Cytotechnology
DOI 10.1007/s10616-014-9697-0

METHOD IN CELL SCIENCE

Multifactorial interaction of growth factors on *Penaeus monodon* lymphoid cells and the impact of IGFs in DNA synthesis and metabolic activity in vitro

P. Jayesh · Rosamma Philip · I. S. Bright Singh

Received: 19 November 2012 / Accepted: 30 January 2014

Abstract Development of continuous cell lines from shrimp is essential to investigate viral pathogens. Unfortunately, there is no valid cell line developed from crustaceans in general and shrimps in particular to address this issue. Lack of information on the requirements of cells in vitro limits the success of developing a cell line, where the microenvironment of a cell culture, provided by the growth medium, is of prime importance. Screening and optimization of growth medium components based on statistical experimental designs have been widely used for improving the efficacy of cell culture media. Accordingly, we applied Plackett–Burman design and response surface methodology to study multifactorial interactions between the growth factors in shrimp cell culture medium and to identify the most important ones for growth of lymphoid cell culture from *Penaeus monodon*. The statistical screening and optimization indicated that insulin like growth factor-I (IGF-I) and insulin like growth factor-II (IGF-II) at concentrations of 100 and 150 ng ml^{-1}, respectively, could significantly influence the metabolic activity and DNA synthesis of the lymphoid cells. An increase of 53 % metabolic activity and 24.8 % DNA synthesis could be obtained, which suggested that IGF-I and IGF-II had critical roles in metabolic activity and DNA synthesis of shrimp lymphoid cells.

Keywords Growth factor optimization · Response surface methodology · Plackett–Burman · Central composite design · *Penaeus monodon* · Lymphoid cell culture · Shrimp cell culture medium

Introduction

In spite of several years of attempts continuous cell lines from crustaceans, including shrimps, have not been established to enable investigations on viral pathogens. This has hindered the study of viral morphogenesis and development of prophylactic and therapeutic measures (Jayesh et al. 2013). The situation is such that even a complete growth medium for shrimp cells, which provides the necessary factors for growth and spontaneous transformation in vitro, has not yet been achieved. The reasons could be incomplete understanding on the requirements of shrimp cells in vitro and the factors required for transformation. Nevertheless, improved

Electronic supplementary material The online version of this article (doi:10.1007/s10616-014-9697-0) contains supplementary material, which is available to authorized users.

P. Jayesh · I. S. B. Singh (✉)
National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Fine Arts Avenue, Kochi 682016, Kerala, India
e-mail: isbsingh@gmail.com

R. Philip
Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Fine Arts Avenue, Kochi 682016, Kerala, India

Published online: 25 February 2014

growth media and protocols could be formulated by a few groups (George and Dhar 2010; Jose et al. 2012; Jayesh et al. 2013). However, the experimental protocols used for such sorts of media optimization for shrimp cells were lacking systematic approach, perhaps the reason that delimited the success. In this context, the development of a novel shrimp cell culture medium (SCCM) by Jayesh et al. (2013) has importance as it helps maintain lymphoid cell culture from *Penaeus monodon* for longer duration. However, statistical experimental design based investigation of the most important transforming growth factors on shrimp cells has not been performed so far. As the cells of different lineage requires specific growth factors for growth and proliferation (Freshney 2000), an investigation on growth factors has been found vital to achieve improved growth and multiplication of lymphoid cells of *P. monodon* in vitro.

Growth factors are proteins or steroid hormones with cell stimulating property on growth, proliferation and differentiation, and have been considered as the choice of ingredients in shrimp cell culture media (Nadala et al. 1993; Hsu et al. 1995; Fan and Wang 2002). In addition, growth factor co-induction (Fan and Wang 2002) has been considered as the leverage for establishing cell lines, alternative to transgenic technology through oncogenic induction (Jayesh 2013). Besides, the membrane receptors for each growth factor being the major limiting factor for signal transduction (Fan and Wang 2002), single and the multifactorial interaction between the growth factors and their potential impacts on shrimp cell cultures demand optimization of growth factors. In view of the fact that each cell type requires a specific condition for growth and proliferation (Freshney 2000), it is therefore necessary to optimize the growth factors in quality and quantity for each cell type of different lineage. Conceiving the above, application of growth factors has been considered as a promising strategy and in this process the lymphoid cell culture has been chosen owing to its importance in developing shrimp cell lines (Jayesh et al. 2012). Importance of lymphoid cell culture resides in the fact that lymphoid organ is a prime target and the site of replication of most systemic 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 (Wang et al. 2000; Rodríguez et al. 2003), Yellow head 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). Lymphoid cell culture has been demonstrated as a platform for viral shrimp studies (Jose et al. 2012). It was found apparent that the lymphoid cells remained stable for longer period of time in the newly formulated SCCM with consistent growth and proliferation (Jayesh et al. 2013). Moreover, rapid monolayer formation, longevity and stability have been pointed out as the characteristics of lymphoid cells in culture (Nadala et al. 1993), and accordingly the lymphoid tissue has been preferred over others for the development of shrimp cell culture (Chen and Kou 1989; Tapay et al. 1995; Jose et al. 2012; Jayesh et al. 2012) and subsequent transformation into cell line (Jayesh et al. 2013).

