyme (2,500 U ml^{-1}), 4 µl reaction buffer, 0.4 µl bovine serum albumin (BSA) and 36.6 µl MilliQ water, incubated at 37 ºC for 2 h followed by heat inactivation at 65 ºC for 20 min. Restriction digested vector was subjected for gel purification to extract GFP tagged 12S E1A oncogene using GenElute™ Gel Extraction kit (Sigma, USA) and the methodology followed well explained in the previous section (6.2.5.1.1.1) in this chapter.

6.2.5.1.2.2. Ligation of GFP tagged 12S E1A into pBacP2 transfer vector and its purification

The purified, GFP tagged 12S E1A oncogene was ligated with the restriction digested pBacP2 vector at *Sal* I and *Not* I restriction site downstream to P2 promoter. The 10 µl ligation mixture containing 2 µl CIP treated pEGFP-N1 vector, 3 µl gel purified 12S E1A oncogene, 1 µl 10x buffer, 0.5 µl T_4DNA Ligase enzyme and 3.5 µl MilliQ water was incubated at 16 °C, overnight. The

methodology followed for transformation, propagation and plasmid extraction has been explained elsewhere in this chapter.

6.2.5.2. Generation of recombinant bacmid shuttle vector

6.2.5.2.1. Transfection of pBacP2-12SE1A-GFP into DH10Bac™ E. coli to produce recombinant bacmid shuttle vector

To the 10 µl ligation mixture in a 5 ml ice cold screw cap tube, added 50 µl competent DH10Bac™ *E. coli* cells, mixed gently and incubated on ice for 20 min. Heat shock was given for 90 sec at exactly 42 °C. The tube was returned to ice for 2 min. Added 600 µl super optimal broth with catabolite repression (SOC; composition for 10 ml: Tryptone-0.2 g; yeast exytract-0.05 g; NaCl-0.005 g; 1 M KCl- 100 µl; 2 M $MgCl_2$-50 µl; 1M glucose-200 µl. $MgCl_2$ and glucose were added just before transformation) and incubated for 2 h at 37 °C with shaking at 230 rpm. After incubation, 200 µl each was plated onto LB plate containing (LB medium- 2 g 100 ml^{-1}; Agar- 2 g 100 ml^{-1}) kanamycin (50 µg ml^{-1}), gentamicin (7 µg ml^{-1}), tetracycline (10 µg ml^{-1}), IPTG (40 µg ml^{-1}), and X-gal (100 µg ml^{-1}), and incubated for 24 to 48 h at 37 °C to select for DH10Bac™ transformants.

6.2.5.2.1.1. Propagation, isolation and PCR confirmation of recombinant bacmid

The individual colonies developed (white) were propagated in 100 ml LB broth containing kanamycin (50 µg ml^{-1}), gentamicin (7 µg ml^{-1}), tetracycline (10 µg ml^{-1}), and incubated at 37 °C with shaking (225 rpm) for plasmid extraction. The recombinant bacmid DNA containing GFP tagged 12S E1A was isolated and purified using PureLink, HiPure Plasmid Miniprep kit (Invitrogen, Germany) by following manufacturer's instruction. Briefly, centrifuged an aliquot of 8 ml overnight culture of *E. coli* DH10Bac containing recombinant bacmid at 15,000xg at RT for 15 min, removed the entire medium. Resuspended the pellet in 0.4 ml of cell suspension buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.2 mg ml^{-1} RNase A. Added 0.4 ml of cell lysis solution containing 200 mM

NaOH and 1% SDS, mixed gently by inverting the capped tube five times and incubated at RT for 5 min. After incubation, added 0.4 ml of neutralization buffer containing 3.1 M potassium acetate (pH 5.5) and mixed immediately by inverting the tube five times. Centrifuged at 15,000xg at RT for 10 min and transferred the supernatant onto the equilibrated column and allowed the solution in the column to drain by gravity flow. The column equilibration was performed by adding 2 ml equilibration buffer containing 600 mM NaCl, 100 mM sodium acetate (pH 5.0) and 0.15% Triton X-100 and the buffer was drained off by gravity. After complete removal of supernatant from the equilibrated column, washed the column two times with 2.5 ml wash buffer containing 800 mM NaCl, 100 mM sodium acetate (pH 5.0) and allowed the solution in the column to drain off. The recombinant bacmid DNA attached on the column membrane was eluted by adding 0.9 ml of elution buffer containing 1.25 M NaCl, 100 mM Tris-HCl (pH 8.5) and allowed the solution in the column to drain to a 2 ml sterile micro centrifuge tube (MCT). The recombinant bacmid DNA was precipitated by adding 0.63 ml isopropanol, mixed and placed on ice for 10 min, centrifuged at 15,000xg at RT for 30 min. Carefully discarded the supernatant and washed the bacmid DNA pellet with 1 ml of ice cold 70% ethanol and centrifuged at 15,000xg at RT for 5 min. Carefully drained off the ethanol and air dried the pellet for 10 min at RT, dissolved in 50 µl of TE buffer containing 10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA. The concentration of recombinant bacmid DNA was calculated fluorometrically using Qubit fluorometer (Invitrogen) and stored at -20 °C. The orientation of bacmid DNA carrying PH-P2-12S E1A-GFP fragments (cassettes) were confirmed by PCR amplification using the M13F (5' CCC AGT CAC GAC GTT GTA AA ACG 3') bacmid primer and GFP specific primer (NP266R-5' CAC GAA CTC CAG CAG GAC CAT G 3'). The 25 µl PCR reaction mixture containing 2.5 µl 10x buffer, 2.5 µl dNTP (2.5 mM), 1 µl Taq polymerase (0.5 U μl^{-1}), 0.2 µl bacmid , 1 µl of each primer (10 pmol μl^{-1}) and the mixture was made up to 25 µl with MilliQ. The hot start PCR programme used for the amplification was 95 °C for 5 min followed by hold at 80

°C, 32 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec, extension at 72 °C for 2 min, followed by final extension at 72 °C for 10 min. An aliquot of 10 µl of PCR products was analyzed by 1% Agarose gel electrophoresis, stained with ethidium bromide, visualized and documented using gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA).

6.2.5.3. Generation of recombinant baculovirus expressing GFP tagged 12S E1A oncogene under the control of PH-P2 hybrid promoter

6.2.5.3.1. Transfection of recombinant bacmid containing PH-P2-12S E1A-GFP expression cassettes into Sf9 cells

The Sf9 cells at a cell density 1.5 x 10^6 cells ml^{-1} grown in TNM-FH medium (Sigma, USA) without antibiotics and serum in 35 mm culture dish was selected for transfection. To generate the recombinant virus, Sf9 cells were transfected with the confirmed bacmid DNA using Cellfection®II reagent (Invitrogen) by following manufacturer's instruction. Briefly, an aliquot of 1 µl (500 ng ml^{-1}) recombinant bacmid DNA was diluted with 100 µl antibiotic and serum free TNM-FH medium and mixed with Cellfectin® II which was previously diluted by adding 8 µl into 100 µl TNM-FH medium (antibiotics and serum free). The lipid-bacmid mixture (transfection mixture) was mixed gently and incubated at RT for 45 min, added dropwise onto the cells and incubated at 28 °C for 6 h. After incubation, the transfection mixture was replaced with TNM-FH medium containing 15% fetal bovine serum (FBS) and antibiotics. The cells were further incubated at 28 °C until the signs of viral infection (occlusion bodies) and fluorescent signals from green fluorescent protein could be seen.

6.2.5.3.2. Isolation, amplification and storage of recombinant baculovirus containing GFP tagged 12S E1A oncogene

Recombinant baculovirus virus released in the TNM-FH medium was collected from each culture dish and re-infected on to another sets of Sf9 cells (1.5

x 10^6 cells ml^{-1}) to re-amplify the viral stock. After re-amplification, the medium containing concentrated virus was collected into sterile centrifuge tube and centrifuged at 500xg for 5 min to remove the cell debris. The clear supernatant was transferred to fresh cryovials, covered with aluminum foil to protect from light and stored at -80 °C until transduction experiment in lymphoid cell culture from *P. monodon*.

6.2.5.4. Analysis of oncogenic 12S E1A induced protein expression in Sf9 cells

After 72 h of post-transduction with oncogenic 12S E1A, Sf9 cells were harvested from the culture dish and protein was extracted using 400 µl of 1x SDS-PAGE lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS) and boiled for 5 min. The extracted protein was subjected to reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) following the method of Laemmli (1970). Briefly, protein extract was (10 µl) mixed with 10 µl of gel loading dye, boiled for 5 min. The protein was separated and analyzed using 4% stacking gel and 15% resolving gel prepared in 10 x 10.5 cm vertical gel plate of miniVE™ mini vertical electrophoresis unit (Hoefer-Amersham, India). Electrophoresis was performed in 1x Tris-glycine SDS (pH 8.3) buffer (tank buffer) at a voltage of 12 mA (EPS 301, Amersham, India). After electrophoretic separation, gel was stained in coomassie brilliant blue stain R-250 (0.025 % coomassie brilliant blue R-250, 40% methanol and 7% acetic acid in distilled water) followed by de-stained in de-staining solution I (40% methanol and 7% acetic acid in distilled water) and de-staining solution II (5% methanol and 7% acetic acid in distilled water), photographed using Gel Doc™ XR+ imaging system (Bio-Rad, USA). 12S E1A induced protein expression was determined by comparing with the protein profile of un-transduced Sf9 cells (control). Molecular weight of protein band was determined by comparing with that of standards (Genei, India).

6.2.5.5. Transduction of lymphoid cells with recombinant baculovirus encoding 12S E1A oncogene

Recombinant baculovirus encoding adenoviral 12S E1A oncogene was prepared as a 48 h supernatant from infected Sf9 cells grown in 35 mm culture dish containing TNM-FH medium. The supernatant was centrifuged at 500xg for 5 min to remove the cell debris. Lymphoid cell culture for transduction experiment was prepared in 35 mm culture dish in shrimp cell culture medium (SCCM) by following the methodology explained in the previous chapter (Chapter 1). After 24 h incubation of lymphoid cells at 25 °C, the medium was replaced with 1 ml SCCM and 1 ml supernatant containing recombinant baculovirus (1:1) and incubated for 6 h at 25 °C, replaced with growth medium (SCCM) containing 15% fetal bovine serum (FBS). The medium was changed every 2 days for 14 days. Un-transduced lymphoid cells were used as the control.

6.2.5.6. Analysis of lymphoid cells transduced with recombinant baculovirus encoding 12S E1A oncogene

As the GFP tagged at the 3' end of 12S E1A, the expression of green fluorescent protein confirmed the viral transcription and transduction of 12S E1A into lymphoid cells. GFP expression was evaluated by microscopic examination using Fluorescence-Inverted phase contrast microscope (DMIL, Leica) with GFP filter, and controlled by image acquisition software (LAS, Leica). Transduced cell cultures were observed in every 3 h for 24 h, subsequently in every 24 h for subsequent days for the *in vitro* transformation mediated phenotypic changes. Normal cells without infection were also subjected to microscopic observation and this served as the control.

6.3. Results

6.3.1. Transfection mediated oncogenic SV40-T expression in lymphoid cell culture from *P. monodon*

Despite its deteriorative effect on cells, electroporation conditions for lymphoid organ cells could be standardized in the present study. After electroporation, the transformed cells which were epithelioid with large nucleus divided promptly within 6 h of post transfection and continued for 72 h (Fig. 5). The cells were rounded-off from the surface of the culture flask at 5th-day post-transfection. Co-transfection experiment with pEGFP-C1 and pSV3-neo in HeLa cells as control (Fig. 6), and lymphoid cells proved the transfection by green fluorescence signals from GFP reporter gene in the pEGFP-C1 vector. This experiment has proven the transformation capability of the cells. Lipofection based gene delivery in lymphoid cells was also found successful with very few transformed cells (>5%). Meanwhile, co-transfection with pEGFP-C1 containing expression of transcription marker (GFP) confirmed the successful gene delivery in lymphoid cells (Fig. 7). However, cells transfected by lipofection method survived for 14 days with less percentage of dead cells, however they degenerated thereafter. Whilst on performing electroporation on lymphoid cell cultures with pSV3-neo vector, most of the cells were found dead and survived cells lived for only 7 days. From these results, it was observed that lipofection was more efficient than electroporation techniques for transfection in primary lymphoid cell culture. Despite the phenotypic variation and the expression of GFP from co-transfected cells, in the immunofluorescence assay, SV40-T antigen in the transfected lymphoid cells was not detected using anti SV40-T antigen. Moreover, multiplication of pEGFP-C1 transfected lymphoid cells with GFP expression was not observed during proliferation.

6.3.2. Construction of recombinant baculovirus expressing GFP tagged 12S E1A and its expression in insect cells

Recombinant baculovirus transduction vectors, BacP2-12SE1A-GFP that carried expression cassettes consisting of gene encoding adenoviral oncogene 12S E1A tagged with GFP, linked to the PH-P2 hybrid promoter was successfully constructed. Insertion of 12S E1A oncogene in pEFGP-N1 vector at its *Bam*H I restriction site confirmed the N-terminal in-frame GFP tagging (Fig. 8). The transfer vector pBacP2 provided the strong polyhedrin (PH) promoter from *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) and P2 promoter from the infectious hypodermal and hematopoietic necrosis virus (IHHNV). The P2 promoter was inserted at the position 4032nd base in pFastBac™1 transfer vector to make pBacP2 transfer vector with PH-P2 hybrid promoter. The GFP tagged oncogene construct 12S E1A was inserted at the *Sal* I and *Not* I enzyme site, downstream to PH-P2 promoter which confirmed the expression under the control of the hybrid promoter system. The expression cassettes in pBacP2 transfer vector containing PH-P2 promoter and green fluorescent protein (GFP) tagged 12S E1A oncogene were at the position between transposon elements Tn7R and Tn7L (Fig. 9) which allowed the site-specific transposition of the expression cassettes along with a gentamicin resistance gene into the baculoviral genome. Transposon mediated transposition in bacmid was confirmed using M13 (forward) and GFP (reverse) primers, indicating that the inserts were transpositioned to form recombinant bacmid and was at correct orientation (Fig. 10). Viral transcription and propagation of recombinant virus in Sf9 cells were confirmed by the expression of GFP after transfection with the recombinant bacmid. Expression of GFP was observed in Sf9 cells within 6 h of post transduction. After 6 h of infection with recombinant virus 5% of the cells expressed green fluorescent protein, and this value was increased to 30% within 12 h of post transduction followed by 80% within 24 h (Fig. 11) and 100% within 32 h (Fig. 12). Moreover, typical baculoviral cytopathic effects (CPE)

including the occlusion bodies were observed in 12 h of post infected cells (Fig. 13). The GFP expressing viral occlusion bodies were very clear after 6 h of post infected cells under the fluorescence microscope (Fig. 11). SDS-PAGE analysis of BacP2-12SE1A-GFP transduced Sf9 cells confirmed the adenoviral oncogene 12S E1A mediated protein expression and up-regulation of genes in transduced cells (Fig. 14).

6.3.3. Transduction mediated oncogenic adenoviral 12S E1A expression in lymphoid cell culture from *P. monodon*

Recombinant baculovirus BacP2-12SE1A-GFP containing PH-P2 hybrid promoter cassettes drove the expression of adenoviral oncogene 12S E1A in primary lymphoid cell culture from *P. monodon*, confirmed by the expression of GFP, which was tagged at the 3' end of the oncogene. Moreover, transduced cells showed a typical phenotypic variation within 12 h of post transduction, in comparison with the control (Fig. 15). The fibroblastic cell type turned to round to the elliptical and prominent large nucleus could be seen. There was no detectable level of GFP expression observed within 7 days of post infection in lymphoid cells. However, high level of expression could be observed after 14 days (Fig. 16), and proliferating cells survived for more than 90 days in growth factor optimized shrimp cell culture medium (SCCM) with GFP expression. Despite the very late observable expression, transduction efficiency in primary lymphoid cells with recombinant virus BacP2-12S E1A-GFP was more than 80% within 14 days of post infection. From the results, it could be confirmed that the observed phenotypic variation was due to *in vitro* transformation of the cells. Besides, the stable transduction of oncogene 12S E1A was proven by the long term expression of GFP in lymphoid cell culture, which was tagged at 3' end of the oncogene.

6.4. Discussion

A major impetus for the study was to identify the possibility of transfection and transduction mediated oncogenic expression in lymphoid cells and its *in vitro*

transformation which leads to immortalization. To attain this, two major oncogenes such as simian virus 40-T antigen and adenoviral 12S E1A gene were used in which, SV40-T was transfected (using electroporation and lipofection) whilst, 12S E1A oncogene transduced (by recombinant baculovirus infection) in lymphoid cells. This selection was with the observation that these viral gene products could bind specifically to cellular proteins which provided an important insight into their ability to transform cells (DeCaprio, 2009).

It has long been recognized that the simian virus 40-T antigen (Brodsky and Pipas, 1998) and adenoviral 12S E1A gene (Braithwaite *et al*., 1983; Haley *et al*., 1984) can induce immortalization in cells via stimulating cellular DNA synthesis and *in vitro* transformation (DeCaprio, 2009). Furthermore, it has also been proved to transform mouse, rat, bovine, rabbit, hamster, and human cells (Macdonald, 1990). The major targets of the SV40 large T antigen (SV40-T) and adenovirus E1A protein was found to be the Rb family of pocket proteins that comprises a group of tumor suppressor proteins; pRb, p107, and p130, demonstrating one mechanism by which tumor viruses can interfere with cell cycle progression in cells (Henley and Dick, 2012). Moreover, the SV40-T antigen and adenoviral 12S E1A gene specifically inhibit p53 by binding and stabilizing it (Harms *et al*., 2004).

