An optimization of supplements and physical factors for growth of hemocyte cell culture from Penaeus vannamei in selective medium

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Research Article

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Abstract
Optimization of cell culture medium was carried out with various supplements and physical factors for the growth of hemocyte cell cultures from Penaeus vannamei. Hemolymph-based shrimp culture medium (HBSCM-5 medium). Various concentrations of fetal bovine serum (FBS; 1–25%), shrimp muscle extract (SME; 1–25%) and basic fibroblast growth factor (bFGF; 0.5 to 5 ng mL\(^{-1}\)) were attempted to optimize the media for the development of primary hemocyte cell culture of P. vannamei. 15% FBS was ideal for the healthy morphology of cells with rapid replication. SME supplementation at 5–20% supported the cell growth for 24 hours but only 30% of cell viability was observed after 48 hours. bFGF (0.5 to 5 ng mL\(^{-1}\)) enhanced cell growth in the medium with 15% FBS; Cells were healthy at all concentrations of bFGF and showed enlarged fibroblast-like morphology. HBSCM-5 medium containing 15% FBS was prepared with various levels of osmolality (540 to 1470 ± 20 mOsm kg\(^{-1}\)). The ideal pH level was examined by preparing the HBSCM-5 medium at pH between 6.8 and 8.0. Temperatures ranging from 20°C to 35°C and natural sea water (NSW) concentrations between 1% and 20% were also tested to determine their effect on cell growth and proliferation. 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, at salt concentrations of more than 2%, cells did not form fibroblast-like morphology and instead a crystal-like morphology was observed.

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 (Jayesh et al., 2013). 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 (Jiang et al. 2006; Claydon and Owens, 2008; Jose et al. 2012; Li et al. 2014). 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 (Nadala et al., 1993; Mulford et al., 2000). Basic cell culture medium provides essential nutrients like salts, amino acids and vitamins for cell growth (Mothersill and Austin 2000). 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 of hemolymph (7 to 8), thus ensuring the pH of the medium is within this range is essential for successful growth of cells (Hsu et al., 1995). The optimum water temperature for shrimp culture is between 25°C to 32°C (George and Dhar, 2010) 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 (Mitsuhashi, 2001). 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 (Jayesh, 2012). 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 20ng mL\(^{-1}\) promotes cell proliferation and increases the survivability of lymphoid tissue cells of *P. stylirostris* (Tapay et al., 1995).

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 (Jose et al., 2010). The present study aimed to optimize the cell culture medium with various supplements, growth factor and physicochemical factors for growth of hemocyte cell culture from *P. vannamei* (White shrimp).
Material And Methods

Shrimp acclimation and tissue dissection

Apparently healthy _P. vannamei_ weighing approximately 5 to 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) and mixed well. The samples were increased to 10 mL with L-15 basal medium, then mixed well and stored immediately in ice for 5 to 10 minutes. The cells were pelleted by centrifugation at 200 xg for 10 minutes at 25°C and resuspended in selective medium, then seeded in 12-well plates (5x10^3 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 1x antibiotic mixture (Penicillin 1,000IU/ml, Streptomycin 1,000µg/ml, Gentamicin 250µg/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. (2019).

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 hours to check contamination and stored at 4°C 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 hemocyte cells were seeded in each of the different concentrations of FBS with the medium at a concentration of 5x10^3 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 (Mulford and Austin 1998; Mulford et al., 2000).

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 1x 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 20x magnification.

Effect of temperature (°C)
Temperature is another important physical parameter essential for cell growth. Hemocyte cells, 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 hemocyte cells were seeded into the 12-well culture plate at concentration of (5×10\(^3\) cells/well) and then incubated at 28°C for 48 hours.

