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Creation of Insect Cell Line Using *Periplaneta americana*

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ABSTRACT
Insect cell systems constitute a major bio-manufacturing platform for production of commercially important molecules in the field of bio-therapeutics & agro-based industries. Insect cell lines are good target cells which can be utilized in cell suspension cultures to produce various therapeutics & economically important biologically active target molecules. We have developed the primary culture from insect cells and characterized a cell line with focus on a development of insect cell clone which could be utilized to express target proteins. Indian species of cockroaches, Periplaneta americana were used to create two primary cell cultures, derived from thoracic and coxal regions of adult cockroaches. A standard Sf-9 cell line was used to study various physiological factors involved in the growth and maintenance of insect cells. A comparative study between Sf-9 cell line and primary cell line isolated from Periplaneta americana was carried using flow cytometry to obtain FSC-SSC analysis, cell count, and cell size of different cells.

Keywords: Insect cell culture; Primary culture; Sf-9; Periplaneta americana; FACS

INTRODUCTION
For years, several host organisms have been used for the expression of recombinant protein including bacteria, yeasts, mammalian cell lines, insect cell lines. The importance gained by vertebrate cell culture research led to an urgent need to have a parallel system from arthropods especially from insects. The field of insect cell culture started off after the successful establishment of a cell line from ovarian tissue of pupa of the Lepidopteran species (*Bombyx mori*).[1] This major breakthrough was a result of patience, the availability of antibiotics, and improved media. As the field matured, diverse insect cell lines have been established (>500) creating some of the most interesting lines, like those from endocrine glands or neurons. A few cell lines have gained commercial value while the potential of the other established lines has not been fully exploited. Some of them are the cell lines derived from *Bombyx mori* (silkworm), *Mamestra brassiere* (a species of moth), *Spodoptera frugiperda* (army worm), *Trichoplusia ni* (cabbage looper), *Drosophila melanogaster* (house fly). Commercial cell lines include *Spodoptera frugiperda*- 21 (Sf-21), Sf-9 and High Five cell lines. Sf-21, first cell line which was intensively used in industries was extracted from the ovarian tissues of *S. frugiperda*.[1] Sf-9 cell line is derived from Sf-21, being the most widely used insect cell line, finds its applications in most biopharmaceutical industries.

The practice of insect cell culture in vitro is very well established and the application of recombinant baculovirus vectors for abundant production of proteins or for manufacture of virus like particles is robust, safe and scalable.[2] Today, insect cell cultures are widely used in various fields like viral diagnosis and biotechnology, for the production of viral pesticides and vaccines as well as for research in areas of molecular biology, endocrinology, biochemistry, genetics and virology.[3] Sf-9 being a standard and well-studied cell line was used to compare and analyze the properties of cell line from *Periplaneta americana*. The factors like growth, nutritional requirements, stability, etc. were studied to understand the lesser known characteristics of the newly established cell line in a better way.

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MATERIALS AND METHOD

Chemicals and Equipments:

HiMedia-Dextrose, Albeneazole (de-worming agent), Grace's Insect Tissue Culture Medium (TNM-FH), HiMedia-Glucose Supplement, Standard BSA solution, Radioimmuno-precipitation assay buffer (RIPA), 70% Ethanol, 10% fetal bovine serum, 10% Di-methly sulfoxide (DMSO), 0.1 mg/ml Streptomycin, 5000 IU Penicillin, Amphotericin-B, fungizone, Amoxycillin, 50 µg/ml Gentamicin, Bradford's reagent, Propidium Iodide, Pharmaceutical Grade Water, Trypan Blue, FACS consumables. Laminar Flow, Thermo-Scientific EVOS FL Auto-inverted microscope, Dissection kit, Remi Centrifuge R8, Water purification system-Milli Q Millipore, Thermo-Scientific CO_2 Incubator, Spectrophotometer, Thermo-Scientific Attune NxT Flow Cytometer, Magnetic Stirrer, Hemocytometer, Autoclave

Optimization of Sf-9 suspension Culture:

For preparing a suspension cell culture from a previous flask, cell count was taken from the flask. The T-flasks were set on a rocker which was carefully placed inside an incubator thus providing the required conditions for proliferation of cell line. For preparation of suspension culture, the cells were disengaged from the walls of the flasks and their density was reduced 2-5×10^4 cells/ml. The suspension culture was scaled-up in sterile laboratory grade borosilicate glass bottles up to 80mL. The seed volume to be taken from the seeding flask, to have a starting cell density of 4×10^4 cells/ml in the bottle was calculated. Streptomycin antibiotic solution was added in traces at regular intervals to avoid contamination. The bottles were placed on magnetic stirrer at a speed of 80-90 rpm for proper stirring and distribution of the nutrient media components.