In the present study, electroporation and lipofection mediated transfection of lymphoid cells with pSV3-neo vector encoding SV40-T antigen was successfully attempted and confirmed either by GFP expression from co-transfected cells or by phenotypic variation. However, the expressed SV40-T antigen was not detected from transfected cells using anti-SV40-T-antibody, albeit the stable expression of SV40-T antigen required for immortalization. Simian virus-40 tumor (T) antigen (SV40-T) mediated *in vitro* transformation and establishment of the continuous cell line from tissues /organs has been considered as the extensively used techniques (Tapay *et al*., 1995). SV40-T immortalized cell

lines have been originated from human and rodents (Chang *et al*., 1985; Benoit *et al*., 1995; Anastassiadis *et al*., 2010). However, despite the successful attempt of transformation in shrimp cells (Tapay *et al*., 1995; Hu *et al*., 2008, 2010), constitutive expression could not be achieved. Tapay *et al*. (1995) suggested that three clones of lymphoid cells such as OKTr-1, OKTr-23 and OKTr-25 could be developed from *Penaeus stylirostris* transfected with pSV3-neo vector encoding SV40-T antigen. Moreover, replication-defective pantropic retrovirus encoding SV40-T was successfully transduced in lymphoid cells (Hu *et al*., 2008) and ovarian cells (Hu *et al*., 2010), which confirmed the presence of SV40-T gene and its stable mRNA expression in transduced cells. Even though the researchers suggested the increased life span of the cells after transduction with SV40-T antigen, establishment of cell lines could not be achieved. It was also concluded that, the observed lethality in the post-transfected cells found in this study might be due to the toxic protein expression from heterogeneous promoters in the post transformed cells.

For the transduction study, a recombinant baculovirus vector pBacP2-12S E1A-GFP, in which expression of exogenous 12S E1A oncogene tagged with GFP driven by PH-P2 hybrid promoters, has been successfully constructed. The stable transduction achieved in the Sf9 and lymphoid cells, was confirmed by the expression of GFP tagged at the 3' end of 12S E1A oncogene. This is the first ever report of adenoviral oncogenic 12S E1A expression in crustacean cells. Yet, a series of previous experiments have documented the immortalizing capacity of the EIA oncogene in many other cell types and confirmed that the introduction of EIA into primary cell cultures, that exhibit limited proliferation capacity, resulted in the establishment of permanent cell lines (Auley, 1983; Gopalakrishnan *et al*., 1997). According to Houweling *et al*. (1980), ElA alone could generate immortalized cell lines from primary cultures. Weigel *et al*. (1990) reported the Adenoviral 12S E1A gene mediated differentiation in F9 teratocarcinoma cell system that led to immortalization. Conversely, Rao *et al*. (1992) suggested that both E1A and E1B

genes were required to establish continuous transformed eukaryotic cell lines. The mechanism of immortalization by 12S E1A oncoproduct was confirmed by the stabilization of p53 that prevents p53-dependent genes activation (Harms *et al*., 2004). Whyte *et al*. (1988) reported that Adenoviral E1A oncoprotein were capable of inducing expression of E2F (transcription factor) responsive genes by disrupting pocket protein/E2F interactions and this disrupting activity was the reason for induced cellular transformation. Moreover, it has also been confirmed that, despite its immortalization property (Auley, 1983), 12S E1A oncoprotein stimulates several cellular proliferative responses (Quinlan, 1994) including DNA synthesis and cell cycle progression in quiescent epithelial cells (Shimojo and Yamashita, 1968) even in the absence of serum (Quinlan and Grodzicker, 1987).

The study revealed that the transfection and transduction mediated oncogenic expression in lymphoid cells was possible, despite the degenerating effects on cells due to transfection, and such induced immortalization is required for shrimp cell immortalization especially using recombinant baculovirus. Though, the recombinant baculovirus vector pBacP2-12S E1A-GFP mediated oncogenic expression was successfully confirmed in lymphoid cells, successful immortalization could not be achieved. This finding support the observation of Darimont *et al*. (2002) that the stable oncogene (SV40-T) expression alone could not immortalize cells, despite, telomerase was a requirement. In this light, it is anticipated that further improvisation of transduction, which have been successfully attained in lymphoid cells, with 'specific' ongogene, shrimp telomerase reverse transcriptase (*PmTERT*) and the epigenetic induction with histone protein modulators may eventually lead to immortalization of shrimp cells.

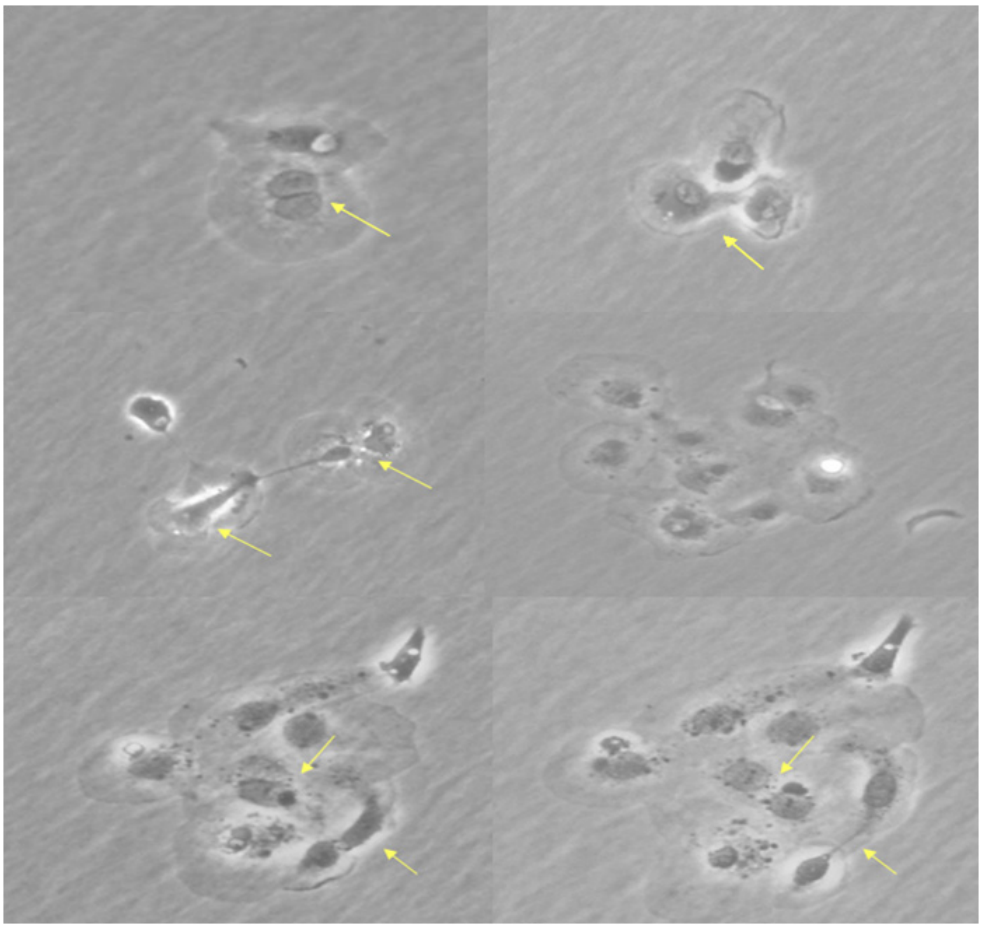


Fig.5. Lymphoid cells transfected (electroporation) with pSV3-neo vector containing SV40-T antigen. Time-lapse image of 12 h post tranfected cells shows enlarged nucleus and active multiplication. Arrow indicates mitotic division (40x magnification).

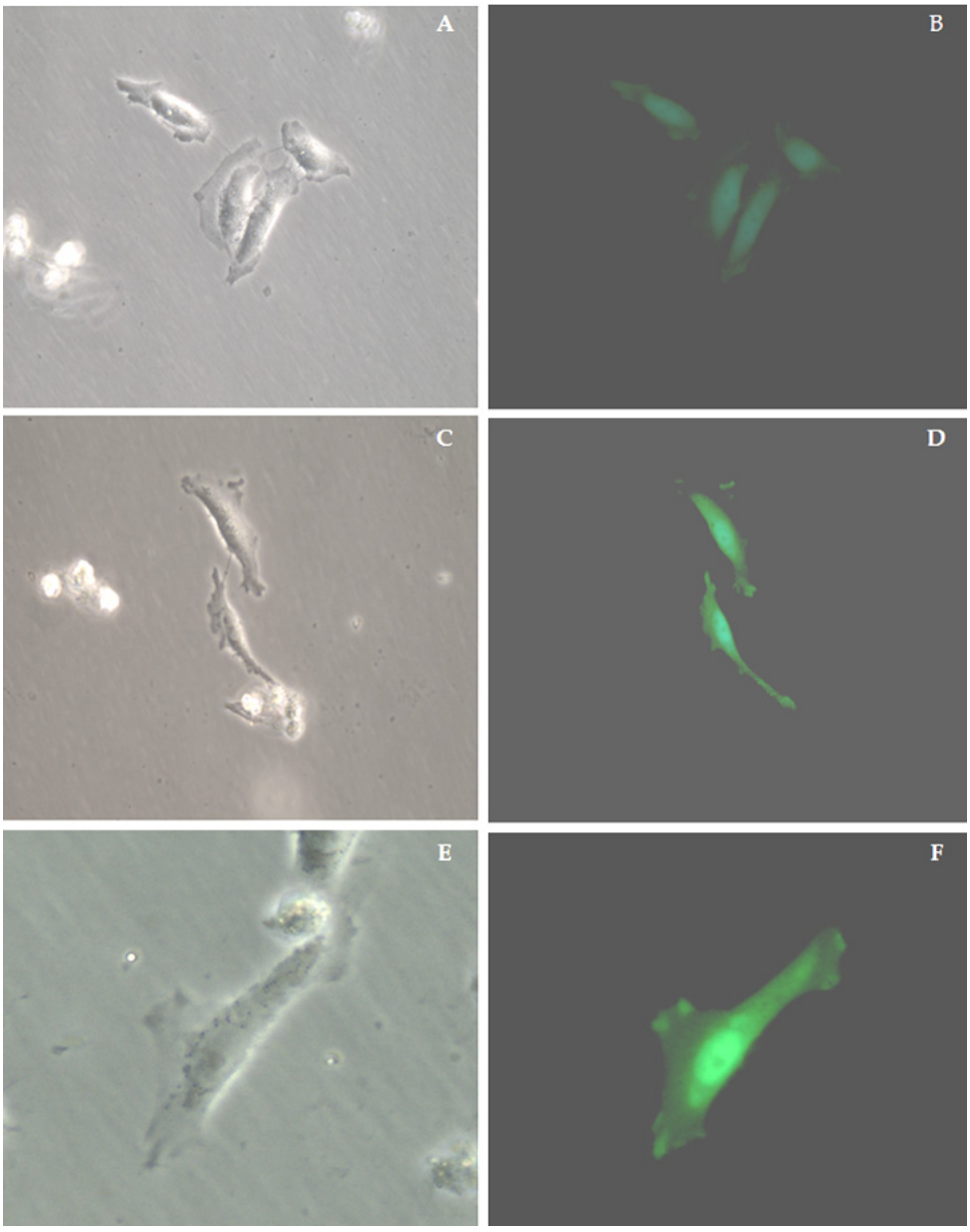


Fig.6. HeLa cells co-transfected (electroporation) with pSV3-neo vector containing SV40-T antigen and pEGFP-C1 vector, used as control. A, C, E: 24 h post tranfected HeLa cells under phase contrast microscopy, and B, D, F: the same cells under fluorescence microscope, indicating GFP expression from pEGFP-C1 vector. A, B, C, D: in 20x and E, F: 40x magnification respectively.

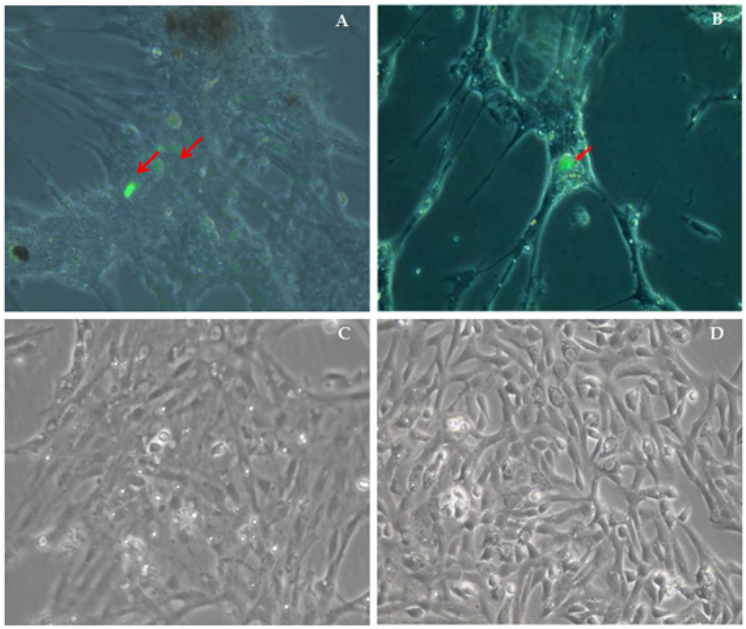


Fig.7. Lymphoid cells co-transfected (lipofection) with pSV3-neo vector containing SV40-T antigen and pEGFP-C1 vector. A, B: 24 h post tranfected cells which show aggregated cell mass and phenotypically different lymphoid cells; C: lymphoid cells treated with lipofection reagent (Cellfectin) without vectors, and D: control cells without any treatment. Arrow indicates expression of GFP from co-transfected pEGFP-C1, confirming successful transfection and expression of foreign gene (20x magnification).

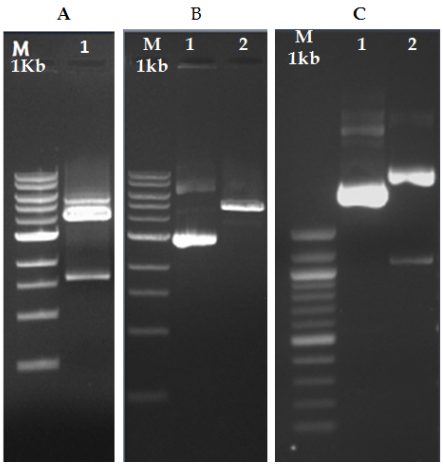


Fig.8. Agarose gel showing A: 12S E1A oncogene restriction digested from pWZL hygro 12S E1A vector with *Bam*H I enzyme, B: 1-Un cut pEGFP-N1 vector, 2- Restriction digested pEGFP-N1 vector with *Bam*H I enzyme and C: 1- 12S oncogene ligated with pEGFP-N1 vector, 2- Release of GFP tagged 12S E1A oncogene after restriction digestion with *Sal* I and *Not* I enzymes. M- molecular markers.

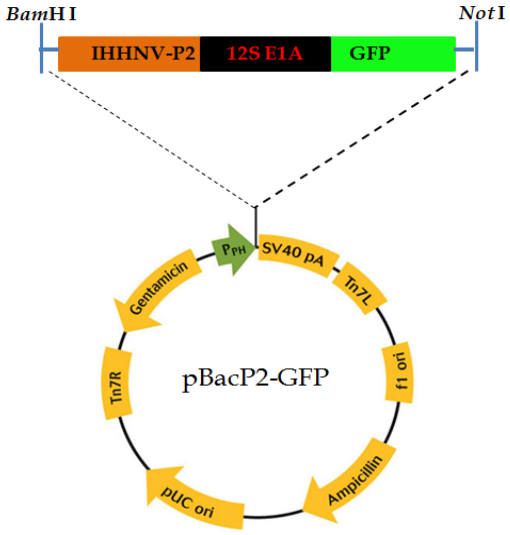


Fig.9. Vector map of pBacP2-GFP with 12S E1A oncogene. This vector was used to generate recombinant baculovirus with hybrid promoter and oncogene tagged with green fluorescent reporter gene. The expression cassette P2-12S E1A-GFP was between *Bam*H I and *Not* I enzyme site.

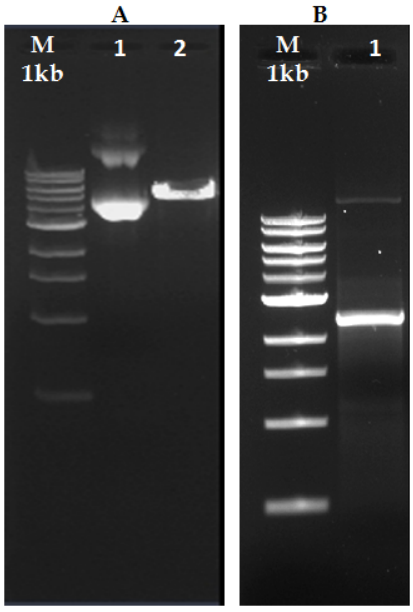


Fig.10. Agarose gel showing A: 1- Uncut pBacP2 transfer vector; 2- pBacP2 transfer vector restriction digestion with *Sal* I and *Not* I enzymes, B: PCR confirmation of GFP tagged 12S E1A oncogene in recombinant bacmid using M13 forward and GFP reverse primers, M: molecular marker of 1-kb size.

