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.,
Lymphoid cell culture has been chosen taking into account its importance as a 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 require 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-1 (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-methoxy-4-nitro-5-sulphophenyl]-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 (CaCO₃) 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 mm² culture flask/dishes (Greiner Bio-One) depending on the experiments to be followed, incubated at 25 °C as an open system without CO₂ 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 mOsms 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:

\[ Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j, \]

where \( Y \) represents the response variable, \( \beta_0 \) represents the interception coefficients, \( \beta_i \) is the coefficient of linear effect, \( \beta_{ii} \) is the coefficient of quadratic effect and \( \beta_{ij} \) 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

\[ \eta = f(x_1, x_2, \ldots, x_n) + \varepsilon \]
where $\eta$ is the response, $f$ is the unknown factor of response, $x_1, x_2, \ldots, x_n$ denotes independent variables, $n$ is the number of independent variables and finally $\varepsilon$ 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 $\varepsilon$ 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:

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

Where, $x$ is the natural variable, $X$ is the coded variable and $x_{\text{max}}$ and $x_{\text{min}}$ 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), $\beta_0$ is the model intercept, $\beta_i$ is the linear coefficient, $x_i$ 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,
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 Montgomery, 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 software, 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 dehydrogenase 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 benzidine 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 10 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 HCl 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 counterpart (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
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 MgCl₂ (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 AT 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 GT 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-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide, XTT) with mitochondrial dehydrogenase 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 H₂SO₄. 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
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, R² 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).

\[
\text{Metabolic activity of the cell} = 0.38 + 0.059*A + 1.924x10^3 *B + 0.077*A^2 + 0.015*B^2 + 0.042*A*B
\]

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 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. 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 incorporation 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 (Rodriguez 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 the 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.
Screening and optimization of growth factors and their potential impacts on lymphoid cell culture: Cellular activity and viral susceptibility

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

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

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

Table 1. Growth factors and their two levels used in the Plackett-Burman design.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Name</th>
<th>Low actual (ng ml⁻¹)</th>
<th>High actual (ng ml⁻¹)</th>
<th>Low coded</th>
<th>High coded</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>IGF-I</td>
<td>50.00</td>
<td>150.00</td>
<td>-1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>B</td>
<td>IGF-II</td>
<td>50.00</td>
<td>150.00</td>
<td>-1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>C</td>
<td>EGF</td>
<td>50.00</td>
<td>150.00</td>
<td>-1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>D</td>
<td>TGF</td>
<td>50.00</td>
<td>150.00</td>
<td>-1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>E</td>
<td>bFGF</td>
<td>4.00</td>
<td>8.00</td>
<td>-1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>F</td>
<td>FGF</td>
<td>2.00</td>
<td>6.00</td>
<td>-1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>G</td>
<td>Interleukin</td>
<td>4.00</td>
<td>8.00</td>
<td>-1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>H</td>
<td>20-HE</td>
<td>0.000</td>
<td>2.00</td>
<td>-1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>J</td>
<td>Dummy</td>
<td>0.000</td>
<td>1.00</td>
<td>-1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>K</td>
<td>Dummy</td>
<td>0.000</td>
<td>1.00</td>
<td>-1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>L</td>
<td>Dummy</td>
<td>0.000</td>
<td>1.00</td>
<td>-1.000</td>
<td>1.000</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Run order</th>
<th>Factors</th>
<th>Metabolic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A B C D E F G H J K L</td>
<td>Experimental value</td>
</tr>
<tr>
<td>1</td>
<td>1 -1 1 -1 -1 1 1 1 0 0 0</td>
<td>0.425433</td>
</tr>
<tr>
<td>2</td>
<td>1 1 -1 -1 -1 -1 1 1 0 0 0</td>
<td>0.475400</td>
</tr>
<tr>
<td>3</td>
<td>-1 1 1 -1 -1 -1 -1 1 0 0 0</td>
<td>0.901467</td>
</tr>
<tr>
<td>4</td>
<td>1 -1 1 -1 1 1 -1 -1 -1 0 0 0</td>
<td>0.388333</td>
</tr>
<tr>
<td>5</td>
<td>1 1 -1 1 -1 1 1 -1 -1 0 0 0</td>
<td>0.652667</td>
</tr>
<tr>
<td>6</td>
<td>1 1 1 1 -1 1 1 -1 1 0 0</td>
<td>0.677800</td>
</tr>
<tr>
<td>7</td>
<td>-1 1 1 -1 1 1 -1 -1 1 0 0</td>
<td>0.704100</td>
</tr>
<tr>
<td>8</td>
<td>-1 -1 1 1 -1 1 1 -1 -1 0 0 0</td>
<td>0.607500</td>
</tr>
<tr>
<td>9</td>
<td>-1 -1 -1 1 1 -1 1 1 -1 0 0</td>
<td>0.696167</td>
</tr>
<tr>
<td>10</td>
<td>1 -1 -1 -1 -1 1 1 -1 -1 0 0 0</td>
<td>0.458767</td>
</tr>
<tr>
<td>11</td>
<td>-1 1 -1 -1 1 1 -1 -1 -1 0 0 0</td>
<td>0.96033</td>
</tr>
<tr>
<td>12</td>
<td>-1 -1 -1 -1 -1 -1 -1 -1 -1 0 0 0</td>
<td>0.639167</td>
</tr>
</tbody>
</table>

