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Investiga Tion of the Sub-Cultivation and Maintenance Conditions of Spodoptera Frugiperda Cell Lines

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Annotation: The current study analyzed growth, survival and regenerative potential of *Spodoptera frugiperda* (Sf9) insect cells *in vitro*. The growth kinetics of Sf9 cells were carried out in SF-900 II medium and allowed to grow at 26-27°C and further analyses included cell density, viability and cell count. After 96 hours, a density of 4.8 x 10^6 cells/ml was reached with respect to cell growth. Within the cryopreservation experiments, a b viability of 95% was achieved with the use of a medium containing 80% SF900 II, 10% DMSO, and 10% FBS after six months of storage. Moreover, a viral titer of 1.3x108 pfu/mL was obtained when the Sf9 cells were infected with *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV). The results support the claim that Sf9 cells have high

growth rate and high cryo-stress resistance, hence useful in the production of recombinant proteins and vaccines in Baculovirus Expression Vector Systems (BEVS). This is because of its high rate of growth, tolerance to crystal stress and the ability to replicate with viruses. In addition, these cells can also be used in the identification of viral pathogens like Human Papillomavirus (HPV) types and hepatitis B virus (HBV)

Keywords: *Spodoptera frugiperda*, Sf9 cell line, baculovirus, BEVS system, cryopreservation, recombinant protein, biotechnology

INTRODUCTION

In the last 10 years, baculovirus-insect cell expression system (BEVS) has proven to be a very effective platform in the discipline of biotechnology. The use of this system enables the generation of high diversity of recombinant proteins, vaccines and gene-therapy vectors. The capability of insect cells to produce complex proteins that can maintain biological activity but are produced under relatively simple conditions is the major benefit of BEVS [1-5].

The Baculoviruses are inherent viral organisms inside the insects and their intracellular multiplication is characterized by very high efficiency. The most common cell line used in BEVS research is thought to be the Sf9 line derived from the fruit-bearing insect, Spodoptera frugiperda. Sf9 cells exhibit a fast growth profile, increased sensitivity to infection by the baculovirus and a significant ability to be adapted to many different culture medium formulations [6-9].

Multiple parameters determine the unfettered proliferation of Sf9 cells, viz, temperature, culture medium content, pH, dissolved oxygen content, and cell density. Only under careful balancing of these variables, stable growth and viability over long periods of storage can be obtained. Therefore, optimization of cultivation and preservation conditions to insect cells does not only enhance the recombinant protein product amounts but also increases the applicability of the latter in biotechnological processes [10–12].

Additionally, *Spodoptera frugiperda* is a phytophagous pest that is able to infest more than 80 various agricultural crops [13]. Feeding activity of the larvae often causes damage to staple crops such as wheat, barley, buckwheat, cotton, sugar beet, corn, millet, sorghum, peanuts, rice, oats, rye and sugarcane, soybean, as well as other species of cereals. This damage is translated into considerable loss of agricultural yields and creates a physical threat to world food security [14].

Figure 1. Caterpillar and butterfly of the moth Spodoptera frugiperda

Considering the use of the insect cells as an in vitro system to determine the insecticidal effect of the bioactive compounds, this study is important in that it can enable the design of next generation insecticidal agents that are environmentally friendly in the agricultural industry.

The main aim of this study accordingly is to observe the growth dynamics, culture parameters, of the cell line Spodoptera frugiperda, Sf9, and determine the best growth conditions and to obtain a stable in-vitro cell culture system. The results obtained through this research should serve as the basis to improve the biotechnological productivity of the baculovirus expression vectors systems (BEVS) technologies and to play a role in developing biopesticidal solutions in a cost-effective manner.

Materials and Methods

In vitro culture and reproduction of insect cell.

The procedures of growing and cloning insect cells *in vitro* have an important role in biology, biotechnology and agricultural disciplines. The accuracy and efficiency of outcomes in experiments is dependent on the proper development of these cells.

In vitro insect cell cultivation has several benefits over other systems including a high level of controllability and stability. The baculovirus expression system is one of the baculovirus spheres that use insect cells and is deemed to be very effective in the production of recombinant proteins. They are regularly cultivated in special media, including the Insect Cell Culture Medium of Grace. This medium offers the best environment to allow cell proliferation, cell growth and also increases the efficiency of production of the recombinant proteins [15, 16].

Cultivation of Sf9 cells conditions.