**Effect of shrimp muscle extract (SME)**

Healthy specimens of *P. vannamei* weighing approximately 10 g were sampled to obtain fresh gill and muscle tissue, which was then homogenized in 100 mL of sterile L-15 medium. The blended mixture was centrifuged at 5,000 xg for 30 minutes at 4°C. The top layer was collected and again centrifuged at 13,000 xg for 15 minutes at 4°C. The supernatant was filtered through 0.45µm paper (Millipore, USA) and stored at -70°C until further use. Different concentrations (01, 02, 05, 10, 20 and 25%) of shrimp muscle extract were added into the HBSCM-5 medium. The hemocyte cells 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 hours. The cell morphology was observed at 20x 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. The hemocyte cells 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 hours. The cell morphology was observed at 20x magnification in inverted microscopy.

**Molecular identification of cell origin**

The DNA was extracted from primary monolayer cells of hemocyte from *P. vannamei* and PCR was carried out (Sivakumar et al., 2019). The fragments of the cytochrome c oxidase subunit I (COI) genes were amplified using universal primers F 5′ TCAACCAACCACAAGACATTGCCAC 3′ and R 5′ TAGACTTCTGGGTGGCACAAGATCA 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. The potential for optimizing the medium used for hemocyte cell 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. Cell culture growth in the optimized medium at different levels of osmolality, pH and temperature was also observed. When the optimized medium was used, in vitro proliferation of hemocyte of *P. vannamei* was significant and found to adhere within six hours and confluent monolayer formation was achieved within 24-48 hours 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.

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

**Effect of temperature**

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

**Effect of Osmolality (mOsm)**

Osmolality of 730 ± 20 mOsm kg\(^{-1}\), previously found to be the optimal range for hemocyte cell 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 cells changed to crystal-like formation, which resulted in decreased viability. The medium color also changed from pink to brown and cell clumping occurred. Similarly, when the osmolality of the medium was decreased 540 mOsm kg\(^{-1}\), the cells 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\(^{-1}\) (Fig.2. A1-A6).

**Effect of basic fibroblast growth factor (bFGF)**

Cells 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 hours (Fig.2. B1-B6).

**Effect of fetal bovine serum (FBS)**

Poor adherence was observed while using 1% and 5% FBS, and the cells were not healthy. Rapid cell proliferation was recorded at 10% to 20% FBS. Optimal growth was observed at 15% to 20%, which resulted in 90% adherence of seeded cells with noticeable formation of a complete monolayer and good cell viability. At 25% FBS, cell growth was inhibited (Fig.3. A1-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 cells, but cell clumping was also observed (Fig.3. B1-B6). Initially there was a change in the color of the medium within 24 hours and cell 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 hours and did not form fibroblast-like morphology in HBSCM-5 medium with a 5 to 20‰ of NSW.

**Effect of shrimp muscle extracts (SME)**

Cells were not attached at 0% or 1% SME. At 5%, 10% and 20% SME the cells adhered very rapidly, and fibroblast-like cell morphology and healthy cells were observed (Fig.4. A1-A6). At 25% SME, the cell growth was inhibited, and cell lysis was observed after 24 hours. Even though 5%, 10% and 20% SME initially promoted cell growth, the cells were completely lysed within 2 to 3 days (Fig.4. B1-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* hemocyte cell mitochondrial DNA sequences. These
sequences thus confirmed that primary hemocyte cells were derived from *P. vannamei*. These sequences have been submitted to the GenBank with accession number KY564433 (Sivakumar et al. 2019).

**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 (Hsu et al., 1995). In order to enhance the growth of the cells from *P. vannamei* previous studies screened various supplements with one or more combinations (Table 2). 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 optimum medium was also prepared at different osmolalities, pH levels and temperatures to explore their effect on cell growth. Proper maintenance of pH in culture media is very essential for the growth of cells. Shrimp are highly sensitive to changes in pH and mortality occurs when the pH is lowered below 6.8 (Han et al., 2013). This was in line with our present study where at 6.5 pH there was a seizure in cell 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 cells. This is similar to many previous studies that suggested an optimum pH range of 6.8 to 7.6 (Toullec et al. 1996; Maeda et al. 2003; Zeng et al. 2010; Han et al. 2013; Jayesh et al. 2013; Li et al. 2014).