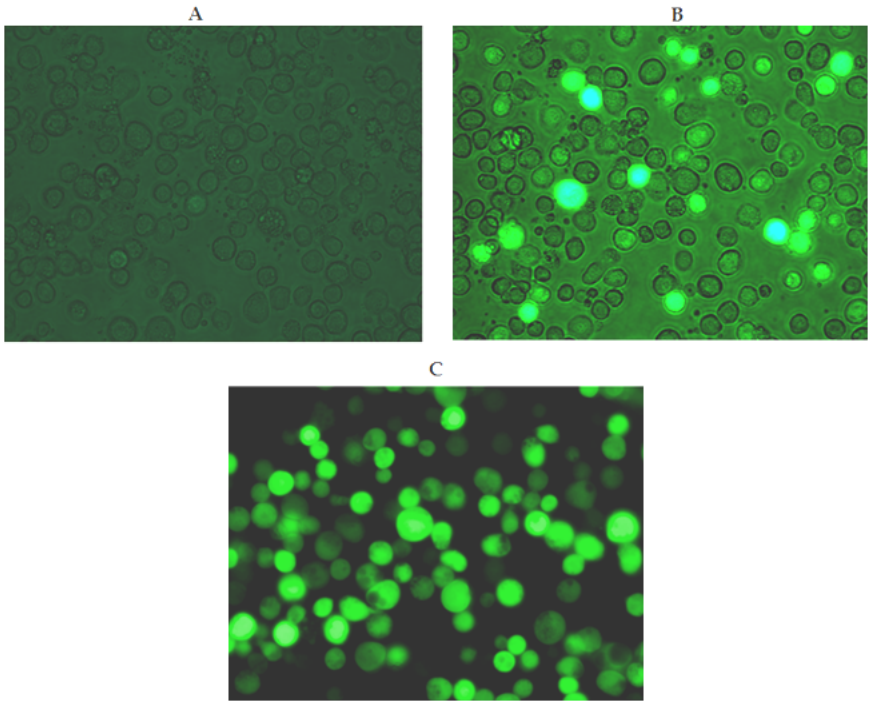


Fig.11. Viral transcription and transduction in Sf9 cells infected with recombinant baculovirus BacP2-12S E1A-GFP containing 12S E1A oncogene. A: 6 h post transduction, B: 12 h post transduction and C: 24 h post transduction. Expression of green fluorescent protein provides evidence of oncogene expression as it is tagged with GFP (40x magnification).

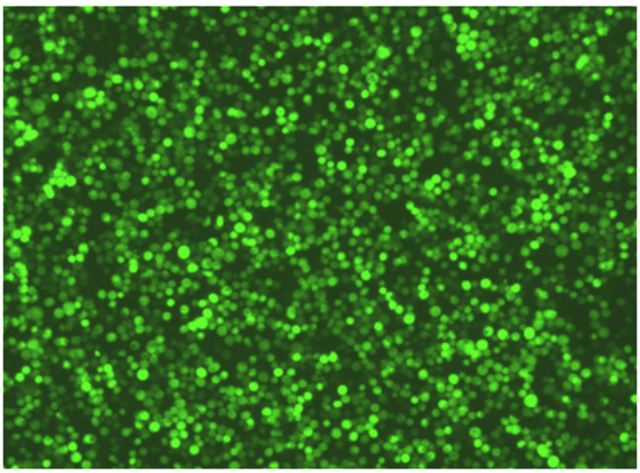


Fig.12. Insect cells (Sf9) transduced with recombinant baculovirus BacP2-12S E1A-GFP containing 12S E1A oncogene. 100% transduction efficiency within 32 h of post infection observed. Images taken by fluorescent microscope (10x magnification).

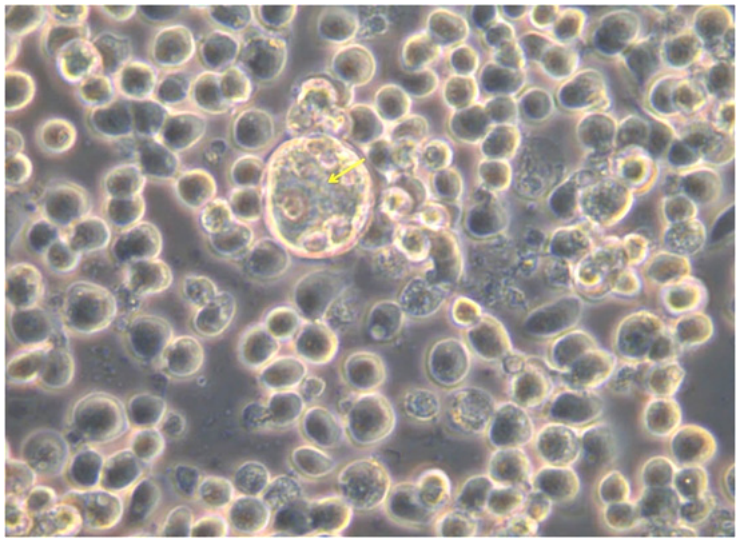


Fig.13. Insect cells infected with recombinant virus. Arrow indicates the presence of viral occlusion bodies inside the 12 h of post infected Sf9 cells (40 x magnifications).

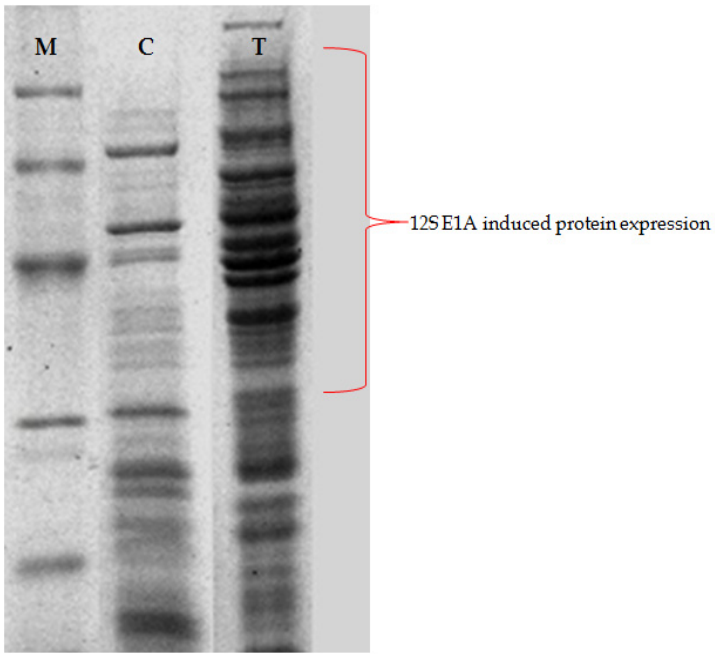


Fig.14. SDS-PAGE analysis of 12S E1A oncogene induced protein expression in Sf9 cells infected with recombinant baculovirus vector BacP2-12S E1A-GFP containing oncogene: T, M: molecular marker, and C: control cells without infection.

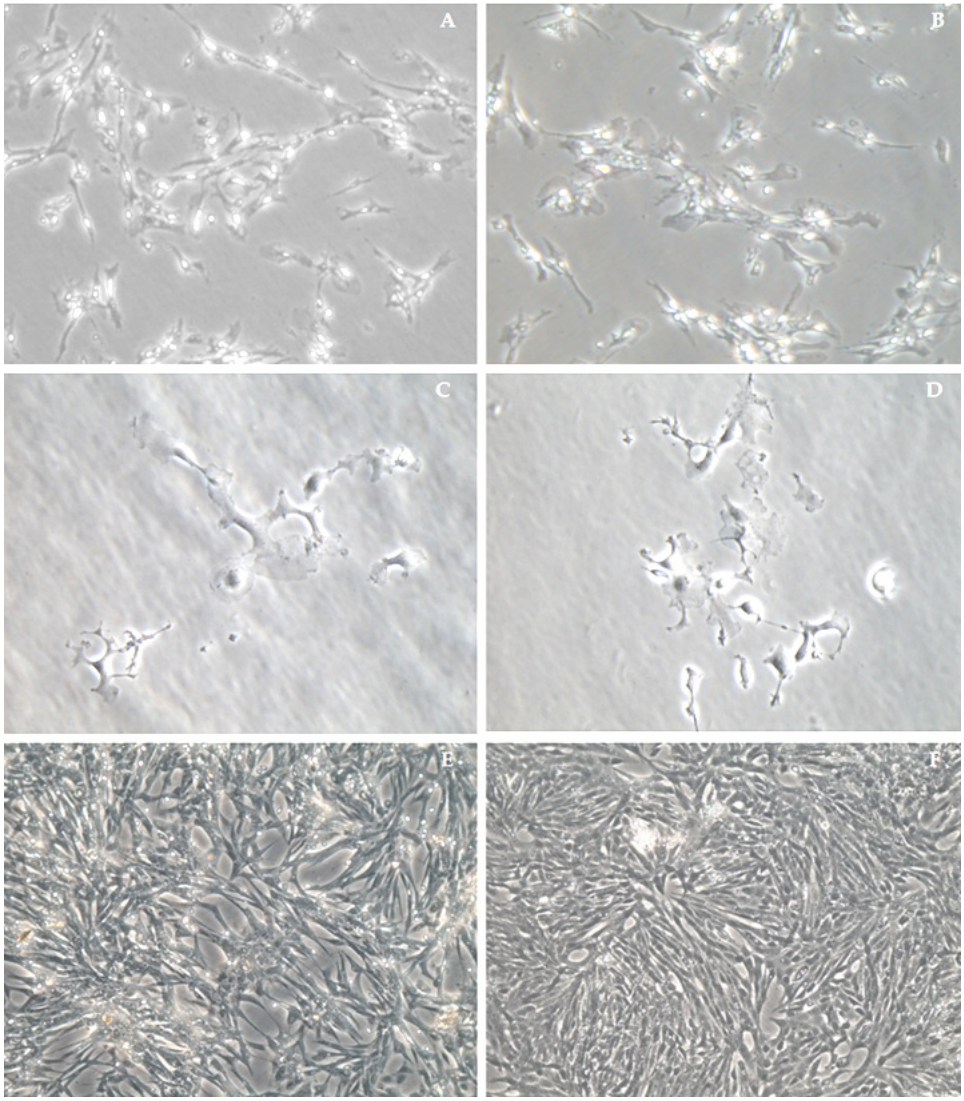


Fig.15. Transduced lymphoid cells with BacP2-12S E1A-GFP recombinant baculovirus containing 12S E1A oncogene showing phenotypic variation. A, B, C, D: the transduced lymphoid cells, and E, F: control cells (magnification 20x).

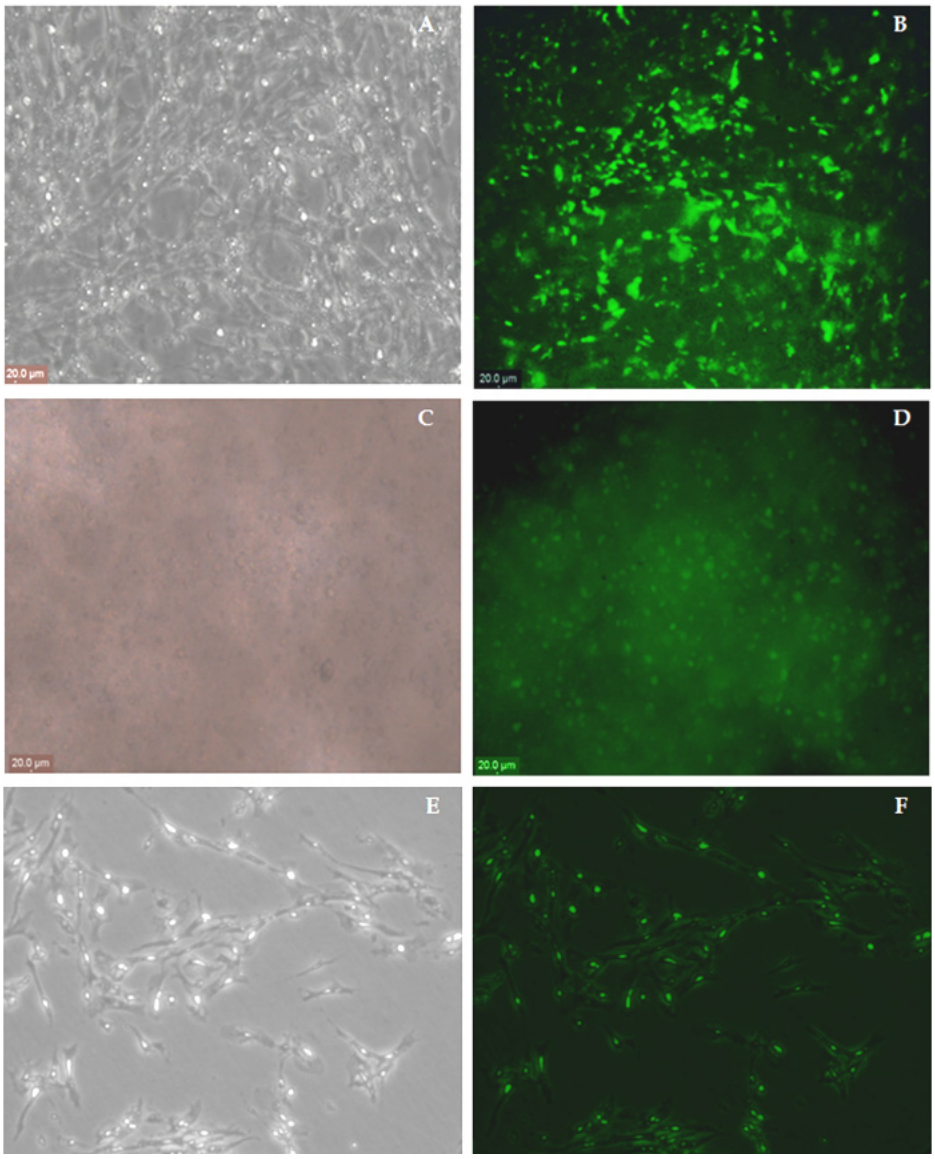


Fig.16. Lymphoid cells from *P. monodon* transduced with recombinant baculovirus BacP2-12S E1A-GFP encoding GFP tagged ocogene. A, C, E: Phase contrast image of BacP2-12S E1A-GFP infected lymphoid cell culture from *P. monodon* and B, D, F: the same under fluorescence microscope. A, E: fibroblastic cells in nature C: explant culture. Expression of GFP confirms the viral transcription inside the cells. As the oncogene tagged with GFP at its N terminal, the GFP expression again confirms the successful transduction of oncogene in lymphoid organ cells. The images taken with 14 days post infected cells (magnification 20x).

Chapter 7

Conclusion and scope for future research

Unveiling the molecular and regulatory mechanisms that prevent *in vitro* transformation in shrimp remains elusive in the development of continuous cell lines, with an arduous history of over 25 years (Jayesh *et al*., 2012). Despite presenting challenges to researchers in developing a cell line, the billion dollar aquaculture industry is under viral threat. In addition, the regulatory mechanisms that prevent *in vitro* transformation and carcinoma in shrimps might provide new leads for the development of anti-ageing and anti-cancer interventions in human (Vogt, 2011) and in higher vertebrates. This highlights the importance of developing shrimp cell lines, to bring out effective prophylactics against shrimp viruses and for understanding the mechanism that induce cancer and ageing in human.

Advances in molecular biology and various gene transfer technologies for immortalization of cells have resulted in the development of hundreds of cell lines from insects and mammals, but yet not a single cell line has been developed from shrimp and other marine invertebrates. With this backdrop, the research described in this thesis attempted to develop molecular tools for induced *in vitro* transformation in lymphoid cells from *Penaeus monodon* and for the development of continuous cell lines using conventional and novel technologies to address the problems at cellular and molecular level.

The first chapter of the thesis has dealt with perception and orientation of the efforts made worldwide in establishing cell lines from shrimp and explained the importance of developing lymphoid cell line from *P. monodon*. Subsequently, the development of a novel medium for lymphoid cell culture and the impacts of this

medium on lymphoid cells at cellular and molecular level have been addressed in the second and third chapters. These results highlighted the importance of lymphoid cell culture being selected for testing the telomerase activity (Chapter fourth) and found that the spontaneous immortalization of lymphoid cell *in vitro* would not be possible due to inadequate expression of telomerase; despite telomerase reverse transcriptase gene (*PmTERT*) has been identified from *P. monodon*. This led to the research intended on oncogene transduction and induction of *in vitro* transformation in lymphoid cells using recombinant baculovirus with hybrid promoter system, which were explained in the subsequent chapters (Chapter fifth and sixth). With this background, the thesis was conceptualized and executed focusing on molecular approaches for the development of cell lines from lymphoid organ of *P. monodon* using most recent techniques. However, continuous cell lines have not yet been achieved through this research, despite the generation of 'stable' lymphoid cell culture and the standardization of molecular tools for *in vitro* transformation with a future perspective.