Growth Curve and Estimation of protein concentration:

Cell density of Sf-9 suspended cells was monitored for around 20 days. Estimation of protein concentration required lysis of the cells so as to carry out the standard Bradford's assay. For lysing the cells, radioimmuno-precipitation assay buffer (RIPA) was added to the cell pellet after centrifugation. The sample was then sonicated with a pulse of 10 seconds. The sample was re-centrifuged and the supernatant was used to carry out Bradford assay. A standard BSA graph was plotted which was employed to estimate the protein concentration (at 595nm) using Bradford's assay. Growth profile of cells and protein concentration was expressed together.

Development of New Cell Line:

Before dissecting the cockroaches, they were kept on a diet consisting of dextrose and deworming agents so that chances of contamination could be reduced. This would prevent the culture from being contaminated by the worms or other contaminants which are generally present in the gastrointestinal tract. Using aseptic techniques and sterile instruments, the cockroaches were dissected and the cells from their thoracic, abdominal and coxal regions were extracted. The cells were collected in 15ml centrifuge tubes and were centrifuged at 3000 rpm for 10 minutes after which they were filtered using a membrane. Spent medium was removed and fresh media was added to the cells and they were resuspended into 96 well-plate and were kept in CO_2 incubator. Grace supplemented medium containing gentamicin was used for further growth of cells. When un-supplemented Grace's media is used, it is supplemented with 10% FBS for optimal growth whereas 5% of FBS was used with TNM-FH insect media. [7] Optimum pH (6.1-6.2) and sodium bicarbonate levels were maintained for healthy growth of the cells. Serum free media is generally preferred for insect cell line development. In spite of this, serum was an essential component of media in order to maintain the cells for longer periods of time.[8,9] Streptomycin was used 0.1 mg/ml and penicillin was used 5000 IU. Also, Amphotericin-B (fungi zone) and amoxicillin was supplemented and the cell growth was found to be healthier. The cells were maintained at 27^0C and 5% CO_2 in a non-humidified incubator. This temperature range is critical for maintaining robust cell growth.

Primary Cell Culture:

The cockroaches were fed on a controlled diet of dextrose and deworming agents for about ten days to reduce the chances of contamination. They were disinfected by wiping with 70% ethanol under laminar flow hood.[10] The cockroaches were rinsed twice with distilled water. Using aseptic techniques and sterile instruments, the cockroaches were dissected and the cells from their thoracic, abdominal and coxal regions

were extracted.[11] The cells were collected and were centrifuged at 3000 rpm for 10 minutes after which they were filtered using a membrane. Spent medium was removed and fresh media was added to the cells and they were re-suspended into 96 well-plate and were kept in CO_2 incubator. The cell count was taken before the cells were kept in the incubator. The plate was kept in the incubator for three days at the specific temperature of 27^0C and CO_2 concentration. After three days the cells were taken out to check the viability and growth, the cell count was taken again using a hemocytometer.[12] Since insect cells do not adhere to the plate, trypsinization was not required. When confluent growth was observed and the cell count per well became high, the cells were passaged. The cell lines were sub-cultured numerous times and were checked for contaminants on regular time interval.

Flow Cytometry:

Propidium iodide (PI) is a DNA binding dye which stains the dead cell proportion within a cellular population. It was used to stain the cells from the established thoracic culture of *Periplaneta americana.* 10 µl of PI was added with the cells and sample for FACS was prepared. Forward and side scatter plot were used to set-up the populations for PI staining analysis. By gating on different sub-populations their properties with regard to different parameters were determined.

RESULTS AND DISCUSSION

Standardization of Sf-9 cell line suspension culture:

Before development of *P. americana* cell line we decided to standardize the Sf9 cell line. After receiving the cells from master cell bank, efforts were made to maintain the cell lines. The cells were maintained and passaged after observing confluent growth (Fig 1a,b). After the fifth passage, once the cell density reached 1×10^7 the cells were cryopreserved. The cells were revived and were checked for viability using trypan blue dye. The cells were transferred into borosilicate glass bottles to study their growth in suspension culture. After achieving confluent growth, samples were taken and analysed for estimation of protein using Bradford's assay and a growth curve was plotted (Fig.2) It was observed that protein concentration was in association of Cell growth in suspension culture. It indicates that normal cell growth was maintained in suspension culture, which is otherwise not a natural growth state of insect cells. This work has built basic foundation for further successful recombinant production of proteins using suspension insect cell culture.