Temperature plays a vital role in the survival and morphology changes and healthy cells of hemocyte. In our study the optimum temperature was 28°C, which resulted in the formation of a confluent monolayer of cells and intact fibroblast-like and round morphology, both of which concur with the results observed by Goswami et al., (2010).

Osmotic pressure is another important physical parameter to be considered for the growth of cells, which not only affects cell proliferation but also the metabolites produced. In this study, promising growth and intact cell morphology were observed at 730 ± 20 mOsm kg\(^{-1}\) osmolality. A previous study had shown better growth at osmolalities between 470 mOsm kg\(^{-1}\) and 770 mOsm kg\(^{-1}\) (Li et al., 2014). In our study, the osmolality range where maximum growth was achieved is in concurrence with the osmolality of hemocyte cells 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 (Sashikumar and Desai, 2008). 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 (Jayesh et al., 2012). Additionally, hydrocortisone present in FBS was found to promote cell attachment and the growth hormone somatomedin has been observed to have a mitogenic effect (Freshney, 2005). 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 (Kasornchandra et al., 1999; Goswami et al., 2010). Some studies reported that 20% FBS highly promoted the cell growth (Kasornchandra et al., 1999; Jiang et al., 2006; Jose et al., 2011). Usage of 10% FBS has also shown improved cell growth (Maeda et al. 2003; George and Dhar, 2010).

Hemocyte cell attachment and proliferation was observed with SME supplementation in HBSCM-5 medium. Even though the cells were healthy morphology observed. Hemocyte cells had good morphology in the medium containing only SME (no FBS), after which the cells got lysed within 48 hours. When 20% SME was incorporated in the medium, cell 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 (Fraser and Hall, 1999; Luedeman and Lightner, 1992). In contrast, SME prepared from juvenile shrimp had a beneficial effect on the growth and proliferation of hemocytes and ovaries (George and Dhar, 2010).

Growth factors are vital elements essential to maintain normal cellular activities. In the present study, all concentrations of bFGF in the medium enhanced the cell growth. Enlarged morphology of the cells was observed when compared with normal cells. bFGF binds to the heparin-like molecule situated in the extracellular matrix of the endothelial cells, thus stimulating cell
proliferation and differentiation (Ma et al., 2017). 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 (Hsu et al., 1995).

**Conclusion**

The hemocyte cell 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. Hemocyte 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 cell morphology, growth and tendency for cell proliferation and also cell viability were observed. An optimized media with known concentrations of supplements, growth factors and also physico-chemical factors will be significant for a researcher. The *in vitro* hemocyte cell culture system grown in our study would be useful for further research and for developing an immortal cell line and specific culture medium.

**Declarations**

**Acknowledgments**

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**Conflict-of-interest statement**

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.

**Ethics statement**

Not applicable for shrimp cell culture

**Consent to participate**

Not applicable

**Author Contributions**

Conceived and designed the experiments: Dr.N.Kalaimani, and performed the experiments and wrote the Manuscript: Dr.S. Sivakumar.