The experiment was done on the cell line of *Spodoptera frugiperda* (Sf9). Cells were cultured in Grace Insect Medium (or Sf-900 2 SFM) at 26 ±1°C and in a CO_2-free incubator. Sterile cultures were cultivated in flasks with a working volume of 25-50 ml. Subculturing was done once in a week where cells were transferred at 1:5 ratio to fresh medium in order to sustain the lag phase.

Sub-culturing of Sf9 cells.

In the process of subculturing, the culture flask was incubated at 4°C and 20 minutes after which the previous medium was taken off. The cells were incubated in 0.05% of trypsin solution between 5 and 7 minutes. After the addition of trypsin, Sf-900 II SFM medium was added and the cells were scraped off the wall of the flask with a pipette. A 0.5 ml aliquot of the ensuing suspension was inoculated into new flasks with 4ml of fresh medium and then incubated at room temperature of 26°C.

Evaluation of cell viability.

Viability of cells was assessed by the exclusion assay of Trypan Blue. A 10 μL of cell suspension was combined with an equivalent volume of tryptan blue solution obtained out of each sample. The counting of

the live cells (unstained) and the dead cells (blue-stained cells) was performed under the microscope with a hemocytometer using 10-x magnification.

The number of viable cells was calculated as follows:

$N = n \times D \times 10^4$

where n is the number of cells per 1 mm^2, and D is the dilution factor.

Determination of the cell growth kinetics and the optical density (OD_{600}).

The Sf9 cells were grown at 27-28°C and 100 rpm in 100 ml spiner flasks. The original cell concentration was also set to 2 x 10^5 cells/mL. After 12 and 24 hours, samples (1ml) were removed to identify the viable cells by use of tryptan blue stain. At the same time, the optical density (OD600) of the cell suspension was determined using a spectrophotometer.

The growth curve was drawn by plotting the logarithm of cell density [log(N)] vs. time (hours) and therefore allowed to distinguish lag, exponential and stationary phases. Table of data on cell density and OD at every time point were plotted and plotted to produce the growth graph (Y-axis: cell density; X-axis: time, hours).

Lag phase: first adjustment of the cells.

Exponential phase: the phase of the high rate of cell proliferation.

Stagnation: deceleration or stagnation of growth.

The OD at 600nm was measured (OD= light absorbance of the cell suspension) in each sample. The actual cell number was obtained with the use of a calibration curve of OD versus cell density. In case OD exceeded 0.5 the sample was diluted so that the measurements could be linear.

Preservation of cells in the long-term.

Exponential growth cells were harvested and centrifuged at a speed of 1000 rpm and in 5 minutes. The pellet was suspended once again in a cryoprotective medium with 10% DMSO. The suspension was aliquoted at 1ml in cryovials and cooled down slowly at 4°C (30 min) and then at -170°C or in liquid nitrogen. In such circumstances, cell preservation was possible within 6-12 months.

Recovery of Frozen Cells

To recover, the cryovials were thawed at 30°C of water bath during 1-2 minutes and centrifugation was done at 1000 rpm. Cells were resuspended and placed in fresh Sf-900 II medium then left to incubate at 26°C over 24 hours. The next day, the dead cells were eliminated, the fresh medium was poured and the culture was incubated under the normal growth conditions.

Results and Discussion

Assessment of the *in vitro* growth characteristics of *Spodoptera frugiperda* cells.

In the present study, Sf9 cells were cultivated in standard 25 ml flasks that were maintained at temperatures ranging from 26°C to 27°C within thermostatically controlled incubators. The cells were grown in the serum-free SF900 II medium, which proved sufficient to satisfy their nutritional requirements. The cultures were kept under static conditions, relying upon natural air exchange for oxygen delivery. Temperature was sustained at 26 - 27°C, which corresponds to the physiological range of the insect cells. Throughout the experiment, key parameters - cell density, viability, and growth rate - were continuously monitored and are discussed in detail below.

Table 1
Sf9 cell growth kinetics

Time (hours)	Viable cell density (×10^6 cells/ml)	OD_{600}	Viability (%)
0	0.20	0.05	98

24	0.40	0.12	96
48	1.40	0.40	94
72	3.80	1.00	90
96	4.80	1.25	85

During the lag phase the density increased gradually, reflecting cellular acclimatization to the new environment, while viability decreased only marginally. In the exponential phase, cells proliferated rapidly, attaining a density of 3.8×10^6 cells/ml and an OD_{600} of 1.0, with viability falling to 90%. In the stationary phase the density plateaued at 4.8×10^6 cells/mL, OD_{600} stabilized at 1.25, and viability decreased to 85 %, indicating that nutrient limitation had begun to restrain growth. These data demonstrate that Sf9 cells exhibit optimal growth in SF900 II medium at approximately 28°C, achieving high densities within four to five days.