There are two ways by which selection of appropriate growth factors for 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. In the statistical screening protocol, the widely accepted medium optimization tool, Plackett–Burman design (Plackett and Burman 1946) is used as multifactorial statistical design (Stanbury et al. 1986) that efficiently screens the important factors among a large number of variables and accounts for the interactions between them (Castro et al. 1992; Lee et al. 1999; González-Leal et al. 2011; Zhang et al. 2013). However, Plackett–Burman design for screening growth factors for enhanced growth and proliferation of shrimp cells in vitro has not been adapted.

To accomplish this target, eight growth factors were screened employing Plackett–Burman design and the most significant ones were selected. Central composite design (CCD) (Box and Wilson 1951) of response surface methodology (RSM) was used to optimize the concentration of the selected growth factors for formulating the modified growth medium. Multifactorial interaction of the growth factors and the induced metabolic activity during the classical as well as statistical screening were evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The performance of mitotic activity of the cells in the optimized medium was evaluated using DNA synthesis markers (5-Bromo-2′-deoxyuridine) in

comparison with that of the control (medium without growth factors) as well as in the routinely used commercial medium, Leibovitz's—L 15.

Materials and methods

Experimental animals

Shrimps for the experiments were maintained in recirculating aquaculture system (RAS) integrated with nitrifying bioreactor (Kumar et al. 2009, 2011) maintained in seawater having 27 ‰ salinity. Post larvae (nested PCR negative to WSSV) were stocked in the system and reared for 3 months maintaining water quality parameters within a narrow range (pH 6.8–7.8; total ammonia <0.1 mg l^{-1}; nitrite <1.0 mg l^{-1}; total alkalinity ($CaCO_3$) 75–125 mg l^{-1}; total hardness 5,000–6,000 mg l^{-1}) fed on pelleted diet 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 tissue (Jose et al. 2012; Jayesh et al. 2013).

Cell culture medium

Shrimp cell culture medium was used for the experiment following the protocol by Jayesh et al. (2013). It contained (mg l^{-1} in natural seawater (27 ‰)) 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. Further, the medium was supplemented with an antibiotic mixture containing penicillin (100 U ml^{-1}), streptomycin (100 µg ml^{-1}), chloramphenicol (0.06 µg ml^{-1}) along with filter sterilized (150 mg l^{-1}) glutamine and 10 % fetal bovine serum (FBS) just before use. All chemicals used for the preparation, unless specifically stated otherwise, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Development of primary lymphoid cell cultures

Lymphoid organ, 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 and sacrificed in ice, surface disinfected by immersion in sodium hypochlorite solution having 800 mg l^{-1} available chlorine in chilled seawater for 10 min followed by washing in sterile seawater. Lymphoid organ was removed aseptically and transferred to holding medium of 720 mOsm kg^{-1} (SCCM without FBS), washed three times with PBS and minced into 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 India Pvt. Ltd., Ahmedabad, India) 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, growth factors were screened based on their contribution to metabolic activity using MTT assay (Jose et al. 2012). The lymphoid cell culture in SCCM without growth factors served as control. MTT assay measures the mitochondrial dehydrogenase 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) to water-insoluble formazan crystals (Xia and Laterra 2006) which can be solubilized in dimethyl sulphoxide (DMSO) yielding a purple-colored solution (Mosmann 1983).

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-β (TGF-β) from porcine 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

Table 1 Plackett–Burman design matrix of the variables (ng ml^{-1}) along with the experimental (n = 3) and predicted values of multi-factorial impact of growth factors on cellular metabolism

Exp no.	Factors								Metabolic activity	
	IGF-I	IGF-II	EGF	TGF	bFGF	FGF	IL-2	20-HE	Experimental	Predicted
1	150	50	150	50	4	6	8	2	0.425433	0.324833
2	150	150	50	50	4	2	8	2	0.475400	0.555278
3	50	150	150	150	4	2	4	2	0.901467	0.827089
4	150	50	150	50	8	2	4	0	0.388833	0.408289
5	150	150	50	150	4	6	4	0	0.652667	0.666622
6	150	150	150	150	8	2	8	0	0.677800	0.667789
7	50	150	150	50	8	6	4	2	0.704100	0.799200
8	50	50	150	150	4	6	8	0	0.608500	0.678933
9	50	50	50	150	8	2	8	2	0.696167	0.698845
10	150	50	50	150	8	6	4	2	0.458767	0.456089
11	50	150	50	50	8	6	8	0	0.986033	0.881489
12	50	50	50	50	4	2	4	0	0.639167	0.649878

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, *IL-2* interleukin-2, *20-HE* 20-hydroxyecdysone

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. All growth factors were purchased from Sigma Aldrich. Primary stock solutions of bFGF and EGF were prepared in the growth medium containing 10 % foetal bovine serum (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), IL-2 in 100 mM acetic acid, TGF-β in 4 mM HCl containing 0.1 % BSA and 20HE in ethyl alcohol (Jose et al. 2012).