**References**


Tables

Table 1. Supplement and physical factor combinations tested on HBSCM-5 medium and associated observations.
<table>
<thead>
<tr>
<th>No</th>
<th>Supplement and/or Physical factor</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1% FBS</td>
<td>Very few cells attached</td>
</tr>
<tr>
<td>2</td>
<td>5% FBS</td>
<td>Partial cell attachment but no replication</td>
</tr>
<tr>
<td>3</td>
<td>HBSCM-5 medium with 10% FBS</td>
<td>Cell proliferation</td>
</tr>
<tr>
<td>4</td>
<td>HBSCM-5 medium with 15% FBS</td>
<td>Rapid proliferation and healthy cells</td>
</tr>
<tr>
<td>5</td>
<td>HBSCM-5 medium with 20% FBS</td>
<td>Proliferation and healthy cells</td>
</tr>
<tr>
<td>6</td>
<td>HBSCM-5 medium with 25% FBS</td>
<td>Cell attachment but no replication</td>
</tr>
<tr>
<td>7</td>
<td>HBSCM-5 medium with 15% FBS</td>
<td>Rapidly attachment and enlarged the cell morphology was observed and 0.5 ng mL(^{-1}) bFGF</td>
</tr>
<tr>
<td>8</td>
<td>HBSCM-5 medium with 15% FBS</td>
<td>Rapidly attachment and enlarged the cell morphology was seen and 1 ng mL(^{-1}) of bFGF</td>
</tr>
<tr>
<td>9</td>
<td>HBSCM-5 medium with 15% FBS</td>
<td>Rapidly attachment and enlarged the cell morphology was observed and 3 ng mL(^{-1}) of FGF</td>
</tr>
<tr>
<td>10</td>
<td>HBSCM-5 medium with 15% FBS</td>
<td>Rapidly attached and enlarged the cell morphology was seen and 5 ng mL(^{-1}) of FGF</td>
</tr>
<tr>
<td>11</td>
<td>HBSCM-5 medium with 15% FBS</td>
<td>Cells attached but most of the cells showed round morphology and 540 ± 20 mOsm kg(^{-1})</td>
</tr>
<tr>
<td>12</td>
<td>HBSCM-5 medium with 15% FBS</td>
<td>Rapid cell attachment, fibroblast-like morphology and cell proliferation was observed and 730 ± 20 mOsm kg(^{-1})</td>
</tr>
<tr>
<td>13</td>
<td>HBSCM-5 medium with 15% FBS</td>
<td>Cells attachment, medium color changed and cells formed clump was observed and 870 ± 20 mOsm kg(^{-1})</td>
</tr>
<tr>
<td>14</td>
<td>HBSCM-5 medium with 15% FBS</td>
<td>Cells attachment, medium color changed and cells formed mass clump was observed and 1075 ± 20 mOsm kg(^{-1})</td>
</tr>
<tr>
<td>15</td>
<td>HBSCM-5 medium with 15% FBS</td>
<td>Medium color changed and most of the cells formed like-cluster were seen and 1270 ± 20 mOsm kg(^{-1})</td>
</tr>
<tr>
<td></td>
<td>Medium Details</td>
<td>Observations</td>
</tr>
<tr>
<td>---</td>
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</tr>
<tr>
<td>16</td>
<td>HBSCM-5 medium with 15% FBS and 1470 ± 20 mOsm kg(^1)</td>
<td>Medium color also changed and most of the cells formed mass cluster.</td>
</tr>
<tr>
<td>17</td>
<td>HBSCM-5 medium with 15% FBS and pH 6.8</td>
<td>Cells attached but most of the cells got lysis within 24 hours</td>
</tr>
<tr>
<td>18</td>
<td>HBSCM-5 medium with 15% FBS and pH 7.2</td>
<td>Rapid cell attachment, healthy cells and good morphology and proliferation was observed</td>
</tr>
<tr>
<td>19</td>
<td>HBSCM-5 medium with 15% FBS and pH 7.5</td>
<td>Less cells attachment and cells got lysis within 2 days</td>
</tr>
<tr>
<td>20</td>
<td>HBSCM-5 medium with 15% FBS and pH 8.0</td>
<td>Few cells attached, proliferation not seen and cells got lysis within 2 days</td>
</tr>
<tr>
<td>22</td>
<td>HBSCM-5 medium with 15% FBS and 20°C temperature</td>
<td>Few cells attached and cell proliferation was not seen</td>
</tr>
<tr>
<td>23</td>
<td>HBSCM-5 medium with 15% FBS and 24°C TM</td>
<td>Very less cells attached and cell proliferation was not observed</td>
</tr>
<tr>
<td>24</td>
<td>HBSCM-5 medium with 15% FBS and 28°C TM</td>
<td>Rapid cells attachment</td>
</tr>
<tr>
<td>25</td>
<td>HBSCM-5 medium with 15% FBS and 32°C TM</td>
<td>Cells partially attachment and cells got lysis</td>
</tr>
<tr>
<td>26</td>
<td>HBSCM-5 medium with 15% FBS and 35°C TM</td>
<td>Few cells attached and no further progress</td>
</tr>
<tr>
<td>27</td>
<td>HBSCM-5 medium with 01% SME</td>
<td>Only few cells attached</td>
</tr>
<tr>
<td>28</td>
<td>HBSCM-5 medium with 05% SME</td>
<td>Some cells attached and no further cell proliferation</td>
</tr>
<tr>
<td>29</td>
<td>HBSCM-5 medium with 10% SME</td>
<td>Moderately attachment the cells and cell proliferation was observed</td>
</tr>
<tr>
<td>30</td>
<td>HBSCM-5 medium with 15% SME</td>
<td>Rapid attachment, healthy cell morphology and proliferation was observed, after 48 hrs the cells got lysis completely</td>
</tr>
<tr>
<td>31</td>
<td>HBSCM-5 medium with 20% SME</td>
<td>Healthy and proliferation of cells was observed, after 48 hrs cells got lysis completely</td>
</tr>
<tr>
<td>32</td>
<td>HBSCM-5 medium with 25% SME</td>
<td>Less cells attached and inhibited the cell growth and cells got lysis within 48 hrs</td>
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<tr>
<td>33</td>
<td>HBSCM-5 medium with 30% SME</td>
<td>Cell proliferation was observed</td>
</tr>
<tr>
<td>Experiment</td>
<td>Medium Composition</td>
<td>Observation</td>
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<td>------------</td>
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<tr>
<td>34</td>
<td>HBSCM-5 medium with 15% FBS and 1‰ NSW</td>
<td>Proliferation of cells seen</td>
</tr>
<tr>
<td>35</td>
<td>HBSCM-5 medium with 15% FBS and 2‰ NSW</td>
<td>Most of the cells showed round type morphology</td>
</tr>
<tr>
<td>36</td>
<td>HBSCM-5 medium with 15% FBS and 5‰ NSW</td>
<td>The medium color changed and clump of cells was seen</td>
</tr>
<tr>
<td>37</td>
<td>HBSCM-5 medium with 15% FBS and 10‰ NSW</td>
<td>The medium color changed and mass clump of cells was seen</td>
</tr>
<tr>
<td>38</td>
<td>HBSCM-5 medium with 15% FBS and 20‰ NSW</td>
<td>The medium color changed and mass clump of cells was seen</td>
</tr>
</tbody>
</table>