The subject matter in this thesis has been divided under the following heads:

1. A novel medium for the development of *in vitro* cell culture system from *Penaeus monodon*
2. Screening and optimization of growth factors and their potential impacts on lymphoid cell culture: Cellular activity and viral susceptibility
3. Differential expression of telomerase in various tissues and primary lymphoid cell culture, and identification and partial sequencing of telomerase reverse transcriptase (*TERT*) gene in *Penaeus monodon*
4. Construction and evaluation of the versatile recombinant baculoviral vector systems with hybrid promoters designed for the expression of foreign gene in shrimp cells

5. Transfection and transduction mediated oncogene expression in lymphoid cell cultures from *Penaeus monodon* for its *in vitro* transformation

Overall achievements of this work are summarized as given below:

- ✓ Haemolymph composition of *P. monodon* including 16 amino acids, 10 metal ions and 15 fatty acids were quantified, hitherto not recorded in this species.
- ✓ Amino acid components in haemolymph could be determined, and they were aspartic acid (17.24%), threonine (4.6%), serine (7.53%), glutamic acid (12.2%), proline (1.3%), glycine (7.55%), alanine (6.12%), cystine (0.18%), valine (8.35%), isoleucine (5.17%), leucine (9.56%), tyrosine (2.32%) , phenyl alanine (7.69%), histidine (6.84%) , lysine (1.18%), and arginine (0.85%).
- ✓ Haemolymph metal ions and the metal ion composition in seawater (27‰) were analyzed and compared. In haemolymph, the metal ions such as sodium (6784.3±785.8 mg l^{-1}), potassium (524.5±157.9 mg l^{-1}) and calcium (488.8±107.9 mg l^{-1}) were found to be within the range of that of 27‰ seawater which contains 8075.5±260.9 mg l^{-1}, 512.935±73.2 mg l^{-1} and 443.7±63.1 mg l^{-1} of the metal ions respectively.
- ✓ It was identified that, out of the 15 fatty acid in haemolymph, 81.63% were found to be contributed by palmitic acid (16:0), linoleic acid (18.2 ω -6), oleic acid (18.1 ω-9) and stearic acid (18:0). Other fatty acids recorded were capric acid (0.07%) lauric acid (0.17%), myristic acid (1.28%), pentadecyclic acid (0.48%), margaric acid (1.95%), linolenic acid (0.16%), nonadecyclic acid (0.53%), arachidic acid (0.46%), eicosenoic acid (0.34%), eicosadienoic acid (0.34%) and arachidonic acid (2.58%).

- ✓ A novel cell culture medium (seawater based) has been designed and formulated based on haemolymph composition and designated as shrimp cell culture medium (SCCM). Techniques were developed for generating primary cell cultures from the tissue/cells/organ of *P. monodon* using this medium.
- ✓ In SCCM, the lymphoid cell culture could be maintained for 85±9 days during which they showed better proliferation among all the cell types tested and exhibited an increase of 107% growth in comparison with 2x L-15, and 59% and 82% in comparison with modified L-15 and Grace's insect medium respectively (p <0.05).
- ✓ Primary cell culture could be generated from most of the organs/tissues and the cell longevity was 63±6 days for cells derived from ovary followed by that of heart (29±1 days) hepatopancreas (25±5 days) testis 21±3 days, haemocytes 10±3 days, eye stalk 9±2 days, muscle 7±1 days, nerve cord and cells from nauplii 6±1 days.
- ✓ The experiments by addition of organic supplements like vitamin mixture, lipid mixture, citric acid cycle intermediates, nitrogenous base and energy precursors over and above incorporated in the basal medium did not bring forth any enhancement in the attachment of cells, their proliferation and confluence.
- ✓ Shrimp cell dissociation 'cocktail' was designed for passaging the primary cell culture and with this, better survival (40%) of lymphoid cells was observed even after two passages.
- ✓ Screening and optimization of growth factors were performed to select most suitable growth factor for lymphoid cells *in vitro* using Plackett-Burman and Central Composite Design of Response Surface Methodology,

which were hitherto not attempted to screen and optimize growth factors in the media for shrimp cell culture.

- ✓ Insulin growth factor –I and II (IGF-I and IGF-II) were found to be effective for the development and maintenance of lymphoid cell culture with a concentration 100 ng ml^{-1} and 150 ng ml^{-1} respectively.
- ✓ IGF-I and IGF-II induced increase of 24.8% in BrdU incorporation, 0.84% in protein synthesis, 16.5% in mitochondrial dehydrogenase activity and 17.5% in glucose assimilation within 48 h of incubation. Moreover, the glucose assimilation rate was elevated to 53.6% (p <0.05) within 72 h of incubation.
- ✓ Immunofluorescence detection of synthesis phase in lymphoid cells was performed, and found that 24±2% of the cells were in S-phase after 48 h of incubation in SCCM.
- ✓ Cell cycle gene expression profile was determined and found that 19.7% increase in the expression of gene encoding transcription elongation factor in lymphoid cells *in vitro*, while comparing with its tissue counterpart.
- ✓ Studies on organization of F-actin filaments were performed and the petaloid nature of F-actin filaments was observed in the lymphoid organ cells grown in SCCM.
- ✓ Mitotic events in lymphoid cells were recorded and confirmed that the cells were mitotically active. This was achieved by time-lapse imaging.
- ✓ The lymphoid cell culture was found to be susceptible to WSSV and as cytopathic effect shrinkage, rounding, and detachment of infected cells was the common features in the event. This was further confirmed by immunofluorescence detection of viral protein employing monoclonal antibodies (MAb-C38) against WSSV.

- ✓ Telomeric repeat amplification protocol (TRAP) for measuring the telomerase activity in various tissue/ organ/ primary culture from *penaeus monodon* was standardized.
- ✓ Telomerase activity in the primary lymphoid cell culture was found to be inadequate for maintaining telomere repeats at the chromosome ends during continuous cell division.
- ✓ Internal amplification standard (ITAS) as internal control for TRAP assay was designed using *P. aeruginosa* MCCB 103 16S rRNA gene sequence along with TS and CX-ext primers.
- ✓ TTAGG repeats were found to be the canonical telomeric repeats of *P. monodon*.
- ✓ Identification and partial sequencing of telomerase reverse transcriptase gene (*PmTERT*) from *P. monodon* was performed and found 100% sequence similarity with *Daphnia pulex TERT* gene.
- ✓ Green fluorescent protein expressing recombinant baculovirus was constructed and expressed in insect cell line (Sf9).
- ✓ Two recombinant baculovirus transduction vectors (BacIe1-GFP and BacP2-GFP) that carried expression cassettes consisting of gene encoding GFP as a reporter linked to the hybrid promoter either to PH-Ie1 or PH-P2 was successfully constructed and expressed in insect cell line and shrimp cells *in vivo* and *in vitro*.
- ✓ The method of virus transduction in lymphoid cells was standardized using recombinant baculovirus.
- ✓ Optimized the condition for lipofection mediated gene transfer in lymphoid organ cells using Cellfectin (Invitrogen) and the positive signals of green

fluorescence from the reporter green fluorescence protein (GFP) in pEGFP-C1 vector indicated the transfection efficiency.

- ✓ Transfection of SV40-T oncogene in to primary lymphoid organ cells proved the immortalization potency. The transformed cells showed abnormal nucleus and activated mitotic division.
- ✓ Recombinant baculovirus expressing 12S E1A oncogene tagged with GFP was successfully transduced in to lymphoid cell culture and the successful transformation was confirmed by protein expression and GFP expression from the transduced cells.

- **Scope for future research: following have been identified for future research**
  - Oncogene induced up-regulation of cell cycle gene (s) in cells *in vitro*.
  - Application of RACE (Rapid Amplification of cDNA Ends) in characterization of *PmTERT* gene from *P. monodon*.
  - Telomerase activation in lymphoid cells by ectopic expression of *PmTERT* gene.
  - Impact on co-transfection and expression of oncogene and telomerase reverse transcriptase (*TERT*) gene in cells *in vitro*.
  - Epigenetic control in shrimp cells that prevent spontaneous *in vitro* transformation.
  - Development of fusion (hybrid) cell lines from *P. monodon*.

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List of Publications and patent

1. Peer reviewed publications

1. **Jayesh P**, Jose S, Philip R, Singh ISB (2012) A novel medium for the development of *in vitro* cell culture system from *Penaeus monodon*. Cytotechnology DOI 10.1007/s10616-012-9491-9. (*IF - 1.301*)
2. **Jayesh P**, Jose S, Singh ISB (2012) Establishment of shrimp cell culture: perception and orientation. Indian J of Virol. doi 10. 1007 / s 13337-012-0089-9. (*IF- 1.133*)
3. Manjusha K, **Jayesh P**, Jose D, Sreelakshmi B, Priyaja P, Gopinath P, Saramma AV, Singh ISB (2012) Alkaline protease from a non-toxigenic mangrove isolate of *Vibrio* sp. V26 with potential application in animal cell culture. Cytotechnology. doi 10.1007/s10616-012-9472-z (*IF- 1.301*)
4. Jose S, **Jayesh P**, Sudheer NS, Poulose G, Mohandas A, Philip R, Singh ISB (2012) Lymphoid organ cell culture system from *Penaeus monodon* (Fabricius) as a platform for white spot syndrome virus and shrimp immune-related gene expression. J Fish Dis 35: 321-334 (*IF- 2.00*)
5. Jose S, **Jayesh P**, Mohandas A, Philip R, Singh ISB (2011) Application of primary haemocyte culture of *Penaeus monodon* in the assessment of cytotoxicity and genotoxicity of heavy metals and pesticides. Mar Environ Res 71: 169-17 (*IF-2.276*)
6. Narayanan TN, Mary APR, Swalih PKA, Kumar DS, Makarov D, Albrecht M, **Jayesh P**, Anas A, Anantharaman MR (2011) Enhanced Bio-Compatibility of Ferrofluids of Self-AssembledSuperparamagnetic Iron Oxide-Silica Core–Shell Nanoparticles. J Nanosci Nanotech. 11: 1958–1967. (*IF- 1.563*)

2. Papers under preparation

1. **Jayesh P**, Vrinda S, Priyaja P, Rosamma Philip, I.S. Bright Singh Inadequate telomerase activity in lymphoid cell culture from *Penaeus monodon* prevents spontaneous transformation and immortalization. (Under preparation)
2. **Jayesh P**, Rosamma Philip, I.S. Bright Singh. Screening and optimization of growth factors to achieve enhanced growth of lymphoid cell culture from *Penaeus monodon* using Plackett-Burman Design and Response Surface Methodology. (Under preparation)

3. **Jayesh P**, Priyaja P, Rosamma Philip, I.S. Bright Singh. Lymphoid cell culture grown in novel medium: Metabolic activity, transfection ability and virus susceptibility. (Under preparation)

4. **Jayesh P**, Rosamma Philip, I.S. Bright Singh. IGF-I and IGF-II enhance glucose assimilation and induce transdiffentiation in lymphoid cell culture from *Penaeus monodon*. (Under preparation)

3. Book Chapter

Jose S, **Jayesh P**, Philip R, Mohandas A, Singh ISB (2007) Shrimp Cell Culture: A review. In: Singh ISB, Joseph V, Philip R, Mohandas A (eds) Aquaculture and Marine Biotechnology. Cochin University of Science and Technology Press, India, ISBN 81-900724-20, pp 76-89

4. Patent

Patent filed on "Development of a novel cell culture medium SCCM (Shrimp Cell Culture Medium) exclusively for shrimp cell culture BT/BPFC/04/012/2011 PID dated 23.08.2011. Inventors: **Jayesh P**, Seena Jose, Rosamma Philip and I.S. Bright Singh

Indian J. Virol.
DOI 10.1007/s13337-012-0089-9

REVIEW ARTICLE

Establishment of Shrimp Cell Lines: Perception and Orientation

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Received: 3 January 2012 / Accepted: 26 June 2012

Abstract Development of continuous shrimp cell lines for effective investigation on shrimp viruses remains elusive with an arduous history of over 25 years. Despite presenting challenges to researchers in developing a cell line, the billion dollar aquaculture industry is under viral threat. Advances in molecular biology and various gene transfer technologies for immortalization of cells have resulted in the development of hundreds of cell lines from insects and mammals, but yet not a single cell line has been developed from shrimp and other marine invertebrates. Though improved growth and longevity of shrimp cells in vitro could be achieved by using modified growth media this did not make any leap to spontaneous transformation; probably due to the fact that shrimp cells inhibited neoplastic transformations. Oncogenic induction and immortalization are considered as the possible ways, and an exclusive medium for shrimp cell culture and an appropriate mode of transformation are crucial. In this review status of shrimp cell line development and its future orientation are discussed.

Keywords Shrimp cell culture · Culture medium · Cell line · Transformation · Transduction · Immortalization

Introduction

Development of continuous shrimp cell lines has been a challenging task, for a long period of over 25 years. However, it still remains unattained presenting researchers more questions than answers [9, 65]. Till date, no permanent cell line could be made available from marine invertebrates in general [67] and shrimp in particular. The major fall out of the situation is the impediment which it imposes on the isolation of crustacean viruses [15, 16, 43]. The fact is that the requirement of continuous cell lines is so high to investigate the radiating viral threats to shrimp aquaculture [21, 88, 93].

According to 'FAO Status of World Fisheries and Aquaculture, 2010,' in the year 2008, the capture fisheries and aquaculture production of decapods was 10,230 tonnes, corresponding to 41 billion US$ [20, 86]. This trend in production is unlikely to perpetuate, because there are more than 20 [6] among the 1,100 recognized invertebrate viruses [1] now known to occur in shrimps which include nine that pose serious threat to their culture [14], resulting in huge loss to shrimp industry [21, 50, 88]. This highlights the importance of developing shrimp cell lines for their isolation, and to bring out effective prophylactics.

In the realm of cell line development, despite the current advancements in decoding the nutritional requirements of cells in vitro, molecular approaches at genomic level for transformation and immortalization of shrimp cells remain unknown and un-attempted. This might be due to the lack of information on the molecular mechanisms that inhibit neoplastic transformations in shrimp. Besides, tumours have only rarely been observed in the decapod crustaceans [87]. Therefore, a thread bear analysis on the very successful history of insect and mammalian cell line development might open up new vistas for focused research towards establishment of shrimp cell lines. Moreover, uncovering the underlying molecular and regulatory mechanisms of the absence of neoplasia and carcinoma in shrimps might provide new leads for the development of

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Published online: 14 August 2012

anti-ageing and anti-cancer interventions in humans as well [86].

In this review, we try to compile the current status and trends on shrimp cell line research and orient towards the prospects of development of continuous cell lines from penaeids.

History of Shrimp Cell Culture

The earliest attempts on shrimp cell culture development appeared as published document in 1986 by Chen and colleagues from National Taiwan University, Taiwan [9]. They had chosen *Penaeus monodon* as the species of choice from which several cell culture systems could be generated using various tissues and organs. Three years after the first publication in shrimp cell culture in 1989, researchers published an attempt [12] of shrimp cell culture development from *Penaeus penicillatus* and on the same year first report on the susceptibility of primary lymphoid cell culture to monodon-type baculovirus was published [8]. This is considered as the first report on in vitro cultivation of penaeid virus in shrimp cell culture. Although only limited success could be obtained, several researchers commenced attempting to develop cell cultures from various tissues and organs of different penaeid species [13, 19, 28, 35, 39, 44–46, 49, 57, 63, 70, 72, 80, 90], and this included test of their susceptibility to shrimp viruses as well [40, 51, 52, 56]. In 2000, report on the ultra structure of *white spot syndrome virus* (WSSV) grown in primary lymphoid cell culture was published [89], however, its morphogenesis could not be fully elucidated for want of certified shrimp cell lines. Although the morphology and ultrastructure of WSSV have not been fully understood, several characteristics of this virus have emerged in recent years [71]. In addition to the effort on spontaneous transformation and immortalization by continuous maintenance and repeated passage of the cells in vitro and the 'organized neglect' [29] in the process of cell culture development, in the year 1995 researchers attempted to induce transformation in shrimp cells by transfection with oncogene [78]. Accordingly, in 2000 first transgenic expression in shrimp cells could be accomplished [73] followed by the development of vesicular somatitis virus-glycoprotein (VSV-G) pseudotyped retroviral vectors [37] and their successful integration in shrimp primary cell culture genome [36]. However, this also did not lead to immortalization of cell cultures. The lack of success in spontaneous and induced cell line development subsequently paved the way for the attempts on developing fusion cell line [15, 16], but, with little success. More recently, researchers have succeeded in viral gene expression [42], determinations of cytotoxicity and genotoxicity [41, 43], and viral multiplication [26] employing primary cell culture systems developed from different species of penaeids.

Cell Culture Medium: A Stepping Stone for Cell Line Development

Several hindrances stand in the way of the development of shrimp cell lines. One among them is the unsettling fact of an appropriate shrimp cell culture medium. The media used for shrimp cell culture development have been mostly the modified commercially available preparations, despite the fact that the media composition happens to be the most important factor which determines the success of any cell line development [60]. To date, a medium exclusively for in vitro growth of shrimp cell cultures has not been designed, and the fact that an appropriate medium is required to establish shrimp cell lines in tune with the quantum change which the Grace's insect cell culture medium [29–32] has brought about; ever since the publication of Grace's insect cell culture medium, over 500 insect cell lines could be established [53, 76]. Likewise, to formulate an exclusive shrimp cell culture medium, in-depth analysis of the biochemistry of body fluids [64, 74] is the prime requirement. Moreover, to tide over the difficulties in developing a complete medium for shrimp cell culture, attention must be directed towards satisfying the nutritional requirements of each cell type.

Despite the necessity of an exclusive medium for shrimp cell culture several researchers, over decades, have been modifying commercially available media to suit the requirements of shrimp cells in vitro [16, 43, 69]. Among the commercial media used, Leibovitz's-15 (L-15) has been the most popular one for shrimp cell culture. Of the 50 selected publications 32 (64 %), were based on L-15 as the basal medium [2–4, 7–11, 13, 15, 18, 25, 37, 40–42, 46, 51, 56–58, 62–64, 69, 73, 74, 78, 80–83, 89], six (12 %) selected Grace's Insect Medium [26, 27, 49, 63, 82, 89], five (10 %) M199 [28, 39, 48, 74, 82], and three (6 %) MPS [19, 36, 80]. A couple of other media such as Pj-2 [55], NCTC 135 [89], MM Insect medium and TC 100 medium [63], were also tested for the development of cell lines from shrimp (Fig. 1). From the review it is rather inappropriate to point out any medium mentioned above as the most effective one as it has been a personal choice.