Selection of optimal conditions for subculture of *Spodoptera frugiperda* insect cells.

Cell growth depends on a number of critical factors. Among these, the composition of the growth medium exerts a primary influence on both growth rate and viability. In static 25 ml flasks, oxygen availability becomes limiting as cell density rises, and this limitation was a major focus of our assessment.

Table 2

Selecting optimal growth conditions for Sf9 cells

Conditions	Maximum density ($\times 10^6$ cells/ml)	Doubling time (hours)	Viability (%) at 96 hours
25°C, SF900 II	4.2	16.0	86
27°C, SF900 II	4.8	14.4	85
29°C, SF900 II	4.5	15.2	82
27°C, Grace's + FBS	4.0	17.5	85

The parameters that were most conducive to Sf9 cell growth were selected within an experimental series of 96 hours. The cells reached a density of 4.8×10^6 cell/ml, doubling time of 14.4h and a viability of up to 85% when cultured in the SF900 II system at 27°C. The serum-free medium supported faster growth compared with conventional serum-supplemented systems, underscoring its suitability for large-scale insect-cell production.

Analysis of long-term storage and recovery efficiency of *Spodoptera frugiperda* cells.

In contemporary virology and recombinant protein research, baculovirus-infected insect cell lines - such as Sf9 - are widely adopted for their robust protein-expression capabilities [2]. The choice of expression system profoundly influences protein yield, post-translational modification fidelity, and purification efficiency [8]. Mammalian cells offer near-human post-translational features but require more elaborate culture conditions, whereas bacterial systems deliver high yields rapidly but lack complex modifications [19]. Insect cells strike a balance, enabling the production of multi-protein complexes with near-human folding and glycosylation patterns [20].

In this way, we decided to identify the best conditions in long-term cell line storage in the early period of our work. Cryopreservation was used in our experimental design, with the possibility of freezing cells at a low temperature of liquid nitrogen (-196°C).

Table 3

Selecting optimal long-term storage conditions for Sf9 cells

Freezing medium	Viability (%) after recovery	Doubling time (hours)	Maximum density ($\times 10^6$ cells/ml)	Growth onset (hours)

90% SF900 II + 10% DMSO	92	15.0	4.7	12
80% SF900 II + 10% DMSO + 10% FBS	95	14.8	4.8	10

Sf9 cells were assessed based on their longevity of storage and recovery efficiency after six months of cryopreservation. Cells that had been cultured in a medium containing 80% SF900 II, 10% DMSO and 10% FBS reached a 95 percent viability, doubling time of 14.8 hours and density of 4.8 10 to the 6 cells/ml and began growing after 10 hours. This research has proven that Sf9 cells are more resistant to cryostress and they can be grown to high densities. To ensure maximum storage, it is advisable to use a medium supplemented with FBS thus, offering useful information to the storage of cell stocks in the baculovirus insect-cell studies.

We furthered our research by determining the effects of different cryoprotectants such as dimethyl sulfoxide (DMSO), glycerol, and Versene buffer on the viability of Sf9 (*Spodoptera frugiperda*) cells, the effects of the length of storage (six and twelve months) on the functionality of the cells and the effects of cell density (2×10^6, 5×10^6 cells/ml) on the preservation efficiency.

In our experiment, in the case of Sf9 cells, we used a medium that consisted of 80% SF900 II, 10% DMSO, and 10% FBS. We also tested 90% nutrient medium and 10% glycerol and 90% nutrient medium and 10% versene buffer (EDTA NaCl, KCl, KH_2PO_4, Na_2HPO_4, used as a cryoprotectant). The storage times were 6 months and 12 months at -196°C (liquid nitrogen). The densities of 2×10^6 and 5×10^6 cells/ml were selected. The thaw was done through dissolution at high rate in a water bath of 37°C and incubation in fresh medium of 26-27°C.

