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ORIGINAL ARTICLE

An optimization of supplements and physical factors for growth of hemocytes culture from *Penaeus vannamei* (White shrimp) in selective medium

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Abstract

Background Standardization of cell culture medium plays a vital role in the development of primary or continuous cell line. Apart from the basal media, supplements in the medium and various physical factors promote the cell growth. With this context, the study was carried out to optimize the culture medium using various supplements and physical factors for the growth of hemocytes culture from *Penaeus vannamei*.

Methods Various concentrations of Fetal Bovine Serum (FBS; 1–25%), Shrimp Muscle Extract (SME; 1–25%) and basic Fibroblast Growth Factor (bFGF; 0.5–5 ng mL $^{-1}$) were attempted to optimize the cell culture media for the development of primary hemocytes culture of *P. vannamei*. Various pH, temperature and osmolality was also screened to optimize the medium.

Results 15% FBS was ideal for the healthy morphology of cells with rapid replication. SME supplementation at 5–20% supported the cell growth for 24 h but only 30% of cell viability was observed after 48 h. bFGF (0.5–5 ng mL^{-1}) enhanced cell growth in the medium with 15% FBS; The ideal pH level was examined by preparing the HBSCM-5 medium at pH between 6.8–8.0. Osmolality of 730 ± 20, pH of 7.2 and temperature of 28 °C resulted in the healthy cells with good morphology. NSW supplement supported the cell growth at low concentrations of salt; however, more than 2% salt concentrations cells did not form fibroblast-like morphology and instead a crystal-like morphology was observed.

Conclusion The hemocytes culture were optimized for use as an in vitro cell culture system by testing cell growth on HBSCM-5 medium with various supplements, growth factors and physical parameters.

Keywords Shrimp cell culture · *Penaeus vannamei* · Optimizing culture media · Temperature · Salinity · pH · bFGF and HBSCM-5 medium

Introduction

Development of shrimp cell culture systems is a major challenge for the foremost researchers all over the world. For the successful development of cell line, culture media is the prime factor for survival of cells. Till now commercially available medium has been modified with permutation and combination to grow the primary cells [1]. Several investigators attempted to develop the primary cell culture system, however, permanent shrimp cell lines were unsuccessful due to the lack of specific medium and supplements [2–5]. Based on these difficulties, the present study aimed to improve the media formulation to enhance the growth and proliferation of cells. Among various commercially available medium, L-15 media showed potential results in promoting the cell growth [6, 7]. Basic cell culture medium provides essential nutrients like salts, amino acids and vitamins for cell growth [8]. For successful development of the cell culture system, the osmolality, pH and temperature in the culture medium must also be known and based on the host conditions it needs to be optimized because these three physical parameters are vital for the in vitro cell culture development. Similarly, shrimp cells culture grow predominantly at the pH

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of hemolymph (7–8), thus ensuring the pH of the medium is within this range is essential for successful growth of cells [9]. The optimum water temperature for shrimp culture is between 25 and 32 °C [10] and for the better growth of cells temperature within this range should be optimized.

Apart from the physical parameters, the growth medium is often supplemented with various nutrients, tissue extracts, fetal bovine serum (FBS) and growth factors to promote cell proliferation [11]. These supplements are selected based on the salt, amino acid, sugar and lipid profile of hemolymph of the specific species being studied. The basal composition of media, including proteins, lipids, carbohydrates, vitamins, amino acids, tissue extract and growth factors, are selected by trial and error by applying various combinations of supplements [12]. Many reports indicated that FBS at 5–20% is essential for cell replication, but it fails to provide complex nutrients like hormones and growth factors that are needed for shrimp cells to grow. Epidermal growth factor (EGF) in the media at 20 ng mL^{-1} promotes cell proliferation and increases the survivability of lymphoid tissue cells of *P. stylirostris* [13].

The greatest advantages in developing a cell culture system are rapidity in forming a monolayer and maintaining a uniform cell number in the culture plate, both of which are essential for most application studies [14]. The present study aimed to optimize the culture medium with various supplements, growth factor and physicochemical factors for growth of hemocytes culture from *P. vannamei* (White shrimp).