Isolation and development of *P. americana* cell line:

Direct Observation:

Characterization and morphology of insect cells was studied in order to maintain a pure cell line. [10] For this purposes, the cells were first observed directly under inverted microscope and then qualitative analysis was done. After direct observation, it was found that the cells were circular in nature and cluster of cells were formed. Cell count was compiled in table 1.

After dissecting the cockroaches and isolating the cells from the organs, the cells were maintained by providing optimum growth conditions.

Use of Flow Cytometer:

Cells were isolated from abdominal region, thorax region and coxal region of *Periplaneta americana* and studied their viability and stability. The cells were observed using scientific EVOS™ FL cell imaging system, and the cell number was found to increase gradually. Maximum cell number was observed for cells isolated from abdominal region. In comparison to abdominal and coxal region, cells found in thorax region were having more viability and purity of that cell source was good. The cells were seen to be circular in shape with a diameter of 10-15 µm. The cells were further analyzed using flow cytometry so as to understand the morphology of the cell like the cell size, granularity and viability. Following percentage was obtained for PI staining given in Figure 3. Standard Sf9 cells were compared thorax region cells isolated from *P.americana*. In this figure it was observed that though Sf9 cells were from standard cell line single distinct population was not observed. Thorax region isolated cells have shown single distinct population in given figure. In figure 4

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Cells were stained with propidium iodide and then were subjected to flow cytometry.[15] Comparative study was done by subjecting a control sample of cells to flow cytometry. (1, 2) Control sample and PI stained sample was plotted for FSC/SSC. Gating was done to check the viable cells from control sample and PI stained sample. R1 is showing first population in the culture with 99.521% of live cells. (3, 4) R2 is the second population in the culture which is of dead cells having a percentage of 8.893. (5, 6) Further gating was done to obtain exact population of dead cells which came to be 0.116%.

CONCLUSION

Standardization of suspension culture of Sf-9 cell line was carried out and scaled up to 80 mL of Grace's medium. Protein estimation was done using Bradford's assay and it was found that protein concentration was increasing with cell growth, which indicated normal cell growth in adapted suspended culture. To further extent of study of cell line development a new cell line was developed from *Periplaneta americana*. Cells were isolated from abdominal, thorax and coxal region of *P.americana*. Cells isolated from thorax region were healthy and having high viability when checked using FACS. It is the first successful report of cell line development from thorax region of *P.americana*.

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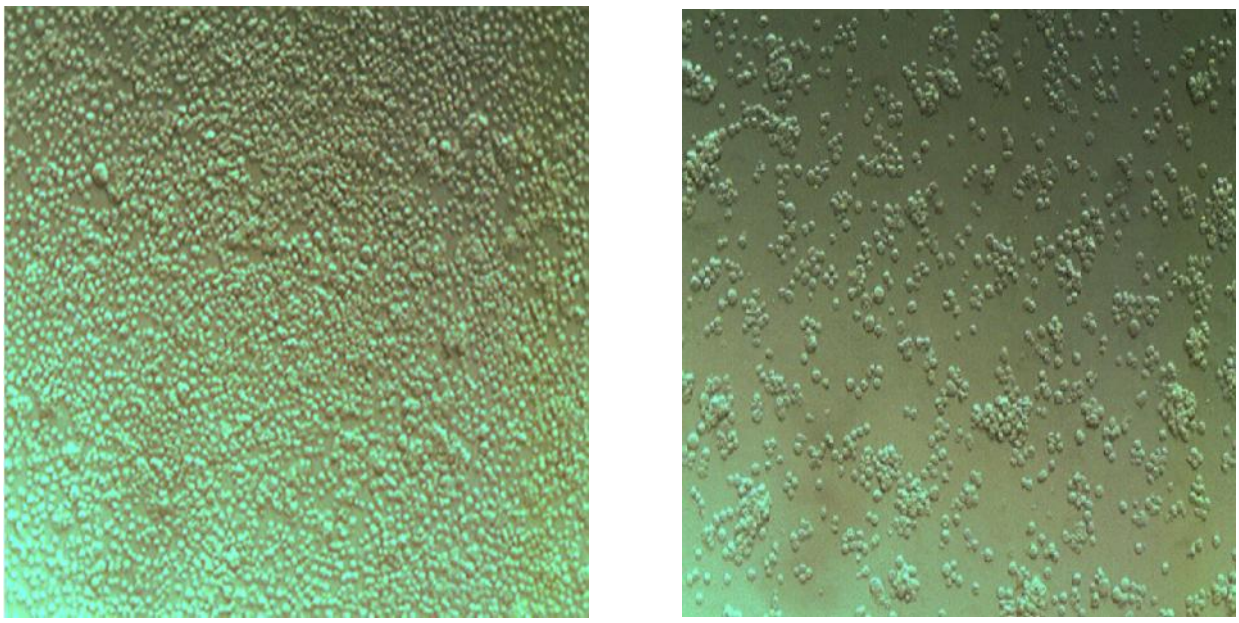