Primary screening of growth factors: One-factor-at-a-time (Classical method)

The eight growth factors were dissolved in appropriate solvents as explained above. Subsequently all of them were diluted with growth medium (SCCM) to get the final concentrations of 2, 4, 6, 8, 10, 25, 50, and 100 ng ml^{-1}. An aliquot of 100 µl growth factor containing 2 × concentration was added to 96 well plate containing a monolayer of lymphoid cell culture with 100 µl growth medium. The plates were incubated at 25 °C for 48 h. After incubation, the medium was changed and 50 µl of MTT solution (5 mg ml^{-1}) prepared in PBS (720 mOsm kg^{-1}) was added and kept for incubation (in dark) at 25 °C for 5 h (Jose et al. 2012). The entire medium was removed and 200 µl of dimethyl sulphoxide (DMSO, HiMedia, Mumbai, India) was added. The wells were mixed using a pipette and the formazan crystals were dissolved, absorbance was measured at 570 nm using a Microplate reader (Infinite M-200 Tecan, Grödig, Austria) and the results obtained were compared with that of control.

Optimization of growth factors by Plackett–Burman factorial design and central composite design using response surface methodology (RSM)

After selecting various growth factors by testing their significance in one-at-a time screening, Plackett–Burman statistical design (Plackett and Burman 1946) was employed to find out the most significant growth factor (s) from among IGF-I, IGF-II, IL-2, EGF, TGF-β, bFGF, FGF-4 and 20-HE based on their multifactorial interaction, which contributed to cellular metabolism (Table 1). Each growth factor was presented at two levels, high (+1) and low (−1) and the response was measured as metabolic activity by way of MTT assay. All trials were carried out in triplicate and the average was used as the response variable. Regression analysis identified the variables that had significant ($p < 0.05$) effect on metabolic activity, and evaluated for further optimization (Table 2).

Response surface approach using CCD was applied to find out the optimum levels of IGF-I and IGF-II and

Table 2 ANOVA for the selected factorial model in Plackett–Burman multi-factorial screening 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	0.18	18.50	0.0077*
B	0.12	1	0.12	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

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

the effects of their interaction on the metabolic activity of lymphoid cells. The design provided 13 combinations 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 (Table 3). 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 demonstrate 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, USA) 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.

Table 3 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

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 sextuplicates and compared with the predicted values and control. Aliquots of 200 µl cell suspensions seeded on to 96 well plates were used and the MTT assay performed by following the procedure described elsewhere in this manuscript.

Mitotic activity of the cells grown in growth factor supplemented shrimp cell culture medium

5-Bromo-2′-deoxyuridine (BrdU) ELISA was performed to confirm DNA synthesis (Gratzner 1982) in the lymphoid cells grown in growth factor (IGFs) supplemented SCCM. The efficiency of the medium was compared with that of the control and L-15. An aliquot of 20 µl 10 mM BrdU solution was added to each well of 96 well plates containing primary lymphoid cell culture grown in the basic SCCM, SCCM supplemented with IGFs, 2X L-15 and the modified L-15 (2X L-15 medium supplemented with 2 % glucose, MEM Vitamins (1X) and tryptose phosphate broth (2.95 g L^{-1}). ELISA was done after 48 h using the Cell Proliferation Biotrak ELISA System (Amersham Biosciences, Little Chalfont, UK). The medium was removed, fixed for 30 min with 4 % paraformaldehyde 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, the reaction was stopped by adding 25 µl of 1 M sulphuric acid into each well and optical density was determined at 450 nm. The medium without cells but with BrdU and the medium with cells but without BrdU were kept as controls. For documenting the images of BrdU incorporated cells, following incubation with peroxidase conjugated anti-BrdU solution in the above step, the wells were rinsed thrice with PBS, and diaminobenzidene (0.6 mg ml^{-1} in 0.05 M tris buffer with 0.03 % hydrogen peroxide) was added as the substrate to the wells. After 20 min, the wells were rinsed with PBS and counterstained with Meyer's Haematoxylin for 30 s. Cells without BrdU were kept as control. The cells were observed under Inverted phase contrast microscope for BrdU incorporation .

Statistical analysis

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

Results

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, and the latter found prominent with elevated lifespan. Mixed cell population was used for screening growth factors and for determining metabolic activity.

Screening and optimization of growth factors and their validation

Screening of one-variable-at-a-time using lymphoid cell culture was performed for the identification of the most effective concentration of the growth factors (Supplimentary data), which were chosen for further statistical screening using Plackett–Burman multifactorial design. Growth factors such as IGF-I, IGF-II, EGF and TGF-β with the concentration at 50 ng to 100 ng ml^{-1} (in a range) were found significant ($p < 0.05$) and produced elevated metabolic activity in lymphoid cell culture. However, FGF-4 and bFGF were found effective ($p < 0.05$) only at lower concentration within a range of 2 ng to 6 ng ml^{-1} and had negative impact at higher concentration. Similarly, IL-2 was found effective ($p < 0.05$) at 6 ng–8 ng ml^{-1} whilst, the insect steroid hormone 20HE supported elevated activity ($p < 0.05$) at 2 ng ml^{-1}. In both cases, at higher concentrations, negative impact was observed in the metabolic activity of the cells. Maximum and minimum concentrations of growth factors which contributed to cellular activity, as determined by MTT assay, were selected for statistical screening, and are given in Table 1. Plackett–Burman statistical screening reduced the number of variables from 8 to 6 with respect to their significance in the interactions among the components. Out of the 6 growth factors, IGF-1 (A) and IGF-II (B) were significant at 95 % confidence level with p value of 0.0077 ($p < 0.01$) and 0.017 ($p < 0.05$) respectively, suggesting the model significant (Table 2). Even though, the other four components such as EGF (C), TGF (D), bFGF (E) and 20-HE (H) did not have any negative effect on metabolic activity of the cells, their interactions proved to be insignificant ($p > 0.05$) with p values of 0.5766, 0.3169, 0.5640 and 0.4284 respectively. The coefficient of determination, R^2 (0.87) showed 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 of 0.0397 ($p < 0.05$) which implied that the model was significant at 95 % confidence level and could be used for further optimization (Table 2).