Material and methods

Apparently healthy *P. vannamei* (White shrimp) weighing approximately 5–10 g were sourced from local suppliers in and around Chennai, Tamil Nadu, India. They were acclimatized and maintained in a wet lab facility at ICAR-Central Institute of Brackishwater Aquaculture, Chennai. All animals were maintained hygienically in clean 250 L fiber-reinforced plastic (FRP) tanks with adequate aeration (Salinity 28 ± 2‰; temperature 30 °C ± 2 °C). *P. vannamei* were fed with commercially available pellet feed and maintained with a water exchange system. Animals were kept in the laboratory in sterile seawater for three days; every day the water was fully exchanged with sterile sea water to reduce the microbial load.

Prior to sampling, the animals were surface disinfected with 70% alcohol prepared in sterile sea water. After surface sterilization, 1 mL of hemolymph was collected aseptically from the ventral sinus located at the base of the third abdominal segment using a 2 mL syringe containing 1 mL of modified Alsever's solution (27 mM Na citrate, 336 mM NaCl, 115 mM Glucose and 9 mM EDTA and made up to 100 mL using sterile de-ionized water) with the pH of 7.2 and mixed well. The osmolality of the alsever's solution was not checked. The samples were increased to 10 mL with L-15 basal medium, then mixed well and stored immediately in ice for 5 to 10 min to avoid the precipitation of cells or cluster formation. The cells were pelleted by centrifugation at 200×*g* for 10 min at 25 °C and resuspended in selective medium, then seeded in 12-well plates (5×10^3 cells/well) (Nunc, Denmark) and incubated at 28 °C. The hemolymph-based shrimp culture medium (HBSCM-5) is composed of L-15 medium with 15% FBS (Sigma-Aldrich, USA) and 1 × antibiotic mixture (Penicillin 1,000 IU/ml, Streptomycin 1000 µg/ml, Gentamicin 250 µg/ml and Amphotericin B250µg/ml (Life Technologies, USA). The medium was formulated based on the hemolymph component of *P. vannamei* and prepared as per the method described by Sivakumar et al. [15]. The pH was adjusted to 7.2 and osmolality was adjusted to the hemolymph osmolality (730 ± 20 mOsm kg^1). The medium was filtered using a 0.2 µm filter on a vacuum pump, then incubated at room temperature for 24 h to check contamination and stored at 4 ºC for further use.

Effect of fetal bovine serum (FBS)

The hemolymph-based shrimp culture medium (HBSCM-5) medium was combined with six different concentrations of FBS (1, 5, 10, 15, 20 and 25%, Sigma-Aldrich, USA). The collected hemocytes were seeded in each of the different concentrations of FBS with the medium at a concentration of 5×10^3 cells/12 well culture plate. The prepared individual medium was screened for cell growth and incubated at 28 °C.

Effect of osmolality (mOsm)

Modified HBSCM-5 media have shown osmolality levels ranging from 400 to 550 mOsm kg^1. To increase the osmolality to that of the hemolymph of *P. vannamei,* a 25% solution of Sodium chloride was added to the HBSCM-5 medium. HBSCM-5 media with different osmolalities such as 540, 730, 870, 1075, 1270 and 1470 ± 20 mOsm kg^1 were prepared and screened. Sodium chloride is the preferred compound used to adjust the osmolality to that of hemolymph of animals [6, 16].

Effect of pH

pH is an important physical parameter essential for cell growth. The pH of the hemolymph of *P. vannamei* was 6.68. In this study, HBSCM-5 medium at 730 ± 20 mOsm kg^1 containing 1 × antibiotic and antimycotic solution with 15% FBS was adjusted to four different pH levels (6.8, 7.2, 7.5 and 8.0) to determine the effect of different pH levels

on cell growth, cell viability and morphological changes. Effects were observed under an inverted microscope at 20 × magnification.

Effect of temperature (°C)

Temperature is another important physical parameter essential for cell growth. Hemocytes, incubated with HBSCM-5 medium at osmolality 730 ± 20 mOsm kg^1, pH 7.2 with 15% FBS at five different temperatures (20, 24, 28, 32, 35 °C) were screened.