**Table 2:** Various studies carried out in *P. vannamei* to develop the primary cell culture
<table>
<thead>
<tr>
<th>S.NO</th>
<th>Authors</th>
<th>Different Tissues</th>
<th>Media</th>
<th>Supplements</th>
<th>Osmolality</th>
<th>TEM</th>
<th>PH</th>
<th>Cell culture/growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Luedeman and Lightner, 1992</td>
<td>Ovary</td>
<td>Grace's sect medium</td>
<td>10% FBS</td>
<td>700-750</td>
<td>25°C</td>
<td>6.8-7.2</td>
<td>80% confluence formed within a 2-day period</td>
</tr>
<tr>
<td>2</td>
<td>Nadala et al., 1993</td>
<td>Oka cells</td>
<td>2x L-15 medium</td>
<td>20% FBS &amp; 8% SME and 20ng/ml EGF</td>
<td>750-770</td>
<td>25°C</td>
<td>Proper pH is essential for cell growth. Cells which used to take 2 weeks to form a monolayer.</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Toullec et al., 1996</td>
<td>Grace's insect medium</td>
<td>10% FBS</td>
<td>750-760</td>
<td>29°C</td>
<td>7.0</td>
<td>Developed primary cell culture maintained up to 2 weeks</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>George and Dhar, 2010</td>
<td>Hemocytes and various tissues</td>
<td>Grace's insect medium</td>
<td>10% FBS and 10% SME</td>
<td>730± 10</td>
<td>26°C</td>
<td>-</td>
<td>Improved methods of cell culture from eye stalk, hepatopancreas, muscle, ovary, and hemocytes of shrimp <em>P. vannamei</em></td>
</tr>
<tr>
<td>5</td>
<td>George et al., 2011</td>
<td>Hemocytes</td>
<td>2x Grace's Insect Medium</td>
<td>10% FBS and 10% SME</td>
<td>730± 10</td>
<td>27°C</td>
<td>-</td>
<td>Hemocytes primary cell culture developed from <em>P. vannamei</em></td>
</tr>
<tr>
<td>6</td>
<td>Li et al., 2014</td>
<td>Lymphoid organ</td>
<td>2x L-15 medium</td>
<td>20% FBS</td>
<td>900 ± 20</td>
<td>27°C</td>
<td>7.5</td>
<td>Promoted the migration of cells from the explants and cell survival. 600 μg/ml cholesterol 1000 μg/ml L-glutathione (GSH) both enhanced cell survival and performance in vitro.</td>
</tr>
<tr>
<td>7</td>
<td>Li et al., 2015</td>
<td>Lymphoid organ</td>
<td>2x L-15 medium</td>
<td>20% FBS &amp; 10% of eye extract</td>
<td>900 ± 20</td>
<td>27°C</td>
<td>7.5</td>
<td>10% of eye extract or 3% of ovary extract to cells for the maximal health of primary cell cultures from the lymphoid organ of <em>P. vannamei</em>.</td>
</tr>
<tr>
<td>8</td>
<td>Vieira-</td>
<td>Hemocytes</td>
<td>2x L-15</td>
<td>20% FBS</td>
<td>720</td>
<td>29°C</td>
<td>-</td>
<td>These</td>
</tr>
</tbody>
</table>
Gira’ et al., 2017