As the Plackett–Burman statistical screening was used in the study to identify the most significant (effective) growth factor components for shrimp cells, factors statistically insignificant were omitted from further investigation. Accordingly, the growth factors such as IGF-I (A) and IGF-II (B) were selected for further optimization by RSM using CCD. Table 3 shows various combinations of IGF-I and IGF-II used and corresponding metabolic activity of the lymphoid cells in terms of MTT assay.

The CCD 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 3 summarizes the metabolic activity of the cells for each combination along with the predicted response. The results obtained after CCD were analysed by standard 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).

$$\text{Metabolic activity of the cell} = 0.38 + 0.059 * A + 1.924 \times 10E + 003 * B + 0.077 * A^2 + 0.015 * B^2 + 0.042 * A * B$$

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 that the model was highly significant at 95 % confidence level (Table 4). 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 of 0.7901 and 0.0841. However, the combination AB (IGF-I and IGF-II) was found significant with the p value of 0.0037 ($p < 0.05$), suggesting the combined positive effect of the growth factors IGF-I and IGF-II, despite the lack of postive effect of IGF-II alone. This indicated that both IGF-I and IGF-II were required to be incorporated in the medium together to improve the metabolic activity and DNA synthesis of 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) which 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 that 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) 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 4). 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. 1), 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 (Table 5). Moreover, during validation, excellent growth of fibroblastic cells from lymphoid tissue was observed in SCCM with optimized growth factors IGF-I and IGF-II (Fig. 2). In addition, while comparing with the control [medium without growth factor (M1)], 53 % increase in metabolic activity was observed in the cells which were grown in the medium with optimized growth factors (M2) (Fig. 3).

Mitotic activity of the lymphoid cells

The BrdU incorporation assay showed that the mitotic activity of lymphoid cells grown in the SCCM (M2) with the optimized growth factors (IGF-I&IGF-II) was significantly ($p < 0.05$) higher than that in the basal

Table 4 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

* Significant model terms

medium (M1, medium 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}, respectively, induced DNA synthesis 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 the medium (M2) with optimized growth factors (IGF-I&IGF-II) than that in the modified L-15 and 2xL15 (Fig. 4a). However, an increase of 26.7 and 27.7 % mitotic activity could be observed in the basal medium (M1) itself while comparing the same with that in the modified L-15 and 2xL-15, respectively, suggesting that the basal medium as such had the required potential to induce mitotic activity. Meanwhile, the additional increase (24.8 %) in mitotic activity was contributed by the growth factors IGF-I and IGF-II. The BrDU incorporation was confirmed by microscopic observation of cells which enterd into S-phase exhibiting brown staining with positive results (Fig. 4b).

Discussion

The development and formulation of a novel SCCM could be accomplished by Jayesh et al. (2013). However, as the cells of different lineage requires specific growth factors (Freshney 2000), an investigation to select the most appropriate ones was undertaken for lymphoid cells of *P. monodon* in vitro.

In the present study, among the vertebrate growth factors and hormones screened by Plackett–Burman statistical design, only IGF-I and IGF-II were found to have significant effect on lymphoid cell culture. This was further optimized by applying CCD and obtained the most effective concentration of 100 ng ml^{-1} and 150 ng ml^{-1} of IGF-I and IGF-II, respectively. Jose et al. (2012) suggested that, IGF-I at a concentration of 10 ng ml^{-1} enhanced proliferation of lymphoid cells in vitro. Fan and Wang (2002) noticed enhanced growth and proliferation in embryonic cells of *P. chinensis* after the administration of IGF-II combining with bFGF. Hsu et al. (1995) reported that lymphoid cells from *P. monodon* treated with IGF-I were capable of being subcultured; however, the cells were eventually discarded due to yeast contamination.

Plackett–Burman is useful in decreasing the number of variables and number of experiments in further optimization step (Cervera et al. 2013). As membrane receptors are the limiting factors for 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 eliminating insignificant ones. Meanwhile, RSM involves graphical representation of the model equation and determination of optimal concentrations of the variables. 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