Effect of natural sea water (NSW ‰)

Six different concentrations of natural sea water (NSW; 01, 02, 05, 10, 15 and 20‰, were added to HBSCM-5 medium with pH 7.2 with 15% FBS. The hemocytes were seeded into the 12-well culture plate at concentration of (5×10^3 cells/well) and then incubated at 28 °C for 48 h.

Effect of shrimp muscle extract (SME)

Healthy specimens of *P. vannamei* weighing approximately 10 g were sampled to obtain fresh muscle extract. The samples were homogenized in 100 mL of sterile L-15 medium. The blended mixture was centrifuged at 5000×*g* for 30 min at 4 °C. The top layer was collected and again centrifuged at 13,000×*g* for 15 min at 4 °C. The supernatant was filtered through 0.45 µm paper (Millipore, USA) and stored at –70 °C until further use. Different concentrations (1, 2, 5, 10, 20 and 25%) of shrimp muscle extract were added into the HBSCM-5 medium. The hemocytes were seeded into the 12-well culture plate at concentration of (5×10^3 cells/well) and the cells were incubated at 28 °C for 48 h. The cell morphology was observed at ×20 magnification in inverted microscopy.

Effect of basic fibroblast growth factor (bFGF)

Basic fibroblast growth factor (bFGF) in various concentrations (0.5, 1, 3 and 5 $ngmL^{-1}$ of bFGF) were added to the HBSCM-5 medium with 15% FBS [6]. The hemocytes were seeded into the 12-well plates at a concentration of (5×10^3 cells/well) and the cells were incubated at 28 °C for 48 h. The cell morphology was observed at 20 × magnification in inverted microscopy.

Molecular identification of cell origin

The DNA was extracted from primary monolayer of hemocytes culture from *P. vannamei* and PCR was carried out [15]. The fragments of the cytochrome c oxidase subunit I (COI) genes were amplified using universal primers F 5′ TCAACCAACCACAAAGACATTGGCAC 3′ and R 5′ TAGACTTCTGGGTGGCCAAAGAATCA 3′. The PCR products of the fragments were sequenced by an ABI 3730 DNA analyzer (Applied Biosystems). The sequences of the mtDNA gene fragments were compared with the published and known sequences in the National Centre for Biotechnology database by the basic local alignment search tool (BLAST).

Results

Due to increase in viral diseases, prophylaxis, and lack of treatment due to non-availability of specific treatment for the respective disease, studies on the improvement in the shrimp cell culture system became significant. Cell culture growth in the optimized medium at different levels of osmolality,