Organic and Inorganic Supplements to Improve Growth of Shrimp Cell Cultures In Vitro

Considering the inadequacy of the available growth media several attempts have been made to improvise the composition by adding supplements in isolation as well as in

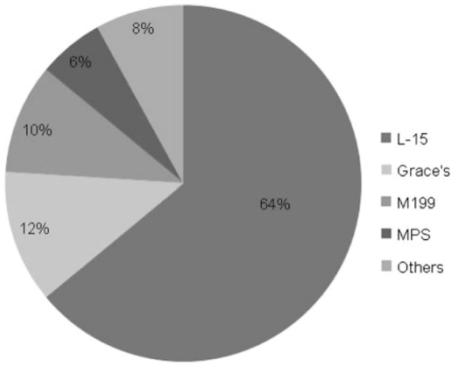

Fig. 1 Growth media used for shrimp cell culture (In % of the 50 selected publications)

multiples. Several investigators selected crustacean body fluids and extracts to improve the basal medium. Among them shrimp extract was the most popular one used in varying concentrations of 4 % [51], 8 % [63, 78], 10 % [8, 26, 27, 82], 27 % [46] and 30 % [9]. Hemolymph of lobsters at 10 % concentration [9] was also used. Moreover, ovary extracts [10] chitosan and nerve nodule extracts [19] were also incorporated in the medium as growth-promoting factors. Fetal bovine serum/fetal calf serum as the supplements with a concentration of 10 % [26, 27, 48, 49, 56, 57], 15 % [2–4], 18 % [9] and 20 % [19, 36, 40–42, 51, 55, 63, 73, 78, 89] were added as the source of minerals, proteins, lipids, hormones [24] and as the growth-promoting substances [59]. Considering the importance of inorganic salts for the maintenance of ionic balance and osmotic pressure [61], researchers have used KCl, $MgSO_4$, $MgCl_2$, and $CaCl_2$ at concentrations ranging from 0.9 to 3 g/l to supplement the required quantity in the growth medium [39, 48, 49]. To adjust osmolality, NaCl at a concentration ranging from 6 to 12 g/l [9, 19, 40, 49] has also been added besides the balanced salt solutions [40, 78].

Addition of vitamins [41, 42], proline [49, 56, 57, 82] and glutamine [28, 82] has been proven to be the choice of supplements in the growth media. In addition, lactalbumin hydrolizate at a concentration of 0.1–1 g/l [2–4, 39, 48, 55–57], tryptose phosphate broth at 2.95 mg/ml [41, 42] and TC Yeastolate at 1 g/l [56, 57] have also been used as the source of peptides, amino acids and carbohydrates. As the additional energy source 0.3–2 g/l glucose [40–42, 55–57] and 0.55 g/l sodium pyruvate [19] have also been supplemented. Buffering agents such as HEPS [28, 48, 82] and $NaHCO_3$ have been incorporated by many researchers [19, 28, 48, 49]. Growth factors such as epidermal growth factor at a concentration 20–30 ng/ml [51, 63, 78] and 10 units/ml of human recombinant interleukin-2 [78] have been used to improve the proliferation of cells in vitro. All these modifications have led to improvisation of growth media with enhancement in growth and multiplication of primary cell cultures, but have never lead to the establishment of any cell line.

Species of Choice: A Major Concern

Since the first attempt on shrimp cell line development, performed in 1986 by Chen et al. [9], *P. monodon* remained the best sought after candidate species among all penaeids in the development of cell cultures; may be due to its availability in all South East Asian Countries and its popularity as the most widely cultured species. Of the 50 selected publications, 17 reported (34 %) *P. monodon* [2–4, 7, 8, 10, 15, 23, 35, 41, 42, 44, 58, 69, 83, 89, 90] as the species of choice, eight researchers (16 %) used *Penaeus japonicus* [10, 39, 47, 48, 55–57, 72], seven (17 %) selected *Penaeus chinensis* [13, 19, 36–38, 40, 80] and *Penaeus vannamei* [18, 26, 27, 49, 51, 63, 82]. Moreover, six authors (16 %) selected *Penaeus stylirostris* [49, 51, 63, 73, 74, 78] as the donor animal of tissues and organs. Besides, in two publications (4 %) *Penaeus indicus* [46, 82] and *Penaeus aztecus* [18, 64] were the species used. There is only one report (2 %) of using *P. penicillatus* [10] for extracting tissues and organs for cell culture development (Fig. 2). This indicated that the species selection was based on availability and personal choice and not on the basis of any advantage which one might obtain by selecting a species.

Tissues and Organs used for Cell Culture Development

Ovary and the lymphoid were the most commonly used donor tissues for cell culture development. Of the 90 selected experiments with 15 different tissues, 20 were conducted with lymphoid [3, 4, 7, 8, 10, 12, 35, 37, 39, 47, 48, 51, 52, 63, 64, 78–80, 89, 90] and 18 with ovary [9–11, 23, 27, 36, 48, 49, 56, 57, 62, 63, 73, 74, 80–82, 90]. Ten experiments were with hemocytes [10, 15, 18, 26–28, 39–42], four with eyestalk [27, 46, 62, 80]. Besides, testis [62, 81], heart [9, 10, 48, 62, 80], hepatopancreas [9, 28, 48, 55, 58, 62, 64, 81, 89], gill [9, 62], nerve [9, 13, 48, 62, 80, 81], muscle [9, 27, 48], hematopoietic tissue [11, 62, 90], embryonic tissue [19, 80, 82], epidermis [81, 82], gut [9, 62] and Y organ [81] were also widely used for cell culture development (Fig. 3). Among the tissues used the most advancement was obtained from lymphoid and ovarian tissues only.

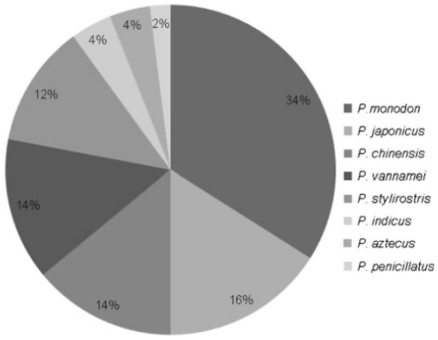

Fig. 2 Penaeid species used for cell culture development (In % of the 50 selected publications)

Longevity and Sub-culturing of the Cell Cultures

The ultimate objective of every shrimp cell culture development programme is the establishment of corresponding cell lines. However, this objective has not been achieved so far. Although unable to be sub cultured, various researchers could maintain cell cultures for different duration. Accordingly, researchers could maintain ovarian cell culture for 66 days [27], 45 days [57], 20 days [10], 10 days [49] and to several months [80, 82] along with single passage [62] and 3 passages [9, 10]. Lymphoid cell cultures were reported to be passaged 2 times [8], 3 times [10], and maintained for 54 days [39], 20 days [10] and for a period

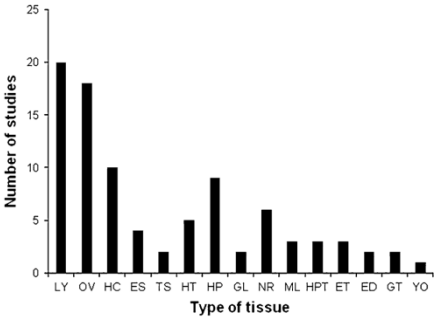

Fig. 3 Various tissues used for shrimp cell culture development. *LY* lymphoid, *OV* ovary, *HC* haemocytes, *ES* eye stalk, *TS* testis, *HT* heart, *HP* hepatopancreas, *GL* gill, *NR* nerve, *ML* muscle, *HPT* haematopoeitic tissue, *ET* embryonic, *ED* epidermis, *GT* gut, *YO* Y organ (results from 90 experiments)

greater than 3 weeks [63] to 3 months [80]. However, Hsu et al. [35] claimed to have attained more than 90 passages for a lymphoid cell culture which was later reported as Thraustochytrid contamination by Rinkevich [68]. At the same time Tapay et al. [78] reported to have attained even 44 passages of lymphoid cell culture. With eye stalk cell culture several workers reported to have maintained them for 12 days [27], 3 months and attained 4 passages [46]. Besides, haemocyte cultures were maintained for 48 days [27], 20 days [40], 10 days [39], 8 days [41, 42], and 4 days [10]. Embryonic cell cultures could be maintained for several months [82] and attained 10 passages [19]. Moreover, researchers could maintain nerve cells from 15 days [13] and to up to 3 months [63], heart tissue for 4 days [10] and hepatopancreas for 30 days [27]. The striking observation was that there existed no consistency in the number of days which a cell culture could be maintained by different workers.

Virus Susceptibility in Various Cell Culture Systems Developed from Shrimp

Penaeid lymphoid organ cell culture system has been claimed as the best option for in vitro growth of several pathogenic viruses. Many researchers claimed the in vitro growth of monodon-type baculovirus in lymphoid cell culture from *P. monodon* [7, 8]. Susceptibility of Yellow head virus in lymphoid cell culture from *P. monodon* [2, 4, 10, 79], *P. japonicus* and *P. penicillatus* [10], and from *P. vannamei* [51, 52] have been reported. Moreover, Lu et al. [52] suggested the in vitro growth of yellow head virus in cell culture from nine different tissues and organs including gill, hepatopancreas, head soft tissue, abdominal muscle, eyestalk, lymphoid organ, heart, nerve cord, and midgut. Susceptibility of WSSV in lymphoid cell culture from *P. monodon* [89], from *P. monodon, P. japonicus* and *P. penicillatus* [10], ovarian cell culture from *P. japonicus* [56], hepatopancreatic cell culture from *P. monodon* [83] haemocytes from *P. chinensis* [40] have also been reported. Recently, Jose et al. [42] conducted a detailed investigation on the viral titration and viral gene expression in *P. monodon* haemocyte culture. Still more recently, George et al. [26] investigated the multiplication of taura syndrome virus in haemocytes from *P. vannamei*. Inspite of the successful attempts by several researchers to grow a few shrimp viruses in cell culture systems from penaeids, strangely enough, there has not been any attempt by other laboratories either to validate the methodology or to uses them as the protocol for shrimp virus cultivation. However, with the available techniques it is possible to generate and maintain primary cell cultures from shrimp and use them for virus titration and viral gene expression.

Molecular Approaches for Shrimp Cell Immortalization

Given the tremendous advancements in human and veterinary virology thanks to the availability of a variety of cell lines, any radical change in crustacean virology would be possible only if appropriate cell lines for in vitro cultivation of intracellular pathogenic agents [17] could be made available. Considering the past experience in this realm more focus should be on the molecular approaches to immortalize shrimp cells by disrupting cell cycle regulator genes and the telomere maintenance.

Usually somatic cells do not spontaneously immortalize in culture, but instead enter replicative senescence after a finite number of population doublings [33, 34]. In contrast to mammals and most insects, decapod crustaceans can enlarge their organs in the adult life period and regenerate lost appendages, organs with indeterminate growth [86]. The high regeneration capability of the crustacean cells (including shrimp) do not show neoplastic transformation and thus it prevents spontaneous immortalization. Neoplastic transformation can be achieved by transfection with active oncogenes [66], the technique which has not yet been fully applied to crustacean and aquatic invertebrate cells [17]. Moreover, unveiling the molecular and regulatory mechanisms that prevent neoplastic transformation in shrimp cells (decapod crustaceans) might provide new leads for the development of anti-ageing and anti-cancer interventions in humans [86].

To date, oncogenic mammalian virus gene, simian virus 40 large T antigen [36, 37, 78] has only been used for transformation of primary shrimp cell culture. The first transformation attempt in lymphoid organ primary cell culture of *P. stylirostris* was made in 1995 [78] with the pSV-3 neo plasmid vector encoding SV40-T antigen gene from Simian virus-40 by lipofection. Further, retroviral vectors pseudotyped with the envelop glycoprotein of VSV was proved to be infective to primary cell cultures from *P. stylirostris* [73], however, without any direct evidence of integration. Even though, researchers [36, 37] proved the use of VSV-G pseudotyped pantropic retroviral vectors by confirming the stable expression of SV40T gene in post transfected cells, the attempts failed to induce in vitro transformation. Moreover, Claydon and Owens [17] transfected human papillomaviruses (HPV) E6 and E7 genes into the *Cherax quadricarinatus* cells by lipofection and the successful transfection was demonstrated by the presence of oncogene mRNA by RT-PCR. While transfection of the oncogenes was successful and transfected cells survived more than 150 days, cell proliferation was stagnant due to the lack of telomere maintenance.

Telomerase activity in cultured cells is a limiting proliferating factor, as inactivation of pRb and p53 pathways [77] in combination with activation of a telomere maintenance mechanism is suggested to be necessary for immortalization of somatic cells [5, 84]. Ablation of cell cycle checkpoint genes through mutation or viral oncogene expression is necessary to lead escape from senescence, additional doublings, and entrance into crisis phase, and finally the emergence of immortal clones. In the vast majority of cases, telomerase is reactivated and telomeres are stabilized [22]. Moreover, researchers proved that the introduction of telomerase activity in normal human cells caused an extension of replicative life span [5, 75, 85]. In our study (un-published data) we could not find any telomerase activity in primary lymphoid cell culture using telomeric repeat amplification protocol assay. Even though, this is contradictory to the reported active telomerase activity in cultured lymphoid organ cells for up to 30 days [47], till date, no additional report has been seen in literature to confirm the telomerase activity in the cultured shrimp cells.

As spontaneous and induced transformation of somatic penaeid cells has not taken place [15], attempts to create hybrid cells by fusing cells from an immortal cell line of insects (*Epithelioma papulosum cyprinid* and *Spodoptera frugiperda*) with haemocytes from *P. monodon* were made and accordingly three fusion-cells could be produced (F11, F12 and F13). However, shrimp genes and viral susceptibility could not be observed in the fusion-cells; this happens to be the first attempt to produce hybrid cells from shrimp cells.

25 Years of Futile History of Cell Line Development: What Went Wrong and What Could be Done?

The 'futile attempts' in shrimp cell line development might be the outcome of the neglect on 'know your animal' [54] philosophy, as the successful history of insect cell lines started from the in-depth knowledge gained on the insect biochemistry with which an appropriate and exclusive insect cell culture medium could be developed [91, 92]. Despite the modification of commercially available medium based on hemolymph analysis [18, 74] an exclusive medium for the growth and development of shrimp cells in vitro has not been accomplished. Even though, Wyatt [91] was not totally successful, her contribution was essential to Grace's ultimate success in the development of Grace's insect cell culture medium [30–32] which resulted in the development of over 500 insect cell lines [53, 54]. Such a scientific temper should be imbibed in the shrimp cell culture research for successful development of a continuous cell line. Moreover, lack of third party validation and confirmation of results achieved by researchers in sister institutions has also hampered the progress of research in

shrimp cell culture development with a diminishing output. The transformation studies include identification of a putative promoter system to construct transformation and transduction vectors specific to shrimp. Development of hybrid cell line might also will pave way for the development of penaeid virus susceptible fusion cell lines. Moreover transgenic expression of oncogene and telomerase reverse transcriptase might also lead to a successful outcome of a valid shrimp cell line.

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Cytotechnology
DOI 10.1007/s10616-012-9491-9

METHOD IN CELL SCIENCE

A novel medium for the development of in vitro cell culture system from *Penaeus monodon*

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Received: 31 March 2012 / Accepted: 11 August 2012

Abstract Lack of a valid shrimp cell line has been hampering the progress of research on shrimp viruses. One of the reasons identified was the absence of an appropriate medium which would satisfy the requirements of the cells in vitro. We report the first attempt to formulate an exclusive shrimp cell culture medium (SCCM) based on the haemolymph components of *Penaeus monodon* prepared in isosmotic seawater having 27 ‰ salinity. The SCCM is composed of 22 amino acids, 4 sugars, 6 vitamins, cholesterol, FBS, phenol red, three antibiotics, potassium dihydrogen phosphate and di-Sodium hydrogen phosphate at pH 6.8–7.2. Osmolality was adjusted to 720 ± 10 mOsm kg^{-1} and temperature of incubation was 25 °C. The most appropriate composition was finally selected based on the extent of attachment of cells and their proliferation by visual observation. Metabolic activity of cultured cells was measured by MTT assay and compared with that in L-15 (2×), modified L-15 and Grace's insect medium, and found better performance in SCCM especially for lymphoid cells with 107 % increase in activity and 85 ± 9 days of longevity. The cells from ovary and lymphoid organs were passaged twice using the newly designed shrimp cell dissociation "cocktail".