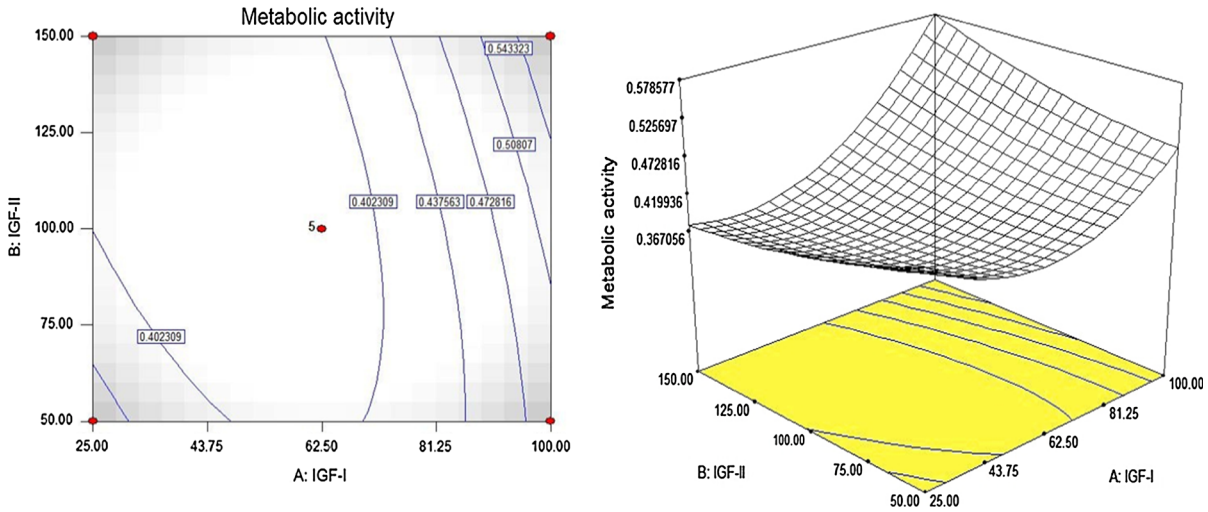

Fig. 1 Contour and response surface plots showing the relative effects of IGFs on the metabolic activity of lymphoid cell culture

Table 5 Solution for the model with predicted response and the experimental response from the model validation

Solution	Growth factors		Metabolic activity		
	IGF-I (ng ml^{-1})	IGF-II (ng ml^{-1})	Predicted response	Experimental response	
1	100	150	0.578577	0.5811	0.581367 *
				0.5801	
				0.5829	
				0.5821	
				0.5798	
				0.5822	

* Average value of absorbance (570 nm) from MTT assay during model validation (n = 6). IGF: Insulin like growth factor

relationship between the response and the independent variables (Wang and Lu 2004).

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 the variabilities 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 having very high correlation and a model with an R^2 value between 0.7 and 0.9 as having high correlation (Guilford and Fruchter 1973; Haaland 1989; 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. The observed and predicted responses of metabolic activity (MTT assay) in lymphoid cell culture were found to be 0.581 and 0.579 (Abs_{570}). These observations confirmed that the model used for screening and optimizing growth factors was valid.

BrdU incorporation in the lymphoid cell culture of *P. monodon* was first reported by Jose et al. (2012), with a few BrdU positive cells in the culture grown in modified Leibovitz's L-15 medium. Well before that Braasch et al. (1999) and Jose et al. (2010) had reported haemocyte cultures from *P. vannamei* and *P. monodon* BrdU positive (1–2 and 22 ± 7 % respectively). Maeda et al. (2003) confirmed 35 % BrdU positive cells in primary ovarian cell culture developed from *Penaeus japonicus*. In the light of above observations BrdU incorporation in lymphoid cells of

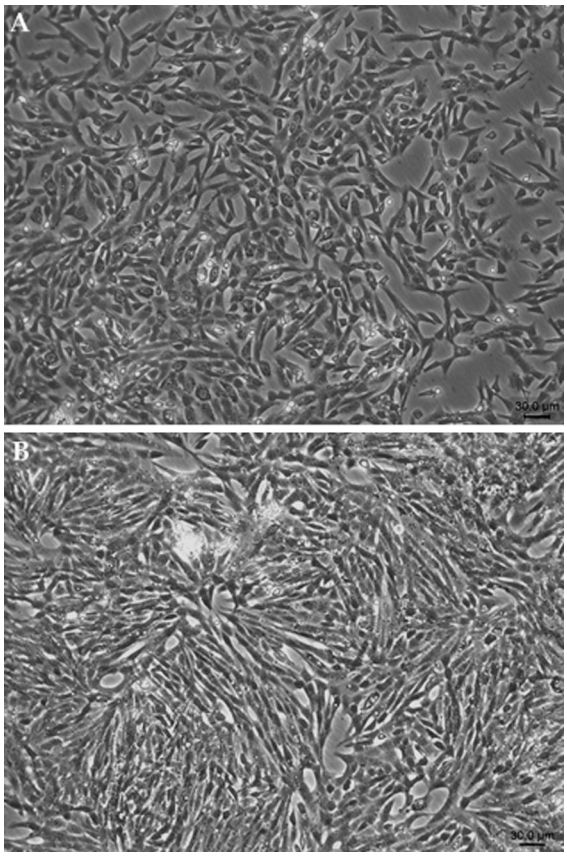

Fig. 2 Images showing lymphoid cell culture from *P. monodon,* 48 h post seeded in SCCM without growth factors (**a**) and with optimized IGFs concentrations (**b**) (IGF-I 100 and IGF-II 150 ng ml^{-1})