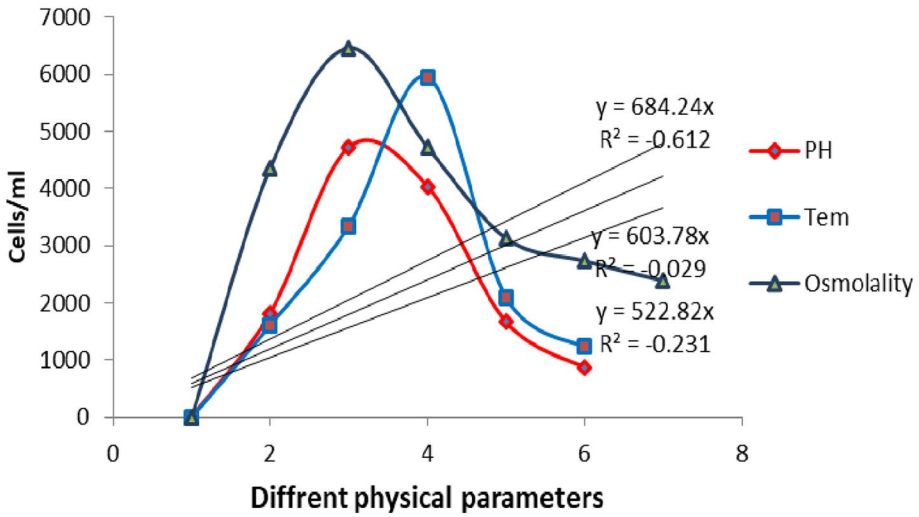

Fig. 1 The effect of pH, Temperature and Osmolality showed cells viability/ml

pH and temperature was also observed (Fig. 1). The potential for optimizing the medium used for hemocytes culture growth of *P. vannamei* by adding different supplements, including fetal bovine serum, shrimp muscle extract, natural sea water, and basic fibroblast growth factor was explored (Fig. 2). When the optimized medium was used, in vitro proliferation of hemocytes of *P. vannamei* was significant and found to adhere within six hours and confluent monolayer formation was achieved within 24–48 h of plating. This monolayer culture was maintained, and healthy cells have shown prominent morphological characteristics. Fibroblast-like and round cells of hemocytes with higher growth and multiplicity were observed in the HBSCM-5 medium (Supplementary Table 1).

Effect of pH

Growth patterns were recorded under different pH levels. At pH 7.2 a large number of healthy hemocytes with good morphology were observed after 24 h of incubation. Formation of hemocytes debris and granulation were observed at pH 7.5 and 8.0, which consequently resulted in cell lysis after 48 h (Supplementary Fig. 1A1–A5). Decreased cell viability and poor attachment were recorded below pH 6.8.

Effect of temperature

Hemocytes observation have shown that the hemocytes incubated at 28 °C were healthy, forming a confluent monolayer and fibroblast-like and round morphology observed (Supplementary Fig. 1B1–B5). Incubation at 20 °C and 24 °C resulted in less hemocytes adherence, no prominent formation of a confluent monolayer and hemocytes were not healthy. At 32 °C and 35 °C the hemocytes were partially attached in the culture plate, lost their original morphology and were lysed within 48 h.

Effect of Osmolality (mOsm)

Osmolality of 730 ± 20 mOsm kg^{-1}, previously found to be the optimal range for hemocytes culture, resulted in confluent monolayer formation and observation of intact fibroblast-like and round morphology. When the osmolality was increased from 730 ± 20 mOsm kg^{-1} to 1470 ± 20 mOsm kg^{-1}, the morphology of the hemocytes changed to crystal-like formation, which resulted in decreased viability. The medium color also changed from pink to brown and hemocytes clumping occurred. Similarly, when the osmolality of the medium was decreased 540 mOsm kg^{-1}, the hemocytes were not healthy and a confluent monolayer formed and also did not form a fibroblast-like morphology in HBSCM-5 medium with 540 ± 20 mOsm kg^{-1} (Supplementary Fig. 2A1–A5).

Effect of basic fibroblast growth factor (bFGF)

Hemocytes were attached uniformly at all concentrations in the range between 0.5 and 5 ng/mL^{-1}, forming a confluent monolayer and fibroblast-like morphology. Healthy elongated cells were observed after 48 h (Supplementary Fig. 2B1–B5).

Effect of fetal bovine serum (FBS)

Poor adherence was observed while using 1% and 5% FBS, and the hemocytes were not healthy. Rapid cell proliferation was recorded at 10% to 20% FBS. Optimal growth was