Table 3. Analysis of variance (ANOVA) for the selected factorial model of growth factor induced metabolic activity in lymphoid cell culture

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
<th>F value</th>
<th>Prob&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>0.32</td>
<td>6</td>
<td>0.053</td>
<td>5.56</td>
<td>0.0397*</td>
</tr>
<tr>
<td>A</td>
<td>0.18</td>
<td>1</td>
<td>0.177</td>
<td>18.50</td>
<td>0.0077*</td>
</tr>
<tr>
<td>B</td>
<td>0.12</td>
<td>1</td>
<td>0.116</td>
<td>12.16</td>
<td>0.0175*</td>
</tr>
<tr>
<td>C</td>
<td>3.403E+003</td>
<td>1</td>
<td>3.403E+003</td>
<td>0.36</td>
<td>0.5766</td>
</tr>
<tr>
<td>D</td>
<td>0.012</td>
<td>1</td>
<td>0.012</td>
<td>1.24</td>
<td>0.3169</td>
</tr>
<tr>
<td>E</td>
<td>3.642E+003</td>
<td>1</td>
<td>3.642E+003</td>
<td>0.38</td>
<td>0.5640</td>
</tr>
<tr>
<td>H</td>
<td>7.089E+003</td>
<td>1</td>
<td>7.089E+003</td>
<td>0.74</td>
<td>0.4284</td>
</tr>
<tr>
<td>Residual</td>
<td>0.048</td>
<td>5</td>
<td>9.554E+003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.37</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

R² = 0.8697, Adjusted R² = 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.
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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.

<table>
<thead>
<tr>
<th>Standard order</th>
<th>IGF-I (ng ml⁻¹)</th>
<th>IGF-II (ng ml⁻¹)</th>
<th>Experimental value</th>
<th>Predicted value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>50</td>
<td>0.4636</td>
<td>0.4570</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>50</td>
<td>0.4811</td>
<td>0.4905</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>150</td>
<td>0.3810</td>
<td>0.3767</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>150</td>
<td>0.5669</td>
<td>0.5786</td>
</tr>
<tr>
<td>5</td>
<td>9.47</td>
<td>100</td>
<td>0.4458</td>
<td>0.4545</td>
</tr>
<tr>
<td>6</td>
<td>115.53</td>
<td>100</td>
<td>0.6348</td>
<td>0.6209</td>
</tr>
<tr>
<td>7</td>
<td>62.5</td>
<td>29.29</td>
<td>0.4120</td>
<td>0.4110</td>
</tr>
<tr>
<td>8</td>
<td>62.5</td>
<td>170.71</td>
<td>0.4206</td>
<td>0.4164</td>
</tr>
<tr>
<td>9</td>
<td>62.5</td>
<td>100</td>
<td>0.3991</td>
<td>0.3837</td>
</tr>
<tr>
<td>10</td>
<td>62.5</td>
<td>100</td>
<td>0.3494</td>
<td>0.3837</td>
</tr>
<tr>
<td>11</td>
<td>62.5</td>
<td>100</td>
<td>0.3994</td>
<td>0.3837</td>
</tr>
<tr>
<td>12</td>
<td>62.5</td>
<td>100</td>
<td>0.3699</td>
<td>0.3837</td>
</tr>
<tr>
<td>13</td>
<td>62.5</td>
<td>100</td>
<td>0.4007</td>
<td>0.3837</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
<th>F value</th>
<th>Prob&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>0.076</td>
<td>5</td>
<td>0.015</td>
<td>39.36</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>A</td>
<td>0.028</td>
<td>1</td>
<td>0.028</td>
<td>71.48</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>B</td>
<td>2.963E+005</td>
<td>1</td>
<td>2.963E+005</td>
<td>0.076</td>
<td>0.7901</td>
</tr>
<tr>
<td>A²</td>
<td>0.041</td>
<td>1</td>
<td>0.041</td>
<td>106.49</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>B²</td>
<td>1.569E+003</td>
<td>1</td>
<td>1.569E+003</td>
<td>4.05</td>
<td>0.0841</td>
</tr>
<tr>
<td>AB</td>
<td>7.087E+003</td>
<td>1</td>
<td>7.087E+003</td>
<td>18.29</td>
<td>0.0037*</td>
</tr>
<tr>
<td>Residual</td>
<td>2.712E+003</td>
<td>7</td>
<td>3.875E+004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lack of Fit</td>
<td>5.715E+004</td>
<td>3</td>
<td>1.905E+004</td>
<td>0.36</td>
<td>0.7888</td>
</tr>
<tr>
<td>Pure Error</td>
<td>2.141E+003</td>
<td>4</td>
<td>5.352E+004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cor Total</td>
<td>0.079</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

R² = 0.9657, Adjusted R² = 0.9411, Predicted R² = 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

<table>
<thead>
<tr>
<th>Solution</th>
<th>Growth factors</th>
<th>Metabolic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IGF-I (ng ml⁻¹)</td>
<td>IGF-II (ng ml⁻¹)</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>0.578577</td>
<td>0.5801</td>
</tr>
<tr>
<td></td>
<td>0.5811</td>
<td>0.581367*</td>
</tr>
</tbody>
</table>

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

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).
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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.
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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).
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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.