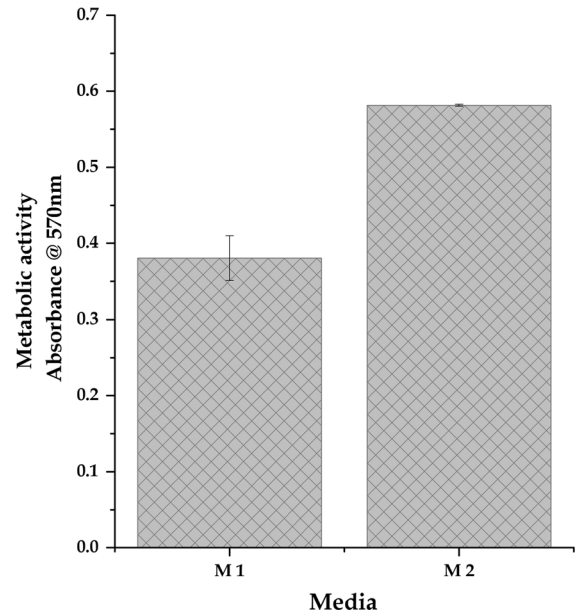

Fig. 3 Metabolic activity of lymphoid cells grown in SCCM with optimized IGFs (IGF-I 100 and IGF-II 150 ng ml^{-1}). MTT assay shows an elevated mitochondrial dehydrogenase activity in lymphoid cells grown in the medium with optimized IGFs concentrations (n = 6). M1: Medium without IGFs; M2: Medium with IGFs

P. monodon was comparatively higher to the tune of 10 ± 1 and 24 ± 2 %, respectively, at the 24th and 48th h on growing them in SCCM supplemented with IGF-I and IGF-II which suggested progression in DNA synthesis over time.

Insulin-like growth factor I (IGF-I) coordinates proliferation and differentiation in a wide variety of cell types, and its stimulatory effects on protein synthesis (Chaulet et al. 2012), growth promoting effects and glucose metabolism (Mathews et al. 1988) have been well documented. Baker and Carruthers (1980) suggested that the bovine insulin stimulated 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*. Chaulet et al. (2012) observed that IGF-I had a stimulatory effect on protein synthesis in the abdominal muscle of *C. quadricarinatus* in vitro. Gutiérrez et al. (2007) reported that the IGF-I induced, elevated in vivo glucose metabolism in *P. vannamei*. Similar to those findings, an elevated mitochondrial dehydrogenase activity through MTT assay and DNA synthesis through BrdU incorporation were observed in the present study with lympoid cell culture grown in SCCM supplemented with IGF-I and IGF-II.

Development of a cell culture system with longevity is the prime requirement towards its in vitro transformation and immortalization. Eventhough, considerable progress has been made towards developing shrimp cell culture systems for the study of viral pathogens, an immortalized cell line from shrimp has not yet been possible. In this context, the present investigation on the multifactorial interaction of growth factors in lymphoid cell culture could yield valuable information about the requirement of critical growth factors for its enhanced growth and proliferation. Moreover, the results may pave the way for better understanding of growth factor receptors in signal transduction. Although it may require further improvements at the level of composition of growth medium for successful in vitro transformation, the protocol developed in this work shall serve as the

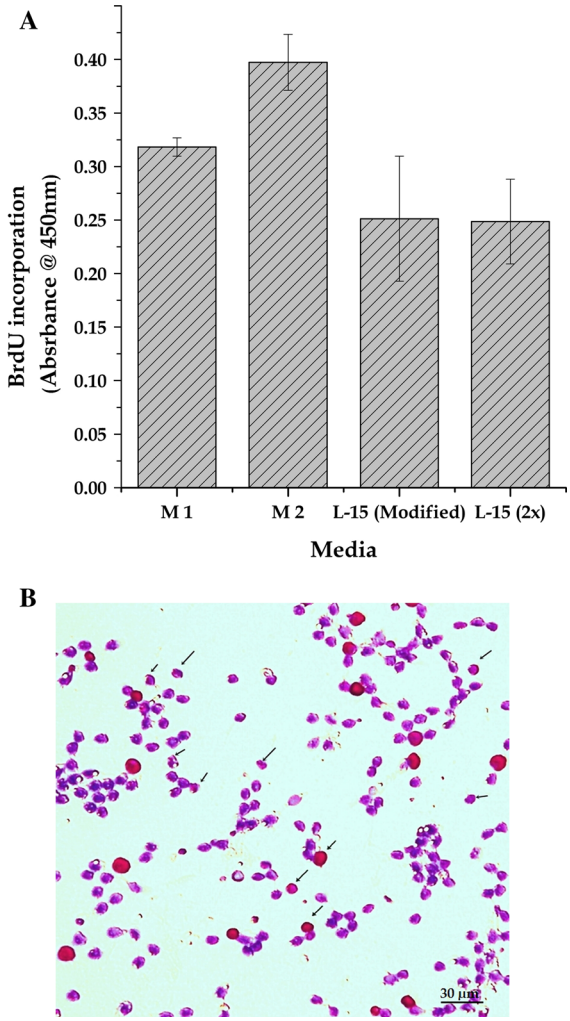

Fig. 4 **a** Mitotic activity of lymphoid cells grown in various media. BrdU incorporation assay shows an elevated DNA synthesis in lymphoid cells grown in SCCM with optimized IGFs concentrations (n = 3) (IGF-I 100 and IGF-II 150 ng ml^{-1}). M1: Medium without IGFs; M2: Medium with optimized concentrations of IGFs; L-15: Leibovitzs L-15 medium. **b** Confirmation of BrdU incorporation in lymphoid cells grown in SCCM with optimized IGFs (IGF-I 100 and IGF-II 150 ng ml^{-1}). *Arrow* indicates BrdU labeled lymphoid cells (*brown*) among unlabelled cells. (Color figure online)

much-needed tool for generating in vitro lymphoid cell culture system from *P. monodon* for cellular and molecular studies as well as for isolation of shrimp viruses.