Keywords Shrimp cell line · *Penaeus monodon* · Shrimp cell culture medium (SCCM) · MTT assay · Lymphoid cell culture

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Introduction

Attempts on the development of continuous cell lines from shrimps have a long and arduous history amidst the absolute requirement of certified cell lines to address the viral diseases which spelled havoc in shrimp industry. Lack of a cell line has been hampering the progress of research in shrimp viruses especially in the study of viral morphogenesis and in the development of prophylactic and therapeutic measures. The most prominent reason for the non attainment of cell lines from shrimps and for that matter from crustaceans altogether might be the lack of an appropriate medium like Grace's insect cell culture medium which paved the way for the development of over 500 insect's cell lines (Lynn 2001). Considering this necessity, several commercially available media have been modified over decades to suit the requirements of cell cultures (Jose 2009). Among them

Journal : Medium 10616 | Dispatch : 16-8-2012 | Pages : 16
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Leibovitz's—15 (L-15) has been the most popular one subjected for modifications (Chen et al. 1986, 1988; Chen and Kou 1989; Nadala et al. 1993; Lu et al. 1995; Tapay et al. 1995; Tong and Miao 1996; Toullec et al. 1996; Mulford and Austin 1998; Chen and Wang 1999; Shike et al. 2000; Wang et al. 2000; Kumar et al. 2001; Shimizu et al. 2001; Chun-Lei et al. 2003; Maeda et al. 2003, 2004; Jiang et al. 2005; Assavalapsakul et al. 2006; Jose et al. 2010, 2011, 2012) They have been modified by supplementing with growth factors in isolation as well as in multiples (Chen et al. 1986, 1988; Machii et al. 1988; Chen et al. 1989; Luedeman and Lightner 1992; Nadala et al. 1993; Ghosh et al. 1995; Lu et al. 1995; Tapay et al. 1995; Toullec et al. 1996; Mulford and Austin 1998; Chen and Wang 1999; Itami et al. 1999; Shike et al. 2000; Wang et al. 2000; Kumar et al. 2001; Mulford et al. 2001; Fan and Wang 2002; Lang et al. 2002; Maeda et al. 2003, 2004; Jiang et al. 2005; George and Dhar 2010; Jose et al. 2010, 2011, 2012). Meanwhile, Shimizu et al. (2001) reported a modified L-15 medium based on the haemolymph composition with some success in prolonging the longevity of primary cell culture from shrimp. More recently, Jose et al. (2010) modified L-15 (2x) by supplementing it with 2 % glucose, MEM vitamins (1×), tryptose phosphate broth (2.95 mg ml^{-1}), 20 % FBS, and 0.2 mM N-phenylthiourea. Mulford et al. (2001) revealed that 2 × Leibovitz's medium supplemented with 10 % (v/v) heat inactivated FBS, 5 % (v/v) muscle extract, 0.06 g l^{-1} of L-proline, 1 g l^{-1} glucose prepared in 25 ‰ seawater was effective for cell migration, survival and longevity. Though the hemolymph composition of *P. aztecus* and *P. stylirostris* had been reported earlier (Najafabadi et al. 1992; Shimizu et al. 2001), a medium exclusively for shrimp cell culture based on it could not be attained so far, other than the modification of the existing media. This might be cited as one of the reasons for the non attainment of immortal cell line from shrimp. In this context we made an attempt to develop seawater based cell culture medium exclusively for shrimp cell culture and named it as shrimp cell culture medium (SCCM). Experiments were carried out using various tissues from *P. monodon* for determining its suitability to develop cell cultures. Primary cell cultures developed by employing this medium from lymphoid and ovarian tissues could be sub cultured twice using shrimp cell dissociation cocktail developed in this study.

Materials and methods

Design of the experiment

The whole experiment was designed to formulate a medium exclusively for shrimp cell culture. The haemolymph components of *P. monodon,* the free amino acids, fatty acids and metal ions were used as background information about the physiological conditions required for in vitro growth of cells. Seawater and artificial seawater were screened for suitable base for the medium. Physical observation was carried out to screen the most suitable combinations initially and further confirmations were done based on MTT assay.

Experimental animals

Shrimps required for the experiments were maintained in Recirculating Aquaculture System (RAS) integrated with nitrifying bioreactor (Kumar et al. 2009) maintained at 27 ‰ salinity. Post larvae, nested PCR negative to white spot syndrome virus (WSSV), were stocked in the system and reared for 3 months, maintaining the water quality parameters within a narrow range (pH 6.8–7.8; total ammonia–nitrogen <0.1 mg l^{-1}; nitrite–nitrogen < 1.0 mg l^{-1}; total alkalinity ($CaCO_3$) 75–125 mg l^{-1}; total hardness 5,000–6,000 mg l^{-1}) and fed pelleted feed (Higashimaru). Shrimps weighing 15–20 g were used as the donor animals for various tissues besides nauplii directly collected from a seed production centre.

Analysis of haemolymph

Collection of haemolymph

For free amino acid and fatty acid analysis, haemolymph was withdrawn aseptically from rostral sinus using capillary tubes containing 100 µl of 10 % sodium citrate (Jose et al. 2011) and the total volume of each sample was measured to calculate the dilution factor (Shimizu et al. 2001). Pooled haemolymph from 20 animals weighing 20–30 g were centrifuged at 1,000 g for 10 min to remove haemocytes, lyophilized the plasma and stored at −20 °C. Haemolymph was collected without anticoagulant also for metal ion analysis. Osmolality of the haemolymph was measured immediately after collection using Fiske 1–10 Osmometer (Fiske Associates, USA).

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Analysis of free amino acids

Aliquot of 160 µg pooled lyophilized haemolymph was collected in a test tube and added 10 ml 6 N HCl. The test tube was filled with nitrogen, sealed and kept at 121 °C for 24 h. The hydrolysed sample was filtered and flash evaporated repeatedly adding distilled water until the traces of chlorine were removed. The residue obtained was made up to 10 ml with 0.05 M HCl.

Samples were filtered through a polyvinylidene fluoride membrane filter (PVDF, Millipore) of 0.45 µm pore size and injected 20 µl to an amino acid analyzer (HPLC-LC 10 AS) equipped with cation exchange column packed with a strongly acidic cation exchange resin, styrene divinyl benzene copolymer, with sulphonic group. The column used was Na type, ISC-O7/S 1504 Na, having a length of 19 cm and diameter 5 mm. The instrument was equipped with Shimadzu FL 6A fluorescent detector and Shimadzu CR 6A Chrompac recorder. A gradient mobile phase was applied with buffer A and buffer B for the effective separation of amino acids. The oven temperature was maintained at 60 °C. The total run was programmed for 62 min. The amino acid analysis was done with non-switching flow method and fluorescence detection after post-column derivatization with o-phthaldehyde. In the case of proline and hydroxyproline, imino group was converted to amino group with hypochlorite. Amino acid standard (Sigma chemical Co., St. Louis, USA) was also run to calculate the concentration of the amino acids in the sample. The amount of each amino acid was expressed as µmol ml^{-1} haemolymph (Antoine et al. 1999).

Analysis of fatty acids

For fatty acid analysis, gas chromatograph with flame ionization detector (GC-FID) was employed (Agilent Technologies, model 6890). The pooled and lyophilized haemolymph was converted into fatty acid methyl esters (FAMEs) by saponification, methylation and extraction (Carvalho and Malcata 2005) into hexane: methyl *tert*-butyl ether. A 25 m (length) × 0.2 mm ID × 0.33 µm film thickness, cross linked 5 % phenylmethyl silicone fused silica capillary column was used to separate the fatty acids. While operation the initial temperature of 170 °C was increased to 310 °C at the rate of 40 °C min^{-1} and held for 1.5 min. Hydrogen was used as the carrier gas at a constant flow rate of 1.3 l min^{-1}. The peaks were analyzed using the software Sherlock (MIDI, Inc., USA) to identify the relative amounts of fatty acids in the sample and were expressed as percentage of the total fatty acids. Identification of the peaks was accomplished by comparison of retention times to those of authentic standards.

Analysis of metal ions

As the analysis of major ions in the hemolymph is crucial to formulate cell culture medium (Najafabadi et al. 1992), metal ion strength of haemolymph and seawater at 27 ‰ salinity were analyzed using Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES Thermo Electron IRIS INTREPID II XSP DUO). Before analysis, seawater was sterilized by autoclaving at 15 lbs for 15 min, filtered through Whatman No. 1 filter paper to remove precipitates. Lyophilized haemolymph (100 µg) from different age group of *P. monodon* was diluted with Milli-Q water to the required volume for analysis (Shimizu et al. 2001; Huang et al. 1999). Identification of the elements was accomplished by comparison with authentic standards (Merck, Germany).

Formulation of shrimp cell culture medium (SCCM) base composition

All chemicals used for the preparation, unless specifically stated otherwise, were purchased from Sigma-Aldrich, USA. The Shrimp Cell Culture Medium contained (mg l^{-1} in artificial/natural seawater) L-alanine 70, L-arginine 45, L-asparagine 15, L-aspartic acid 10, L-cystine 1, L-cysteine 1, L-histidine 15, L-leucine 20, L-lysine 60, L-isoleucine 10, L-methionine 5, L-phenyl alanine 10, L-proline 100, L-serine 15, L-taurine 100, L-threonine 15, L-tryptophan 15, L-glutamine 150, L-glutamic acid 10, glycine 20, L-tyrosine 80, L-valine 20, choline bitartarate 1.8, D-pantothenic acid (hemicalcium) 1, folic acid 1, myo-Inositol 2, pyridoxal-HCl 1, riboflavin 0.1, thiamine 1, niacinamide 1, glucose 1,000, ribose 10, trehalose 10, sodium pyruvate 500, potassium dihydrogen phosphate 2, di-sodium hydrogen phosphate 11.5, cholesterol 0.2, and phenol red 0.01. This composition was considered as the base for SCCM to which additional ingredients such as antibiotic mixture and fetal bovine serum (FBS) were added as

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described elsewhere, and the efficacy was evaluated through development of cell cultures and their subsequent visual observation under inverted phase contrast microscope (Leica DMIL) connected with CCD camera (Leica DFC 420C).

Artificial seawater and natural seawater as liquid base

Based on the report of Dall (1981), the isosmotic point of *P. monodon was* identified equivalent to 27 ‰ salinity. Natural seawater at 27 ‰ salinity having 810 ± 20 mOsm kg^{-1} osmolality was used for the whole experiment. Seawater was sterilized by autoclaving at 15 lbs for 15 min, filtered through Whatman No. 1 filter paper to remove precipitates and stored in a 20 l container at room temperature. Artificial seawater was prepared by dissolving the following ingredients one after the other: NaCl, 2.3926 g; Na_2SO_4, 0.4 g; KCl 0.0677 g, $NaHCO_3$ 0.0196 g, KBr 0.0098 mg, H_3BO_3 0.0026 g and NaF, 0.0003 g in 75 ml MilliQ water with constant stirring. To this solution, 5.327 ml of 1.0 M $MgCl_2 \cdot 6H_2O$, 1.033 ml of 1.0 M $CaCl_2 \cdot 2H_2O$ and 0.09 ml of 0.1 M $SrCl_2 \cdot 6H_2O$ were added and made up to 100 ml with MilliQ water (Kester et al. 1967). The differential effects of artificial and natural seawater were evaluated by visual observation of the extent of attachment and monolayer formation of primary cell cultures from various tissues using an inverted phase contrast microscope (Leica DMIL).

Effect of inorganic salts and trace elements

To equalize the inorganic salt and trace element concentration of SCCM to those of haemolymph, additional inorganic salts and trace elements were added to the tune of 0.518 mg l^{-1} barium chloride, 124.2 mg l^{-1} copper chloride, 37.38 mg l^{-1} zinc sulphate, 8.28 mg l^{-1} ferric citrate and 0.26 mg l^{-1} manganese chloride. The effect of this modification was evaluated visually by observing the extent of attachment and monolayer formation of the cells using an inverted phase contrast microscope (Leica DMIL).

Effect of organic supplements

Five different mixtures of organic supplements were added to the SCCM and the effect of these supplements were evaluated by the extent of cell attachment and monolayer formation and compared with that of the basal SCCM as control. The following were the various supplements in final concentration added to SCCM.

Supplement A: consisted of a mixture of arachidonic acid 0.02 µg l^{-1}, linoleic acid 0.1 µg l^{-1}, linolenic acid 0.1 µg l^{-1}, myristic acid 0.1 µg l^{-1}, oleic acid 0.1 µg l^{-1}, palmitic acid 0.1 µg l^{-1} stearic acid 0.1 µg l^{-1}, cholesterol 2.2 µg l^{-1}, tween-80 20.2 µg l^{-1}, and tocopherol acetate, 0.7 µg l^{-1}.

Supplement B: consisted of precursors of signal molecules at a concentration, 0.83 mg l^{-1} flavin adenine dinucleotide (FAD-Na_2), 0.55 mg l^{-1} adenosine 5′-triphosphate magnesium salt (ATP), 1.14 mg l^{-1} nicotinamide adenine dinucleotide phosphate (NADP), 0.35 mg l^{-1} adenosine 5′-monophosphate (AMP-Na_2), 0.77 mg l^{-1} Coenzyme A (CoA-Na_2),

Supplement C: consists of precursors of nucleic acid synthesis such as, adenosine 0. 27 mg l^{-1}, guanosine 0.28 mg l^{-1}, cytosine 0.11 mg l^{-1}, thymine 0.13 mg l^{-1}, deoxy ribose 0.13 mg l^{-1}, uracil 0.11 mg l^{-1}, uridine 5′-triphosphate (UTP 0.55 mg l^{-1}).

Supplement D: consisted of Kreb's cycle intermediates which included ketoglutaric acid 0.15 mg l^{-1}, malic acid 0.13 mg l^{-1} and succinic acid 0.12 mg l^{-1}.

Supplement E: consisted of vitamins viz., ascorbic acid 0.01 mg l^{-1}, biotin 0.05 mg l^{-1} nicotinamide 0.01 mg l^{-1}, nicotinic acid 0.01 mg l^{-1}, pyridoxin, 0.01 mg l^{-1} calciferol 0.01 mg l^{-1}, tocopherol 0.01 mg l^{-1}, p-aminobenzoic acid 0.01 mg l^{-1} .

Preparation of shrimp cell culture medium (SCCM)

Different constituents of SCCM were prepared separately and mixed as follows. A 100 × concentration amino acid mixture ("amino mix-I") containing L-alanine, L-arginine, L-asparagine, L-cysteine, L-histidine, L-lysine, L-methionine, L-proline, L-serine, L-taurine, L-threonine, glycine, L-valine was prepared in MilliQ water. The "amino mix-II" was prepared by dissolving 100 × concentration of L-aspartic acid, L-cystine, L-leucine, L-isoleucine, L-phenyl alanine, L-tryptophan, L-glutamic acid, L-tyrosine in 1 N HCl. An aliquot of 100 × concentration of "sugar mix" was prepared by dissolving glucose, ribose, trehalose, sodium pyruvate, potassium dihydrogen phosphate and di-sodium hydrogen phosphate in MilliQ water. "Vitamin mix" was prepared by mixing 100 × concentrations of choline bitartarate, D-pantothenic acid

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(hemicalcium), myo-Inositol, pyridoxal-HCl, thiamine and niacinamide in MilliQ water with 100 × concentration riboflavin and folic acid in 1 M NaOH. Cholesterol was prepared (100x) separately in MilliQ water. Phenol red solution was prepared by dissolving 1 mg phenol red (100x) in 1 ml NaOH (1 M). All the above mixtures were separately filtered through PVDF (Millipore) of 0.22 µm pore size and stored in amber colored bottle at −20 °C till use.

The final SCCM (1,000 ml) was prepared by mixing 10 ml each of amino mix I, amino mix II, sugar mix, vitamin mix, cholesterol and phenol red solution in 100 ml double distilled water, mixed with 685 ml sterile seawater of 810 ± 20 mOsm kg^{-1} osmolality (27 ‰ salinity). An aliquot of 100 ml fetal bovine serum was added, Osmolality of the medium was adjusted to 720 ± 10 mOsm kg^{-1} by adding NaCl and measuring by means of osmometer (Fiske One-Ten Osmometer, Fiske Associates, USA). pH of the medium was adjusted to 6.8 using 1 N NaOH or 1 N HCl, the medium was made up to 1,000 ml with distilled water. Further, the medium was supplemented with antibiotic mixture containing penicillin (100 U ml^{-1}), streptomycin (100 µg ml^{-1}) and chloramphinicol (0.0 6 µg ml^{-1}) and added filter sterilized 150 mg l^{-1} glutamine just before use.

Development of primary cell cultures

Prior to dissection, the juvenile shrimp and nauplii were chilled in ice and surface sterilized by immersion in 800 ppm sodium hypochlorate in ice cold seawater for 10 min. followed by thorough washing in sterile seawater. Lymphoid organ, heart, hepatopancreas, nerve code, eye stalk, muscle, testis and ovary were removed aseptically and collected in holding medium (SCCM without FBS) of 720 mOsm kg^{-1} osmolality. The tissues and nauplii were washed three times with PBS and minced into very small pieces using sterile surgical knife. The clumps of tissue were separated using cell dissociation sieve (CD-1, Sigma) with a 60 mesh screen (Mulford et al. 2001); the suspension was mixed thoroughly with the medium and seeded on 25 mm^2 culture flask/wells (Greiner Bio-One) and incubated at 25 °C. For haemocyte culture, haemolymph was withdrawn aseptically from rostral sinus using capillary tubes containing 100 µl anticoagulant (tris HCl 0.01 M, sucrose 0.25 M, tri sodium citrate 0.1 M) and diluted to obtain 5 × 10^5 cells ml^{-1} using SCCM supplemented with N-phenylthiourea (0.2 mM), 0.06 mg ml^{-1} chloramphenicol, 100 mg l^{-1} streptomycin and 100 IU m ml^{-1} penicillin (Jose et al. 2011) and aliquots of 200 µl were dispensed into the wells of 96 well plates (Greiner Bio-One) and incubated at 25 °C.

MTT reduction assay for measuring cellular metabolism

In spite of the visual observation, mitochondrial dehydrogenase activity was measured as the cell viability and metabolic activity which depended on an intact mitochondrial membrane and the respiratory chain. MTT assay measures the mitochondrial dehyrogenase which reflects the metabolic activity of the cells. Succinate dehydrogenase system which belongs to the mitochondrial respiratory chain reduces MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, Sigma-Aldrich Co.) to insoluble formazan crystals, solubilized in dimethyl sulphoxide (DMSO) yielding a purple-coloured solution (Mosmann 1983). Accordingly, aliquots of 200 µl cell suspension of ovary, lymphoid organ, heart, hepatopancreas and the hemocytes were seeded on to 96 well plates and after desired incubation period (2, 4, 6 and 8 days), the medium was pipetted out and replaced with 50 µl MTT solution (5 mg ml^{-1}) prepared in PBS (720 mOsm kg^{-1}), and the plate was kept for incubation (in dark) at 25 °C for 5 h (Jose et al. 2012). The control consisted of the medium alone with MTT added. Removed the MTT solution and added 200 µl DMSO, (HiMedia Laboratories, Mumbai) mixed well to confirm the dissolution of formazan crystals, absorbance measured at 570 nm in a microplate reader (InfiniteM-200 Tecan, Austria).