Fig.1Confluent growth of cells of Sf9 cell line before passaging After passaging their density decreases as observed in

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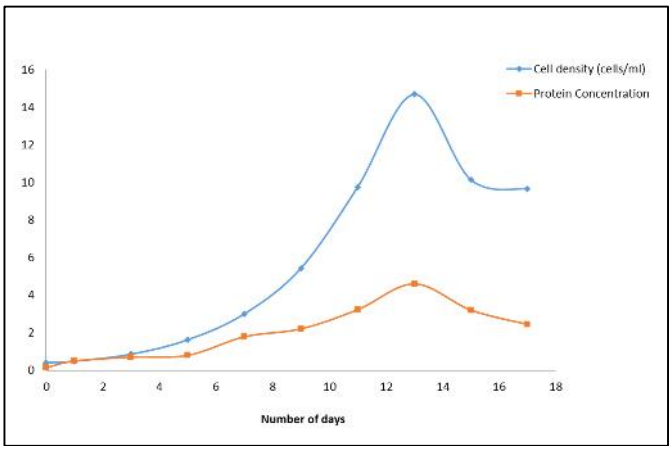

Fig 2: Comparative study of cell growth and protein concentration

	Initial Cell Count	**First splitting**	**Second splitting**
Abdominal region	15.5× 10^6/ml	8 × 10^6/ml	6 × 10^6/ml
Thoracic region	8 × 10^6/ml	2.8 × 10^6/ml	2.1 × 10^6/ml
Coxal region	4×10^5/ml	2.1 ×10^5/ml	1 × 10^6/ml

Table 1. Initial Cell count of *P.americana* insect cell line

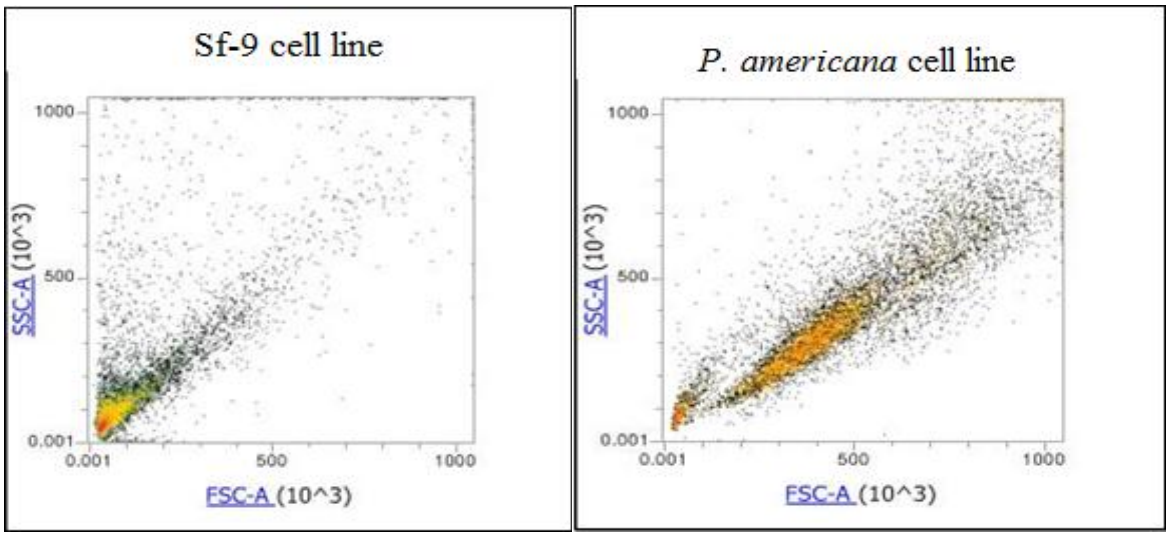

Fig. 3: Above figures show population distribution according to size and granularity of cells. Fig. 4(b) shows a single distinct population of *P. americana* as compared to the standard Sf-9 cell line Fig. 4(a)