In conclusion, through this study significant interaction of the growth factors such as IGF-I and IGF-II on lymphoid cells of *P. monodon* in vitro could be observed. The results confirmed their combinatorial effect at concentrations of 100 and 150 ng ml^{-1}, respectively, in SCCM, which could significantly influence the metabolic activity and DNA synthesis of the lymphoid cells suggesting their critical roles in cellular growth and proliferation.

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.

References

Ahuja SK, Ferreira GM, Moreira AR (2004) Application of Plackett-Burman design and response surface methodology to achieve exponential growth for aggregated shipworm bacterium. Biotechnol Bioeng 85:666–675

Baker PF, Carruthers A (1980) Insulin stimulates sugar transport in giant muscle fibres of the barnacle. Nature 286:276–279

Bell TA, Lightner DV (1988) A handbook of normal penaeid shrimp histology. Special Publication No. 1. World Aquac Soc 52–73

Bonami JR, Lightner DV, Redman RM, Poulos BT (1992) Partial characterisation of a togavirus (LOVV) associated with histopathological changes of the lymphoid organ of penaeid shrimps. Dis Aquat Org 14:145–152

Box GEP, Wilson KB (1951) On the experimental attainment of optimum conditions. J Roy Stat Soc 13:1–45

Braasch DA, Ellender RD, Middlebrooks BL (1999) Cell cycle components and their potential impact on the development of continuous in vitro penaeid cell replication. Methods Cell Sci 21:255–261

Castro PML, Hayter PM, Ison AP, Bull AT (1992) Application of a statistical design to the optimization of culture medium for recombinant interferon-gamma production by Chinese hamster ovary cells. Appl Microbiol Biotechnol 38:84–90

Cervera L, Gutiérrez-Granados S, Martínez M, Blanco J, Gòdia F, Segura MM (2013) Generation of HIV-1 Gag VLPs by transient transfection of HEK 293 suspension cell cultures using an optimized animal-derived component free medium. J Biotechnol 166:152–165

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

Chaulet A, Medesani DA, Freitas J, Cervino A, Cervino N, Rodríguez EM (2012) Induction of somatic growth in juvenile crayfish *Cherax quadricarinatus* (Decapoda, Parastacidae), by ecdysone and insulin growth factor. Aquaculture 370–371:1–6

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

Fan TJ, Wang XF (2002) In vitro culture of embryonic cells from the shrimp, *P. chinensis*. J Exp Mar Biol Eco 267:175–184

Fraser CA, Owens L (1996) Spawner-isolated mortality virus from Australian *Penaeus monodon*. Dis Aquat Org 27:141–148

Freshney RI (2000) Culture of animal cells: a manual of basic technique. Wiley-Liss, New York

George SK, Dhar AK (2010) An improved method of cell culture system from eye stalk, hepatopancreas, muscle, ovary, and hemocytes of *Penaeus vannamei*. In Vitro Cell Dev - An 46:801–810

González-Leal IJ, Carrillo-Cocom LM, Ramírez-Medrano A, López-Pacheco F, Bulnes-Abundis D, Webb-Vargas Y, Alvarez MM (2011) Use of a Plackett–Burman statistical design to determine the effect of selected amino acids on monoclonal antibody production in CHO cells. Biotechnol Prog 27:1709–1717

Gratzner HJ (1982) Monoclonal antibody to 5-bromo and 5-iododeoxyuridine: a new reagent for the detection of DNA replication. Science 218:474–475

Guilford JP, Fruchter B (1973) Fundamental statistics in psychology and education. McGraw-Hill, New York

Gutiérrez A, Nieto J, Pozo F, Stern S, Schoofs L (2007) Effect of insulin/IGF-I like peptides on glucose metabolism in the white shrimp *Penaeus vannamei*. Gen Comp Endocrinol 153:170–175

Haaland PD (1989) Separating signals from the noise. Experimental design in biotechnology. Marcel Dekker, New York, pp 61–83

Hasson KW, Lightner DV, Mohney LL, Redman RM, White BM (1999) Role of lymphoid organ spheroids in chronic Taura syndrome virus (TSV) infections in *Penaeus vannamei*. Dis Aquat Org 38:93–105

Hsu YL, Yang YH, Chen YC, Tung MC, Wu JL, Engelking MH, Leong JC (1995) Development of an in vitro subculture system for the oka organ (Lymphoid tissue) of *Penaeus monodon*. Aquaculture 136:43–55

Jayesh P (2013) Development of lymphoid cell culture system from *Penaeus monodon* and molecular approaches for its transformation. Ph.D. Thesis. Cochin University of Science and Technology, India

Jayesh P, Seena J, Singh ISB (2012) Establishment of shrimp cell lines: perception and orientation. Indian J Virol 23:244–251