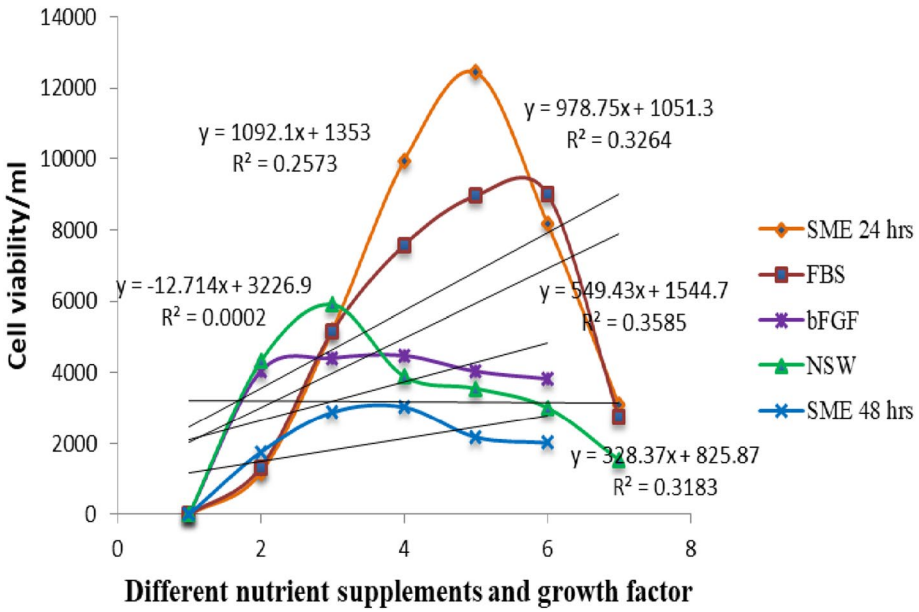

Fig. 2 The effect of SME, FBS, bFGF and NSW showed cells viability/ml

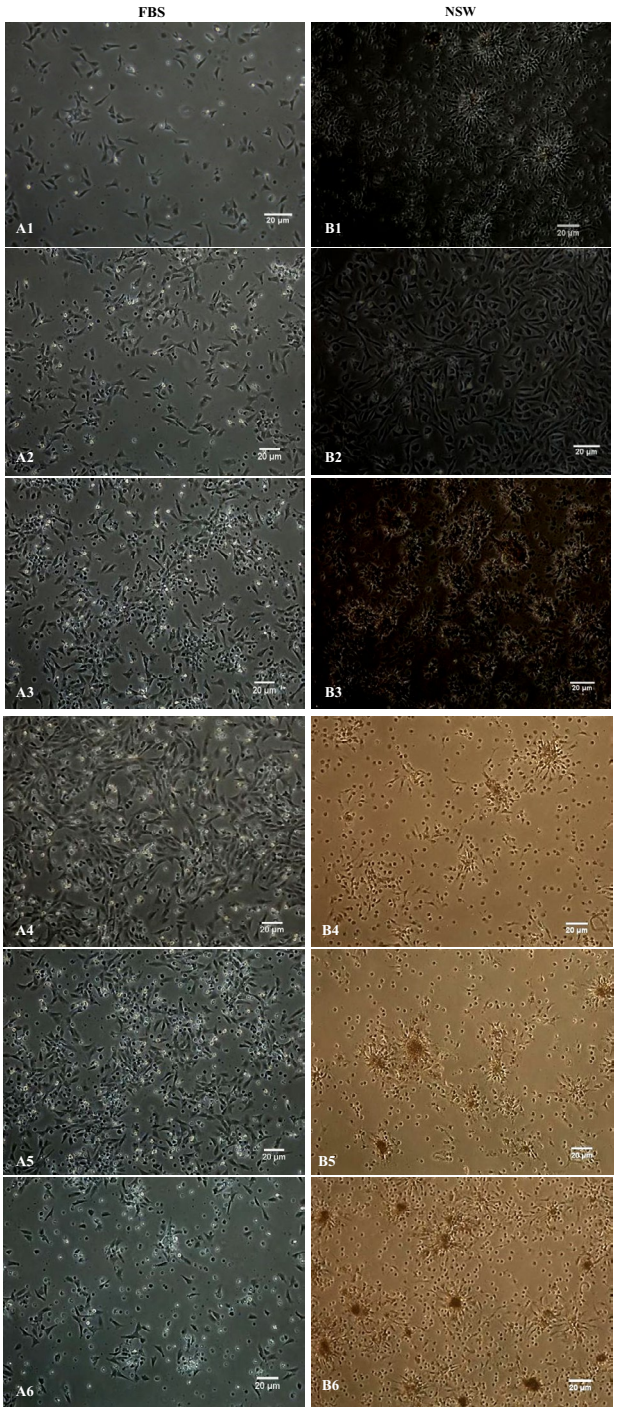

Fig. 3 Hemocytes grown in HBSCM-5 medium with supplemented various concentrations of FBS. Cells grown with FBS concentrations of 2%, 5%, 10%, 15%, 20% and 25% are depicted in **A1**–**A6**, respectively. Hemocytes grown in HBSCM-5 medium supplemented with 15% FBS and prepared with different concentrations of NSW. Hemocytes grown with NSW concentrations of 1‰, 2‰, 5‰, 10‰, 15‰ and 20‰ are shown in B1-B6, respectively

observed at 15% to 20%, which resulted in 90% adherence of seeded hemocytes with noticeable formation of a complete monolayer and good hemocytes viability. At 25% FBS, hemocytes growth was inhibited (Fig. 3A1–A6).