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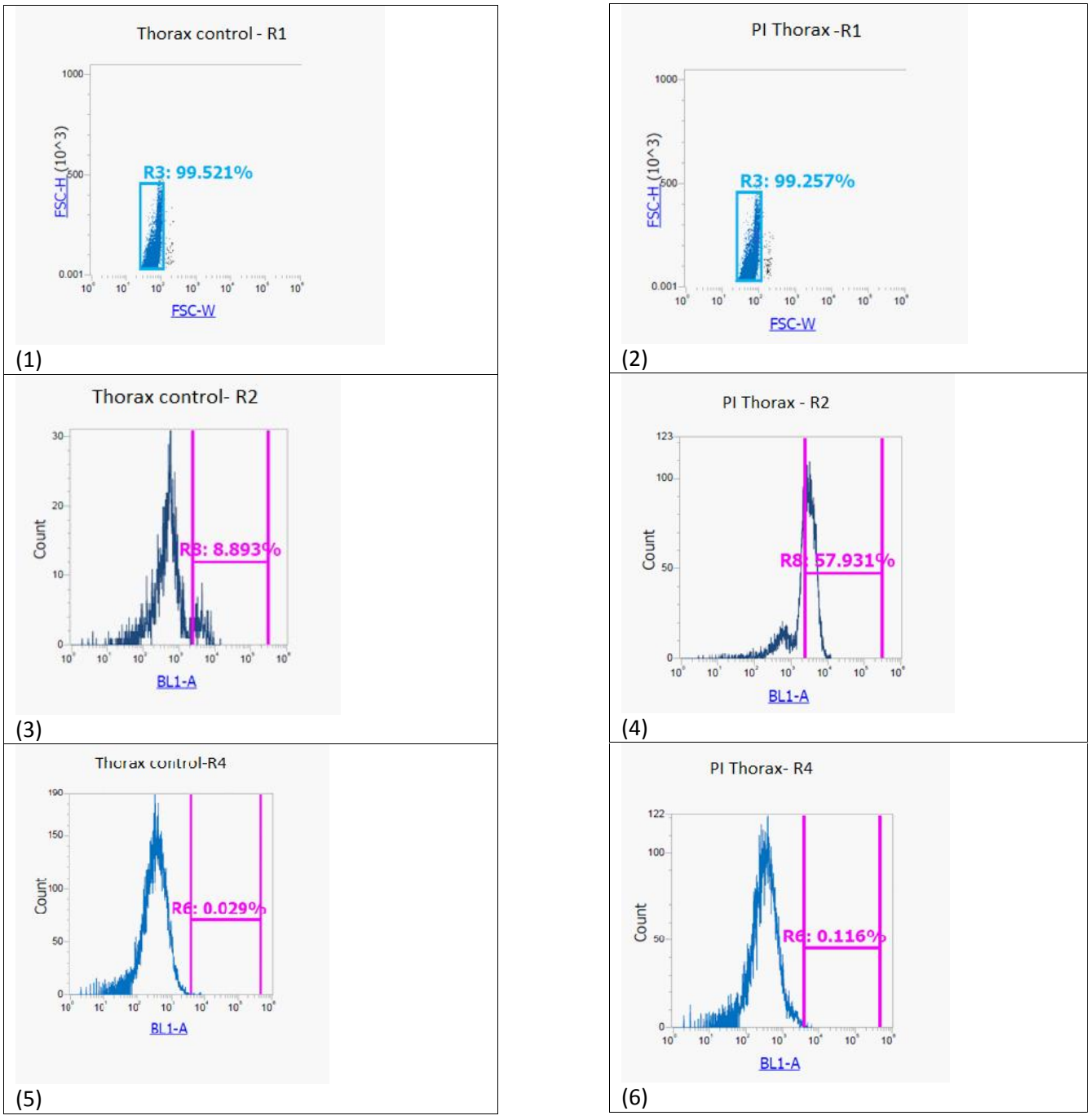

Fig. 4: Flow cytometric anaylsis of cells extracted from thoracic region of *P. americana*.

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