Comparison of SCCM with other selected media

The efficacy of SCCM on in vitro growth and viability of cell types from ovary, lymphoid organ, heart, hepatopancreas and the hemocytes was determined by comparing the same with 2 × L-15 with 10 % FBS (v/v), modified L-15 (Jose et al. 2010) and Grace's insect medium with 10 % FBS (v/v). Metabolic activity (viability) of the cultures in vitro was analyzed on the 4th day of culture using MTT assay, and the percentage difference was compared.

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Cell dislodgement and passaging

Cell dislodgement was performed for passaging the primary cell cultures developed from explants of ovary, lymphoid organ, heart, hepatopancreas and the hemocytes. Trypsin (0.25 %, w/v), collagenase type V, accutase, non enzymatic cell dissociation solution-I (prepared in PBS), non enzymatic cell dissociation solution-II (prepared in HBSS without calcium and magnesium) and shrimp cell dissociation 'cocktail' were tested. The 'cocktail' contained 0.25 g trypsin, 0.02 g EDTA, 0.02 g EGTA, 0.04 g polyvinyl pyroline and 0.05 g glucose dissolved in 100 ml PBS (720 mOsm kg^{-1}). pH was adjusted to 6.8 within a range 6.8-7.2 using 1 N NaOH or 1 N HCl and supplemented with antibiotic mixture containing penicillin (100 U ml^{-1}) streptomycin (100 µg ml^{-1}) and chloramphenicol (0.06 µg ml^{-1}), filtered through 0.22 µm pore size polyethersulfone (PES) (Millex GP, Millipore) membrane using syringe filter.

Statistical analyses

The results in the figures are average values of 3–6 replicates ± standard deviation. The effects of treatments were statistically analyzed by single factor and two factor analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$.

Results

Analysis of haemolymph

Amino acid components from haemolymph recorded were aspartic acid (47.02 ± 28.68 mg l^{-1}) threonine (11.51 ± 0.98 mg l^{-1}), serine (13.89 ± 7.72 mg l^{-1}), glutamic acid (92.17 ± 54.19 mg l^{-1}), proline (26.45 ± 13.51 mg l^{-1}), glycine (14.77 ± 6 mg l^{-1}), alanine (26.55 ± 8.56 mg l^{-1}), cystine (1.64 ± 0.27 mg l^{-1}), valine (18.58 ± 10.64 mg l^{-1}), isoleucine (11.16 ± 3.24 mg l^{-1}), leucine (27.3 ± 7.64 mg l^{-1}), tyrosine (11.08 ± 3.42 mg l^{-1}), phenyl alanine (25.11 ± 10.10 mg l^{-1}), histidine (15.63 ± 10.56 mg l^{-1}), lysine (13.33 ± 11.36 mg l^{-1}), and arginine (20.3 ± 17.96 mg l^{-1}).

Fatty acid profile exhibited long chain fatty acids (up to 20-carbon atoms) along with polyunsaturated fatty acids (PUFA) (Table 1). Among the fatty acid components of the haemolymph lipids, 81.63 % were contributed by palmitic acid (16:0), linoleic acid (18.2 ω-6), oleic acid (18.1 ω-9) and stearic acid (18:0). Other fatty acids recorded were capric acid (0.07 %) lauric acid (0.17 %), myristic acid (1.28 %), pentadecanoic acid (0.48 %), margaric acid (1.95 %), linolenic acid (0.16 %), nonadecyclic acid (0.53 %), arachidic acid (0.46 %), eicosenoic acid (0.34 %), eicosadienoic acid (0.34 %) and arachidonic acid (2.58 %).

Of the ten elements analyzed, concentration of copper, zinc, barium, iron and manganese were lower in the seawater (27 ‰ salinity) than in the haemolymph of *Penaeus monodon*. Copper and manganese levels in the seawater were very low (< 0.01 mg l^{-1}) compared to those of the haemolymph, 148.6 ± 34.50 mg l^{-1} and 0.33 ± 0.10 mg l^{-1} respectively. However, the concentration of sodium, potassium, calcium, boron and strontium were more or less the same (Table 2). Concentration of zinc in seawater was 0.035 ± 0.02 mg l^{-1} and iron 0.053 ± 0.01 mg l^{-1}, whilst in haemolymph the concentrations were 39.415 ± 2.88 mg l^{-1} and 12.98 ± 6.63 mg l^{-1} respectively. Moreover, the barium level in seawater (0.015 ± 0.01 mg l^{-1}) was hundred times lower than that of the haemolymph concentration (15.29 ± 14.27 mg l^{-1}). Inorganic salts and trace elements were added to the SCCM as supplements to adjust these differences. Osmolality of haemolymph and 27 ‰ seawater were found to be 730.5 ± 51.2 and 810 ± 20 mOsm kg^{-1} respectively.

Artificial seawater and natural seawater as liquid base

Based on the visual observation of growth and monolayer formation of various primary cell cultures from different tissues and organs (Table 3), it was clear that the artificial seawater was not promising as the liquid base for SCCM in terms of limited proliferation and less attachment of cells compared to natural sea water inspite of the fact that there was chances of variations in the composition of natural seawater from place to place.

Effect of inorganic salts, trace elements and organic supplements

The inorganic salts and trace elements added to equalize the haemolymph metal ion concentration

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Table 1 Fatty acid profile of *P.monodon* haemolymph

S. No.	Retention time (RT)	Carbon atom	Fatty acid	Percentage
1	2.808	10:0	Capric acid	0.07
2	4.302	12:0	Lauric acid	0.17
3	6.682	14:0	Myristic acid	1.28
4	8.166	15:0	Pendadecylic acid	0.48
5	9.782	16:0	Palmitic acid	29.30
6	11.476	17:0	Margaric acid	1.95
7	13.208	18:0	Stearic acid	13.84
8	12.807	18.1 ω-9	Oleic acid	17.96
9	12.719	18.2 ω-6	Linoleic acid	20.53
10	12.485	18.3 ω-6	Linolenic acid	0.16
11	14.939	19:0	Nonadecyclic acid	0.53
12	16.651	20:0	Arachidic acid	0.46
13	16.250	20:1 ω-9	Eicosenoic acid	0.34
14	16.184	20.2 ω-6	Eicosadienoic acid	1.31
15	15.605	20.4 ω-6	Arachidonic acid	2.58

Table 2 Comparison of the metal ion composition of *P. monodon* haemolymph with *P. stylirostris*, *P. aztecus* and seawater (27 ‰)

S. No	Parameter analyzed	Seawater 27 ‰ (mg l^{-1})	Concentration in haemolymph (mg l^{-1})		
			P. monodon	*P. srylirostris* (Shimizu et al. 2001)	*P. aztecus* (Najafabadi et al. 1992
1	Sodium	8,075.5 ± 260.92	6,784.34 ± 785.83	8,411.1 ± 549.7	6,188.00 ± 795.6
2	Potassium	512.935 ± 73.16	524.535 ± 157.88	328.44 ± 50.8	281.52 ± 62.56
3	Calcium	443.72 ± 63.10	488.84 ± 107.93	439.2 ± 44.3	410 ± 70
4	Copper	BDL (<0.01)	148.595 ± 34.50	81.6 ± 13.9	
5	Zinc	0.035 ± 0.02	39.415 ± 2.88	11.4 ± 1.9	
6	Barium	0.015 ± 0.01	15.29 ± 14.27	NA	
7	Iron	0.053 ± 0.01	12.98 ± 6.63	0.1 ± 0.0	
8	Strontium	7.05 ± 0.44	7.27 ± 1.90	6.3 ± 0.6	
9	Manganese	BDL (<0.01)	0.325 ± 0.10	NA	
10	Boron	4.87 ± 0.65	8.42 ± 7.68	0.4 ± 0.3	
11	Osmolality (mOsm kg^{-1})	810 ± 20	730.5 ± 51.2	784 ± 36	625 ± 46

were found to have negative effect on growth and monolayer formation of various cell types (Table 3). Moreover, the experiments by addition of organic supplements like vitamin mixture, lipid mixture, citric acid cycle intermediates nitrogenous base and energy precursors over and above incorporated in the basal medium did not bring forth any enhancement in the attachment of cells, their proliferation and confluence.

Preparation of shrimp cell culture medium (SCCM)

The base composition of SCCM was finalized by conducting a series of experiments by trial and error method (data not shown) by incorporating different permutation combination of amino acid mix I and II, sugar mix, vitamin mix, cholesterol, FBS and pH.

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Table 3 Effect of different combinations of shrimp cell culture medium (SCCM) on primary cell culture of ovary (OV), lymphoid organ (LY), heart (HT), hepatopancreas (HP), hemocytes (HC), Nerve cord (NC), Testis (TS), nauplii (N), eyestalk (ES) and muscle (MS)

Exp. No	Media combinations pH 6.8, osmolality 720 ± 10 mOsm kg^{-1}	Primary culture's growth and monolayer formation									
		LY	HT	HC	OV	HP	NC	TS	N	ES	MS
I	BASE + ASW + 0 % FBS	–	–	–	–	–	–	–	–	–	–
	BASE + ASW + 1 % FBS	–	–	–	–	–	–	–	–	–	–
	BASE + ASW + 2.5 % FBS	–	–	–	–	–	–	–	–	–	–
	BASE + ASW + 5 % FBS	–	–	–	–	–	–	–	–	–	–
	BASE + ASW + 7 % FBS	+	+	+	–	–	–	–	–	–	–
	BASE + ASW + 10 % FBS	++	+	+	+	+	–	–	–	–	–
	BASE + ASW + 15 % FBS	++	+	+	+	+	–	–	–	–	–
	BASE + ASW + 20 % FBS	++	+	+	–	–	–	–	–	–	–
II	BASE + SW + 0 % FBS	–	–	–	–	–	–	–	–	–	–
	BASE + SW + 1 % FBS	–	–	–	–	–	–	–	–	–	–
	BASE + SW + 2.5 % FBS	+	+	–	–	–	–	–	–	–	–
	BASE + SW + 5 % FBS	+	+	–	–	–	–	–	–	–	–
	BASE + SW + 7 % FBS	++	++	–	–	–	–	–	–	–	–
	BASE + SW + 10 % FBS	+++++	++++	+++	++++	+++	+	+	+	+	+
	BASE + SW + 15 % FBS	++++	++++	++	++++	+++	+	+	+	+	+
	BASE + SW + 20 % FBS	++++	++++	++	+++	++	+	+	+	+	+
III	BASE + SW 5 ‰ + 10 % FBS	+++	+++	++	+	+	–	–	–	–	–
	BASE + SW 10 ‰ + 10 % FBS	+++	+++	++	+	+	–	–	–	–	–
	BASE + SW 15 ‰ + 10 % FBS	+++	+++	++	+	+	–	–	–	–	–
	BASE + SW 20 ‰ +10 % FBS	+++	+++	++	+	+	+	+	+	+	+
	BASE + SW 27 ‰ +10 % FBS (SCCM)	+++++	++++	+++	++++	+++	+	+	+	+	+
	BASE + SW 30 ‰ +10 % FBS	+++	+++	++	+	+	+	+	+	+	+
	BASE + SW 35 ‰ + 10 % FBS	+++	+++	+	+	+	–	–	–	–	–
IV	SCCM	+++++	++++	+++	+++	+++	+	+	+	+	+
	SCCM + inorganic salts & trace elements	+++	++	+	++	+	+	+	+	+	+
	SCCM + supplement A	+++++	++++	+++	++++	++	+	+	+	+	+
	SCCM + supplement B	+++++	++++	+++	++++	++	+	+	+	+	+
	SCCM + supplement C	+++++	++++	+++	++++	++	+	+	+	+	+
	SCCM + supplement D	+++++	++++	+++	++++	++	+	+	+	+	+
	SCCM + supplement E	+++++	++++	+++	++++	++	+	+	+	+	+

BASE: All ingredients except FBS (Fetal bovine serum) and seawater. SCCM: BASE with 27 ‰ SW and 10 % FBS

ASW artificial seawater, *SW* seawater (natural)

The confluence of primary cell cultures was categorized as: +tissues/cells attached; ++ attached tissues/cells started multiplying; +++ and ++++ started monolayer formation, +++++ confluent monolayer formed

Phenol red, antibiotic mix and glutamine were in fixed quantity. Potassium dihydrogen phosphate and di-Sodium hydrogen phosphate were added as the buffering agents incorporated in the sugar mix. Cholesterol, 0.2 mg l^{-1}, was added as the lipid component and phenol red as the pH indicator. The most appropriate composition was finally selected based on the extent of attachment of the explants and proliferation of cells by visual observation (Table 3). The pH, temperature of incubation and osmolarity of the medium were 6.8–7.2, 25 °C and 720 ± 10 mOsm kg^{-1} respectively. Natural seawater as the liquid base at 27 ‰ gave better performance. Concentration of 10–15 % FBS (v/v) was found to be most effective.

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Development of primary cell cultures

Among the combinations studied, SCCM supplemented with 10 % FBS prepared in 27 ‰ seawater was the most effective one for attachment of cells, their proliferation and confluence for all cell types tested (Table 3). Among the cell types tested, lymphoid and ovarian cells were the most promising ones having the cell longevity exceeding 50 days, 85 ± 9 days for lymphoid cells and 63 ± 6 days for ovarian cells followed by heart cells (29 ± 1 days) and hepatopancreas (25 ± 5 days). Meanwhile, the longevity of other cell types were, testis 21 ± 3 days, haemocytes 10 ± 3 days, eyestalk 9 ± 2 days, muscle 7 ± 1 days, nerve cord and cells from nauplii 6 ± 1 days (Fig. 1). Moreover, metabolic activity of lymphoid cells in terms of MTT assay showed a significant increase ($p < 0.05$) from day 2 to day 8 compared to the other cell types which were to a large extent static (Figs 2, 3).

Morphology of lymphoid cells in culture was spherical or elliptical initially, and the cells were found getting attached within 3 h and after 24 h, 90 % of the cells were anchored, judged by visual observation. They were epitheloid with large nucleus and granulated cytoplasm exhibiting mitotic division (Fig. 4). Subsequently, 42 % increase in cell metabolic activity was observed by MTT assay within 48 h (Fig. 2). Moreover, additional increase of 27 and 16 % were observed within 72 and 96 h respectively. A rapid monolayer formation and cell proliferation were characteristic of the lymphoid cells compared to all other cell types. Hemocytes in culture appeared similar shape of lymphoid cells, however within 6 h transformed to spindle shaped cells anchor dependent (Fig. 5). Even though, haemocytes were viable for 10 days (judged through MTT assay) direct mitotic division could not be observed under microscope. In ovarian culture, fibroblastic as well as round cells were found proliferating (Fig. 7). Meanwhile, cells from hepatopancreas (Fig. 8) required longer duration to attach to and proliferate (>24 h) than the heart cells which showed better multiplication within 24 h of seeding (Figs. 6, 7). Cell culture developed from testis was very small compared to all other cell types (Fig. 8, 9). Despite the attachment to the substratum, viability and longevity, cell migration and multiplication were not satisfactory with respect to the cells originated from muscle, eyestalk, nerve cord and nauplii.