Effect of concentration of natural sea water (NSW‰)

Supplementing the HBSCM-5 medium with NSW concentrations greater than 5‰, resulted in rapid attachment of hemocytes, but hemocytes clumping was also observed (Fig. 3B1–B6). Initially there was a change in the color of the medium within 24 h and hemocytes morphology became crystal-like. At 2‰, NSW, a group of crystals-like cells with reduced viability formed. Most of the cells were floating after 48 h and did not form fibroblast-like morphology in HBSCM-5 medium with a 5 to 20‰ of NSW.

Effect of shrimp muscle extracts (SME)

Hemocytes were not attached at 0% or 1% SME. At 5%, 10% and 20% SME the hemocytes adhered very rapidly, and fibroblast-like cell morphology and healthy hemocytes were observed (Fig. 4A1–A6). At 25% SME, the hemocytes growth was inhibited, and hemocytes lysis was observed after 24 h. Even though 5%, 10% and 20% SME initially promoted growth, the hemocytes were completely lysed within 2 to 3 days (Fig. 4B1–B6).

Molecular identification of the cell line

Molecular identification of cell origin was performed by amplification of the COI mitochondrial DNA. Comparative analysis of the sequences revealed a match of 99% for COI to known *P. vannamei* hemocytes mitochondrial DNA sequences. These sequences thus confirmed that primary hemocytes were derived from *P. vannamei*. These sequences have been submitted to the GenBank with accession number KY564433 [15].

Discussion

Generally, crustacean primary cell culture would not survive for more than five days in the basal medium and the addition of appropriate nutrient supplements is essential for cell proliferation [9]. In order to enhance the growth of the hemocytes from *P. vannamei* previous studies screened various supplements with one or more combinations (Table 1). This study optimized HBSCM-5 medium with various supplementary nutrients, including fetal bovine serum, shrimp muscle extract, natural sea water and basic fibroblast growth factor to explore their ability to promote cell growth. The