and 2% glucose hemocytes remained viable for at least 8 days with a slight increase in viability on that period.

**Figures**

**Figure 1**

A) Hemocyte cells grown in HBSCM-5 medium supplemented with 15% FBS and prepared at different pH levels. Cells grown at pH levels of 6.8, 7.2, 7.5 and 8.0 are depicted in A1-A5, respectively. B) Hemocyte cells grown in HBSCM-5 medium supplemented with 15% FBS and incubated at different temperatures. Cells grown at temperatures of 20ºC, 24ºC, 28ºC, 32ºC and 35ºC are depicted in B1-B5, respectively.

**Figure 2**

A) Hemocyte cells grown in HBSCM-5 medium supplemented with 15% FBS and prepared at different osmolalities. Cells grown with 540, 730, 870, 1075, 1270 and 1470 ± 20 mOsm kg⁻¹ are depicted in A1-A6, respectively. B) Hemocyte cells grown in HBSCM-5 medium supplemented with 15% FBS and different concentrations of bFGF. Cells grown at bFGF concentrations of 0, 0.5, 1, 3 and 5 ng/mL are shown in C1-C5, respectively. All cells were observed after 48 hours of incubation using inverted phase contrast microscopy at 20X magnification.

**Figure 3**

A) Hemocyte cells 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. Hemocyte cells grown in HBSCM-5 medium supplemented with 15% FBS and prepared with different concentrations of NSW. Cells grown with NSW concentrations of 1‰, 2‰, 5‰, 10‰, 15‰ and 20‰ are shown in B1-B6, respectively.

**Figure 4**

B) Hemocyte cells cultured in HBSCM-5 medium supplemented with various concentrations of shrimp muscle extract (SME). Cells grown in SME concentrations of 1%, 2%, 5%, 10%, 20% and 25% are depicted after 24 hours of incubation in B1-B6, respectively, and after 48 hours of incubation in C1-C6.
Figure 5

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

Figure 6

The effect of SME, FBS, bFGF and NSW showed cells viability/ml
Supplementary Files

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