Jayesh P, Seena J, Philip R, Singh ISB (2013) A novel medium for the development of in vitro cell culture system from *Penaeus monodon*. Cytotechnology 65:307–322

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

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 71:169–177

Jose S, Jayesh P, Sudheer NS, Poulose G, Mohandas A, Philip R, Bright 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

Kumar VJR, Achuthan C, Manju NJ, Philip R, Singh ISB (2009) Activated packed bed bioreactor for rapid nitrification in brackish water hatchery systems. J Ind Microbiol Biot 36:355–365

Kumar VJR, Joseph V, Vijai R, Philip R, Singh ISB (2011) Nitrification in a packed bed bioreactor integrated into a marine recirculating maturation system under different substrate concentrations and flow rates. J Chem Technol Biotechnol 86:790–797

Lee GM, Kim EJ, Kim NS, Yoon SK, Ahn YH, Song JY (1999) Development of a serum-free medium for the production of erythropoietin by suspension culture of recombinant Chinese hamster ovary cells using a statistical design. J Biotechnol 69:85–93

Mathews LS, Hammer RE, Behringer RR, D'Ercole AJ, Bell GI, Brinster RL, Palmiter RD (1988) Growth enhancement of transgenic mice expressing human insulin-like growth factor-I. Endocrinology 123:2827–2833

Maeda M, Mizuki E, Itami T, Ohba M (2003) Ovarian primary tissue culture of the kuruma shrimp *Marsupenaeus japonicus*. In Vitro Cell Dev-An 39:208–212

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

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

Nadala EC, Loh PC, Lu PC (1993) Primary culture of lymphoid, nerve and ovary cells from Penaeus stylirostris and *Penaeus vannamei*. In Vitro Cell Dev-An 29:620–622

Nadala ECB, Lu Y, Loh PC, Brock JA (1992) Infection of *Penaeus stylirostris* (Boone) with a rhabdovirus isolated from Penaeus spp. Gyobyo Kenkyu 27:143–147

Owens L, Beer SD, Smith J (1991) Lymphoidal parvovirus-like particles in Australian penaeid prawns. Dis Aquat Org 11:129–134

Plackett RL, Burman JP (1946) The design of optimum multifactorial experiments. Biometrika 33:305–325

Rajendran KV, Cowley JA, McCulloch RJ, Walker PJ (2006) A TaqMan real-time RTPCR for quantifying Mourilyan virus infection levels in penaeid shrimp tissues. J Virol Methods 137:265–271

Richardson NA, Anderson AJ, Sara VR (1997) The effects of insulin/IGF-I on glucose and leucine metabolism in the redclaw crayfish (*Cherax quadricarinatus*). Gen Comp Endocrinol 105:287–293

Rodríguez J, Bayot B, Amano Y, Panchana F, Blas I, Alday V, Calderón J (2003) White spot syndrome virus infection in cultured *Penaeus vannamei* (Boone) in Ecuador with emphasis on histopathology and ultrastructure. J Fish Dis 26:439–450

Rusaini, Owens L (2010) Insight into the lymphoid organ of penaeid prawns: a review. Fish Shellfish Immunol 29:367–377

Spann KM, Vickers JE, Lester RJG (1995) Lymphoid organ virus of *Penaeus monodon* from Australia. Dis Aquat Org 23:127–134

Sritunyalucksana K, Apisawetakan S, Boon-nat A, Withyachumnarnkul B, Flegel TW (2006) A new RNA virus found in black tiger shrimp *Penaeus monodon* from Thailand. Virus Res 118:31–38

Stanbury PF, Whitaker A, Hall SJ (1986) Media for industrial fermentations. Principles of fermentation technology. Pergamon, Oxford, pp 93–122

Tang KFJ, Pantoja CJ, Poulos BT, Redman RM, Lightner DV (2005) In situ hybridization demonstrates that *Litopenaeus vannamei*, *L. stylirostris* and *Penaeus monodon* are susceptible to experimental infection with infectious myonecrosis virus (IMNV). Dis Aquat Org 63:261–265

Tapay L, Lu Y, Brock JA, Nadala ECB, Loh PC (1995) Transformation of primary cultures of shrimp (*Penaeus stylirostris*) lymphoid (Oka) organ with simian virus-40 (T) antigen. Proc Soc Exp Biol Med 209:73–78

Wang Y-X, Lu Z-X (2004) Statistical optimization of media for extracellular polysaccharide by *Pholiota squarrosa* (Pers. ex Fr.) Quel. AS 5.245 under submerged cultivation. Biochem Eng J 20:39–47

Wang C-H, Yang H-N, Tang C-Y, Lu CH, Kou GH, Lo C-F (2000) Ultrastructure of white spot syndrome virus development in primary lymphoid organ cell cultures. Dis Aquat Organ 41:91–104

Xia S, Laterra J (2006) Hepatocyte growth factor increases mitochondrial mass in glioblastoma cells. Biochem Bioph Res Commun 345:1358–1364

Zhang H, Wang H, Liu M, Zhang T, Zhang J, Wang X, Xiang W (2013) Rational development of a serum-free medium and fed-batch process for a GS-CHO cell line expressing recombinant antibody. Cytotechnology 65:363–378