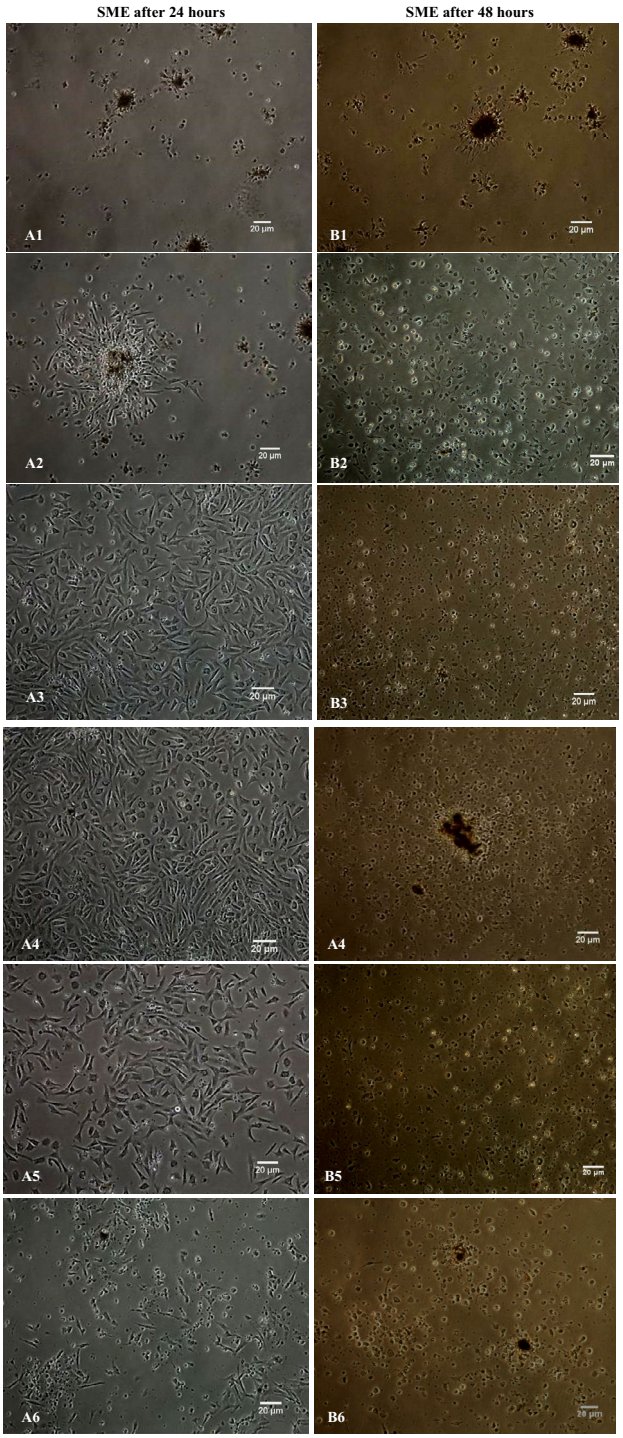

Fig. 4 Hemocytes cultured in HBSCM-5 medium supplemented with various concentrations of shrimp muscle extract (SME). Hemocytes grown in SME concentrations of 1%, 2%, 5%, 10%, 20% and 25% are depicted after 24 h of incubation in **A1**–**A6**, respectively, and after 48 h of incubation in **B1**–**B6**

optimum medium was also prepared at different osmolalities, pH levels and temperatures to explore their effect on hemocytes growth. Proper maintenance of pH in culture media is very essential for the growth of hemocytes. Shrimp are highly sensitive to changes in pH and mortality occurs when the pH is lowered below 6.8 [17]. This was in line with our present study where at 6.5 pH there was a seizure in hemocytes growth. Since the pH of hemolymph was found to be 6.68, the media prepared with pH ranging from 6.8 to 7.2 resulted in clear media without any precipitation and supported better growth of hemocytes. This is similar to many previous studies that suggested an optimum pH range of 6.8–7.6 [1, 5, 17–20].

Temperature plays a vital role in the survival and morphology changes and healthy hemocytes culture. In our study the optimum temperature was 28 °C, which resulted in the formation of a confluent monolayer and intact fibroblast-like and round morphology, both of which concur with the results observed by Goswami et al. [21].

Osmotic pressure is another important physical parameter to be considered for the growth of hemocytes, which not only affects cell proliferation but also the metabolites produced. In this study, promising growth and intact morphology were observed at 730 ± 20 mOsm kg^{1} osmolality. A previous study had shown better growth at osmolalities between 470 and 770 mOsm kg^{1} [5]. In our study, the osmolality range where maximum growth was achieved is in concurrence with the osmolality of hemocyte that was previously measured in the hemolymph sample. In our study, the addition of NSW in the medium as a supplement did not assist in the growth and proliferation of cells. Earlier studies have also shown L-15 medium supplemented with NSW had poor buffering capacity [22]. FBS is the most common and widely used supplement added to enhance cell growth. FBS contains high levels of embryonic growth promoting factors and components that have been shown to satisfy the metabolic requirements of cells during culture [12]. Additionally, hydrocortisone present in FBS was found to promote cell attachment and the growth hormone somatomedin has been observed to have a mitogenic effect [23]. In the present study, FBS used at concentrations between 10 and 20% showed better growth and proliferation of cells, but when increased to 25%, FBS inhibited the cell growth. This is in agreement with earlier reports that also explored the effect of FBS [21, 24]. Some studies reported that 20% FBS highly promoted the cell growth [3, 24, 25]. Usage of 10% FBS has also shown improved cell growth [10, 18].