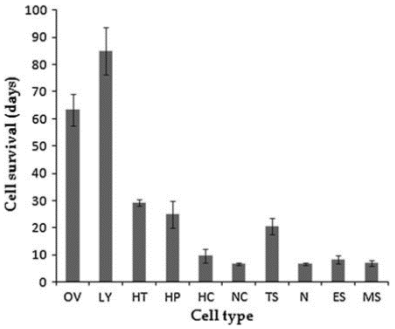

Fig. 1 Survival of primary cell cultures of ovary (*OV*), lymphoid organ (*LY*), heart (*HT*), hepatopancreas(*HP*), hemocytes (*HC*), Nerve cord (*NC*), Testis (*TS*), nauplii (*N*), eyestalk (*ES*) and muscle (*MS*) of *P.monodon* cultured in SCCM supplemented with 10 % FBS

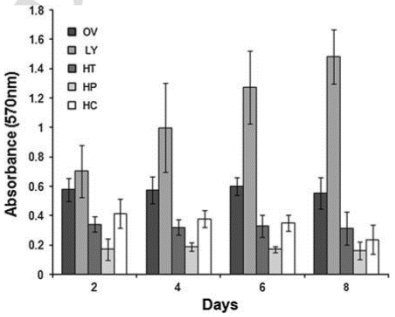

Fig. 2 Viability (in terms of MTT assay) of primary cultures of ovary (*OV*), lymphoid organ (*LY*), heart (*HT*), hepatopancreas (*HP*) and hemocytes (*HC*), of *P.monodon* cultured in SCCM supplemented with 10 % FBS

Comparison of SCCM with other selected media

Lymphoid and ovarian cells ameliorate in SCCM compared to the same in other selected media. An increase of 107 % growth in terms of MTT assay ($p < 0.05$) was observed in lymphoid cells in SCCM in comparison with the same in 2 × L-15, and 59 and 82 % growth in comparison with modified L-15 (Jose et al. 2010) and Grace's insect medium respectively. Ovarian cells showed an increase of 45, 37 and 36 %

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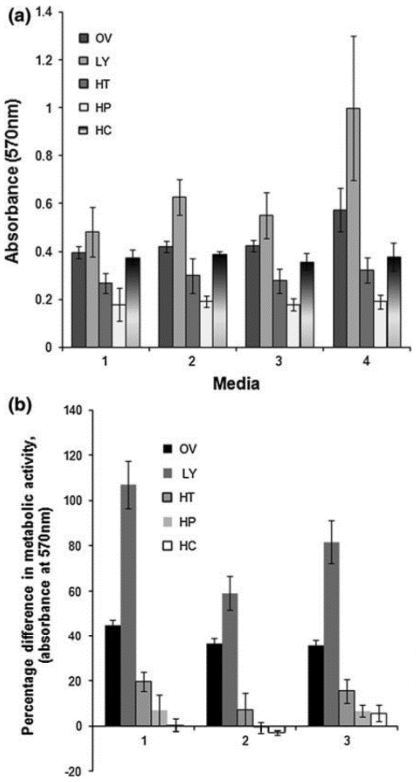

Fig. 3 **a** Effect of different media on in vitro growth (4th day results by MTT assay) of different cell types from *P. monodon*. 1. L-15 + 10 % FBS, 2. Modified L-15, 3. Grace's insect medium +10 % FBS, 4. SCCM + 10 % FBS. OV- ovary, LY-lymphoid organ, HT-heart, HP-hepatopancreas and HC- hemocytes. **b** Percentage difference in the metabolic activity (4th day results by MTT assay) of various cell types grown in SCCM in comparison with selected media. 1. L-15 + 10 % FBS, 2. Modified L-15, 3. Grace's insect medium +10 % FBS. OV-ovary, LY-lymphoid organ, HT-heart, HP-hepatopancreas and HC- hemocytes

($p < 0.05$) in SCCM, 2 × L-15, modified L-15 (Jose et al. 2010) and Grace's medium respectively (Fig. 3a, b). However, increase in growth and multiplication of cells from heart, hepatopancreas and hemolymph in SCCM was less than 20 % compared to the same in the other media.

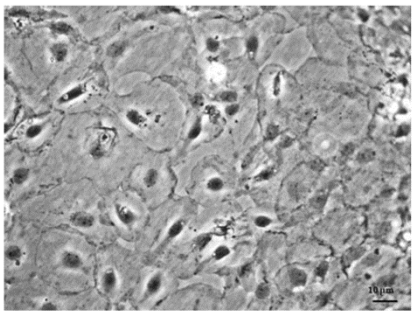

Fig. 4 Primary cell culture developed from *P. monodon* lymphoid cells in SCCM

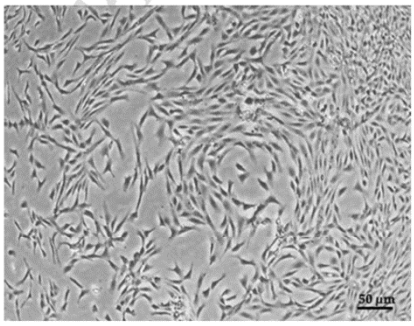

Fig. 5 Primary cell culture from *P. monodon* haemocytes grown in SCCM

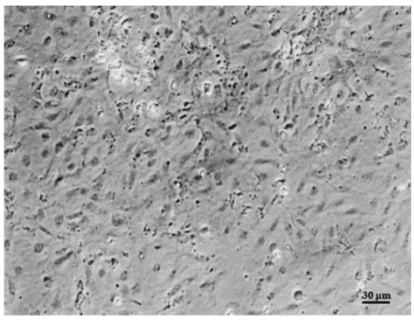

Fig. 6 Primary cell culture developed from *P. monodon* heart cells in SCCM

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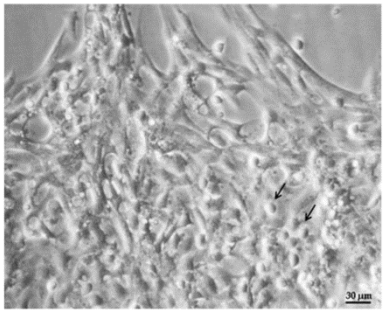

Fig. 7 Primary cell culture in SCCM developed from ovarian cells of *P. monodon*. Proliferating fibroblastic and round cells (*black arrow*) can be seen

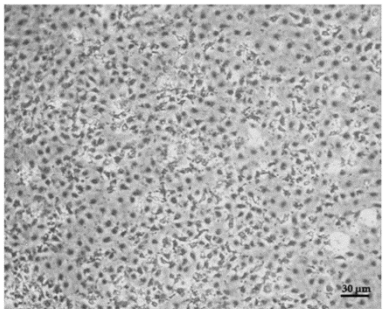

Fig. 8 Primary cell culture developed from hepatopancreatic cells of *P. monodon*.in SCCM

Cell dislodgement and passaging

The results of cell dislodgement and passaging of primary cell cultures from ovary, lymphoid organ, heart, hepatopancreas and haemocytes using different dissociation methods and their efficacy in cell reattachment and growth are summarized in Table 4. Of the six dissociation agents tested shrimp cell dissociation 'cocktail' showed better survival (40 %) of lymphoid cells after two passages. Heart cells and ovarian cells showed a survival rate of up to 30 % where as to hepatopancreas and haemocytes it was

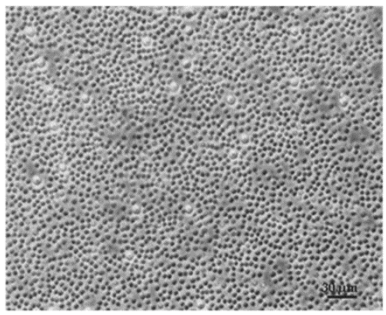

Fig. 9 Primary cell culture developed from *P. monodon* testicular cells in SCCM

toxic with a survival rate 20 % and less than 10 % respectively. Trypsin (0.25 %) and accutase (1 %) were found toxic to cells with a survival rate of less than 10 %. The non-enzymatic solution I and II were proven to be not suitable for shrimp cells altogether. However, in 1 % collagenase V, all cells types showed comparatively better survival (>10 %) except haemocytes, which was less than 10 %.

Discussion

On surveying the literature it has been observed that shrimp cell line development has not yet attained considerable success mainly because of the absence of an appropriate growth medium (Jayesh et al., 2012). Even though medium composition was very important among the factors which affect the proliferation of cells from primary cultures (Mitsuhashi 2001), what has been done so far was to modify and use the available media which otherwise had been designed for mammalian cell culture systems. Among the commercially available cell culture media used Leibovitz's -15 (L-15) has been the most popular one (Chen et al. 1986, 1988; Chen and Kou 1989; Nadala et al. 1993; Lu et al. 1995; Tapay et al. 1995; Tong and Miao 1996; Toullec et al. 1996; Mulford and Austin 1998; Chen and Wang 1999; Shike et al. 2000; Wang et al. 2000; Kumar et al. 2001; Shimizu et al. 2001; Chun-Lei et al. 2003; Maeda et al. 2003,

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Table 4 Effect of different cell dissociation agents on passaging primary cell cultures

z	Cell dissociation agent	Cell type	Cells survived	Passage no
I	Collagenase type V	LY	++	1
		HT	++	1
		HC	+	1
		OV	++	1
		HP	++	1
II	Trypsin	LY	+	1
		HT	+	1
		HC	–	–
		OV	+	1
		HP	+	1
III	Accutase	LY	–	–
		HT	–	–
		HC	–	–
		OV	+	1
		HP	–	–
IV	Non enzymatic solution-I	LY	–	–
		HT	–	–
		HC	–	–
		OV	–	–
		HP	–	–
V	Non enzymatic solution-II	LY	–	–
		HT	–	–
		HC	–	–
		OV	–	–
		HP	–	
VI	"Cocktail"	LY	+++++	2
		HT	++++	1
		HC	+	1
		OV	++++	2
		HP	+++	1

OV Ovary, *LY* lymphoid organ, *HT* heart, *HP* hepatopancreas, *HC* hemocytes

The confluence of primary cell cultures was categorized as: +<10 % of cells passaged, ++10 % of cells passaged, +++20 % of cells passaged, ++++30 % of cells passaged, +++++>30 % of cells passaged

2004; Jiang et al. 2005; Assavalapsakul et al. 2006; Jose et al. 2010, 2011; 2012). Other media such as M199 (Ghosh et al. 1995; Toullec et al. 1996; Itami et al. 1999; Shimizu et al. 2001; Lang et al. 2002), MPS (Tong and Miao 1996; Fan and Wang 2002; Hu et al. 2010;), and Grace's insect medium (Luedeman and Lightner 1992; Nadala et al. 1993; Toullec et al. 1996; Wang et al. 2000; George et al. 2011; George et al. 2011; Jose et al. 2012) were also tested alone as well as with additives for growth of shrimp cells in vitro.

Seawater based shrimp cell culture medium (SCCM) was formulated in 27 ‰ natural sea water as the base since the isosmotic point of salinity for *P. monodon* had indirectly been calculated (Dall 1981) to be 27 ‰ by comparing with its coexist *Penaeus merguiensis*. Earlier to this Mulford et al. (2001) had prepared L-15 medium in 25 ‰ seawater and found effective for cell migration, survival, and cell longevity and claimed rapid migration of cells from explants of hematopoietic tissue of the lobster *Nephrops*

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norvegicus. Meanwhile, in our study the experiment with artificial seawater was not found to be as effective for growth and proliferation of cells as those in natural sea water.

The great success achieved in the development of insect cell line by Grace (Grace 1962) was due to the contributions by Wyatt et al. (1956) in insect biochemistry. May be motivated by them, Najafabadi et al. (1992) and Shimizu et al. (2001) attempted to investigate the biochemistry of haemolymph from *P. aztecus* and *P. stylirostris* and modified commercially available L-15 to suit the requirement of shrimp cell culture in vitro. Following these lines we undertook determination of the composition of haemolymph of *P. monodon* and quantified 15 fatty acids, 16 amino acids and 10 metal ions, which were hitherto not recorded in this species. Taking queues from this information we formulated a novel shrimp cell culture medium having natural sea water as the base. This was due to the fact that the haemolymph metal ions such as sodium (6,784.3 ± 785.8 mg l^{-1}), potassium (524.5 ± 157.9 mg l^{-1}) and calcium (488.8 ± 107.9 mg l^{-1}) were with in the range of 27 ‰ seawater where they were 8,075.5 ± 260.9 mg l^{-1}, 512.935 ± 73.2 mg l^{-1} and 443.7 ± 63.1 mg l^{-1} respectively. These values supported the results of Najafabadi et al. 1992 who observed 6,188.00 ± 795.6 mg l^{-1} sodium, 281.5 ± 62.6 mg l^{-1} potassium and 410 ± 7 mg l^{-1} calcium in the haemolymph of *P. aztecus*. In *P. stylirostris* (Shimizu et al. 2001) the values were 8,411.1 ± 549.7 mg l^{-1}, 328.44 ± 50 mg l^{-1}, and 8,439.2 ± 44.3 mg l^{-1} respectively (Table 2). Concentration of iron, zinc and strontium in the haemolymph of *P. monodon* were 12.9 ± 6.6 mg l^{-1}, 39.42 ± 2.9 mg l^{-1} and 7.3 ± 2 mg l^{-1}, respectively whilst in seawater it was 0.053 ± 0.01 mg l^{-1}, 0.035 ± 0.02 mg l^{-1} and 7.05 ± 0.44 mg l^{-1}. However, Shimizu et al. (2001) estimated the concentrations of these elements in *P. stylirostris* haemolymph as 0.1 ± 0.0 mg l^{-1}, 11.4 ± 1.9 mg l^{-1} and 6.3 ± 0.6 mg l^{-1} respectively.

In our study, the addition of salts such as barium chloride, copper chloride, zinc sulphate, ferric citrate and manganese chloride and the organic supplements such as vitamin mixture, citric acid cycle intermediates, nitrogenous base and energy precursors to 27 ‰ natural sea water did not make any observable changes in the growth and multiplication of primary cell cultures over and above what has been observed in SCCM. This may be due to the availability of sufficient organic and inorganic elements from the supplemented fetal bovine serum (Freshny 2000) and the elemental complexity of the natural seawater (Bruland et al. 1991).

Amino acid and fatty acid constituents in the haemolymph provided a basic knowledge about the concentration to be used in the new medium. Accordingly, various concentrations of the amino acid mixture were tried to find out the most appropriate one which provided maximum cell attachment, multiplicity and survival. Despite the requirement of 0.02 % cholesterol for better performance of the shrimp cell culture in SCCM, the addition of lipid mixture by and large did not make any observable changes. In similar lines Kasorchandra et al. (1992) and George and Dhar (2010) had recommended 0.01 % cholesterol for better performance of shrimp cell culture in media.

Even though the maintenance of proper pH is essential for the successful growth of cells (Nadala et al. (1993) no direct measurements of haemolymph pH of *P. monodon* or any penaeid has been published. However, Huang et al. (1999) calculated in culture flasks, the optimum pH for hepatopancreatocytes of *Penaeus chinesis* as 6.5 with a suggested range of 6.0–7.2. Meanwhile, most researchers selected a pH within the range of 7–7.2 (Tong and Miao 1996; Fan and Wang 2002; Jiang et al. 2005) and 6.8–7.2 (Chen and Wang 1999: Kumar et al. 2001). In the present study pH of SCCM was selected as 6.8 within a range of 6.8–7.2 where the medium remained clear without any precipitation supporting better growth of the cell cultures.

Osmolality of the medium was fixed at 720 ± 10 mOsmol kg l^{-1} in accordance with the haemolymph osmolality, which was found to be 730.5 ± 51.2 mOsmol kg l^{-1} and the cells in vitro were in isosmotic state at this osmolality as judged by visual observation. This was supported by the findings of Kasorchandra et al. (1992) and Fraser and Hall (1999), who used 730 ± 10 and 720 ± 20 mOsmol kg l^{-1} respectively for maintaining cells in vitro from *P. monodon*.

In SCCM, we could maintain ovarian cell culture for 63 ± 6 days, lymphoid cells for 85 ± 9 days during which they showed better proliferation among all the cell types tested, and also as evidenced through MTT assay with significant increase in cell metabolic activity ($p < 0.05$). They exhibited better survival (40 %) after two passages. Further, cell cultures could

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be maintained from hepatopancreas for 25 ± 5 days, heart for 29 ± 1 days, haemocytes for 10 ± 3 days, testis for 21 ± 3 days, eyestalk for 9 ± 2 days, nerve cord, and cells from nauplii for 6 ± 1 days and muscle for 7 ± 1 days in SCCM. Several authors claimed to have maintained ovarian cell culture for 45–66 days (George and Dhar 2010 Maeda et al. 2003), to several months (Toullec et al. 1996; Tong and Miao. 1996). Lymphoid cell culture was reported to be maintained for 54 days (Itami et al. 1999) to 3 months (Tong and Miao. 1996), hepatopancreas for 30 days (George and Dhar. 2010), heart tissue for 4 days (Chen and Wang. 1999) and haemocytes for 48 days (George and Dhar. 2010). Even though, Toullec et al. (1996) maintained embryonic culture for several months, Chun-Lei et al. (2003) maintained nerve cells for 15 days and Nandala et al. (1993) for 3 months such results were not found to have been reproduced by other workers for reasons not known.

Growth and multiplication of cells in SCCM were compared with those in other media based on MTT assay. The lymphoid cells grown in SCCM exhibited an increase of 107 % growth in comparison with 2 × L-15, and 59 and 82 % in comparison with modified L-15 (Jose et al. 2010) and Grace's insect medium respectively ($p < 0.05$). Meanwhile, ovarian cells showed an increased growth of 45, 37 and 36 % ($p < 0.05$) in SCCM in comparison with 2 × L-15, modified L-15 (Jose et al. 2010) and Grace's insect medium respectively. However, increase in growth and multiplication of cells from heart, hepatopancreas and hemolymph in SCCM was less than 20 % compared to the same in the other media.

The passaging of primary shrimp cell cultures and their survival have been found to be the most difficult tasks (Fraser and Hall. 1999; Chun-Lei et al. 2003). However, we could passage lymphoid cell culture twice using shrimp dissociation cocktail. But only about 40 % cells were found surviving after the passage as evidenced by visual observation. Even though, lymphoid cell culture were reported to be passaged 3 times (Chen and Wang. 1999) to 44 times (Tapay et al. 1995), neither confirmation of their results nor adoption of methods by other shrimp research labs could be found reported elsewhere.

From the difficulties experienced by researchers over the last few decades, it is obvious that several untold hindrances stand on the way of the development of shrimp cell lines, for that matter crustaceans altogether. The major unsettling fact was that the media used for the study were mostly by the modification of commercially available ones which were designed mainly for supporting cells from mammalian origin or mostly for the terrestrial animals. Virtually, application of none of the media could end up with a lead in cell line development specific for shrimp cell culture. Availability of growth medium and optimal conditions for supporting cell cultures could be one of the major possible ways to tackle the issue of immortalization. In this context the medium developed here gives the lead to work towards attainment of immortality of shrimp cell culture especially from lymphoid tissue. However, it may require further improvisation.

In conclusion, through this study, a seawater based novel cell culture medium for the development of shrimp cell culture has been formulated and validated with various tissues of *P. monodon*. The success in extending longevity, metabolic activity and the passaging efficiency of the cells grown in SCCM in vitro suggests that this medium should help researchers in the development and establishment of shrimp cell lines for various applications.

Acknowledgments This research was supported by Department of Biotechnology, Government of India (BT/PR8050/AAQ/03/289/2006). The first author thanks DBT for Fellowship.

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