Hemocytes attachment and proliferation was observed with SME supplementation in HBSCM-5 medium. Even though the hemocytes were healthy morphology observed. Hemocytes had good morphology in the medium containing only SME (no FBS), after which the hemocytes got lysed within 48 h. When 20% SME was incorporated in the medium, hemocytes growth was inhibited. This may be due to the presence of an inhibitory substance in SME. This is also in agreement with the study by [26, 27]. In contrast,

Table 1 Various studies carried out in *P. vannamei* to develop the primary cell culture

S.NO	Authors	Different tissues	Media	Supplements	Osmolality	TEM	PH	Cell culture/growth
1	Luedeman and Lightner [27]	Ovary	Grace's sect medium	10% FBS	700–750	25 °C 28 °C	6.8–7.2	80% confluence formed within a 2-day period
2	Nadala et al. [7]	Oka cells	2 × L-15 medium	20% FBS & 8%SME and 20 ng/ml EGF	750–770	25 °C	proper pH is essential	Found to markedly enhance cell growth. Cells which used to take 2 weeks to form a monolayer
3	Toullec et al. [19]		Grace's insect medium	10% FBS	750–760	29 °C	7.0	Developed primary cell culture maintained up to 2 weeks
4	George and Dhar [10]	Hemocytes and various tissues	Grace's insect medium	10% FBS and 10% SME	730 ± 10	26 °C and 28 °C	-	Improved methods of cell culture from eye stalk, hepatopancreas, muscle, ovary, and hemocytes of shrimp *Penaeus vannamei*
5	George et al. [29]	Hemocytes	2 × Grace's Insect Medium	10% FBS and 10% SME	730 ± 10	27 °C	-	Hemocytes primary cell culture developed from *Penaeus vannamei*
6	Li et al. [5]	Lymphoid organ	2 × L-15 medium	20% FBS	900 ± 20	27 °C	7.5	Promoted the migration of cells from the explants and cell survival. 600 µg/ml cholesterol and 1000 µg/ml L-glutathione (GSH) both enhanced cell survival and performance in vitro
7	Li et al. [30]	Lymphoid organ	2 × L-15 medium	20% FBS & 10% of eye extract	900 ± 20	27 °C	7.5	10% of eye extract or 3% of ovary extract to cells for the maximal health of primary cell cultures from the lymphoid organ of *P. vannamei*
8	Vieira-Giro' et al. [31]	Hemocytes	2 × L-15medium	20% FBS and 2% glucose	720	29 °C	-	These hemocytes remained viable for at least 8 days with a slight increase in viability on that period

SME prepared from juvenile shrimp had a beneficial effect on the growth and proliferation of hemocytes and ovaries [10].

Growth factors are vital elements essential to maintain normal cellular activities. In the present study, all concentrations of bFGF in the medium enhanced the hemocytes growth. Enlarged morphology of the hemocytes was observed when compared with normal hemocytes. bFGF binds to the heparin-like molecule situated in the extracellular matrix of the endothelial cells, thus stimulating cell proliferation and differentiation [28]. Similarly, a high concentration of bFGF will down-regulate the bFGF receptor and induce cell transformation. According to a previous report addition of bFGF stimulated cell growth and able to withstand passage for 90 times in the lymphoid culture [9].

Conclusion

The hemocytes culture was optimized for use as an in vitro cell culture system by testing cell growth on HBSCM-5 medium with various supplements, growth factors and physical parameters. Hemocytes culture was optimized for supplements including fetal bovine serum, shrimp muscle extract, and basic fibroblast growth factor, as well as physico-chemical factors including pH, temperature, osmolality and natural sea water concentration. Based on the hemocytes morphology, growth, and tendency for cell proliferation and hemocytes viability were observed. An optimized media with known concentrations of supplements, growth factors and physico-chemical factors will be significant for a researcher. The in vitro hemocytes culture system grown in our study would be useful for further research and for developing an immortal cell line and specific culture medium.

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Author contributions Conceived and designed the experiments: N.K, and performed the experiments and wrote the Manuscript: S.S.

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Declarations

Conflict of interest The authors declare no conflict of interest to research. We certify that the submission is original work and is not under review at any other publication.

Ethical approval Not applicable for shrimp cell culture.

Consent to participate Not applicable.

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