Table 4
Analysis of the effects of cryoprotectants on cells

Cell line	Cryoprotectant	Storage Life (month)	Density ($\times 10^6$ cells/ml)	Viability (%) after recovery	Doubling time (hour)	Maximum density ($\times 10^6$ cells/ml)
Sf9	DMSO	6	2	92	15,2	4,5
Sf9	DMSO	6	5	94	14,8	4,6
Sf9	DMSO	12	2	89	15,8	4,3
Sf9	DMSO	12	5	91	15,4	4,4
Sf9	Glycerin	6	2	84	16,8	4,1
Sf9	Glycerin	6	5	86	16,4	4,2
Sf9	Glycerin	12	2	81	17,4	3,9
Sf9	Glycerin	12	5	83	17,0	4,0

Sf9	Versene buffer	6	2	80	17,5	3,8
Sf9	Versene buffer	6	5	82	17,2	3,9
Sf9	Versene buffer	12	2	77	18,0	3,6
Sf9	Versene buffer	12	5	79	17,8	3,7

Sf9 cells at a density of 5 × 10^6 cell/ml were successfully frozen with DMSO and rewarmed 6 months later with viability of 94% and doubling time of 14.8 hours and a density of 4.6 × 10^6 cell/ml. Glycerol showed 86% viability whereas versene buffer contained 82%. The present findings form a strong basis on further research on the baculovirus infection of insect cell lines.

The second step was the analysis of the appropriateness of insect cell lines to baculovirus replication. An MOI of 72 *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) was put into the insect cell lines to cause a 72 h incubation. Plaque assay was done to determine virus titers (pfu/ml). Sf9 cells were in a position to support replication of AcMNPV in standard flasks with a temperature of 26-27°C over a period of 72 h; the virus titer was 1.3 × 10^8 pfu/ml, with cell viability decreasing to 78 percent, indicating high replication efficiency.

Effect of pH and nutrient composition on cell growth.

At the last step, we evaluated how the medium composition (glucose and glutamine concentrations) and pH influence the Sf9 membrane integrity and activity of the enzyme. Cells were incubated in pH 6.0, 6.5, 7.0, glucose 2 and 4 g/L, glutamine 2-4 mM, 96 h at 26-27°C. The pH medium was brought to 7.5 using HCl or NaOH.

Table 5
pH levels of nutrient media

Cell line	**H**	**Glucose (g/L)**	**Glutamine (mM)**	**Maximum density (×10^6 cells/ml)**	**Doubling time (hour)**	**Viability (%) at 96 hours**
Sf9	,0	2	2	4,2	16,0	88
Sf9	,0	4	4	4,4	15,6	87
Sf9	,5	2	2	4,7	14,5	87
Sf9	,5	4	4	4,9	14,2	86
Sf9	,0	2	2	4,3	15,8	85
Sf9	,0	4	4	4,5	15,4	84

The ideal condition was pH 6.5, 4g/L glucose and 4mM glutamine which produced a maximum density of 4.9 x 10^6 cells/ml and 14.2 h doubling time and 86% viability. At pH 7.0, density fell to 4.5 × 10^6 cells/ml. Higher glucose and glutamine only raised the growth by 4-5% but the pH effect was stronger. In this way, the experiment revealed the optimal growth, preservation, and recovery of the Sf9, which were better in growth rate, reduced doubling time, and better cryostress tolerance as compared to previous reports.

Discussion

Over the last few years, the baculovirus-insect cell systems have been widely used in the production of biotechnological products. The most advanced recombinant protein production system today is now considered to be Sf9 cells that were originally described by Vaughn et al. [17]. Later studies by Kost and Condréy [3] and by Yu-chen HU [21] and associates showed that insect cell culture in serum free medium is economically favorable as well as produces high biosimilarity.

The growth kinetics rate of Sf9 cells was the highest in SF-900 II medium at 27°C in the current study. These data are consistent with the information provided in the past but a cell density of 4.8×10^6 cells/ml and viability 85% was observed. The preservation of natural gas exchange conditions in the unagitated flask conditions can be attributed to the high viability.

Additionally, a recovery efficiency increased by 5-10 percent compared to the recovery efficiency realised by the use of DMSO alone was realised by optimising the composition of the cryopreservation medium. Cells that were stored in a blend of 80% SF-900-II, 10% DMSO, and 10% FBS maintained their morphology and reduced recovery time of growth. Conversely, media containing glycerol or Versene buffer had reduced viability, thus confirming the protective effect of FBS.

The high sensitivity of cell infectivity was confirmed in the virus-replication experiment in which Sf9 cells amplified the stock of the virus of *Autographa californica* MNPV to 1.3×10^8 pfu/ml. The obtained outcome corresponds to the dynamics of replication of baculovirus described by Lu and Miller [18].

Conclusion

The findings obtained in the present study show that, Sf9 cells of the genus *Spodoptera frugiperda* exist in a high stable and adaptable form that can be applied in the biotechnological processes. The optimal growth factors were 27°C in serum free SF-900 II medium, because the cells became 4.8×10^6 cells/ml subculture in 96 h. The presence of DMSO and FBS in the cryopreservation process ensured that 95% of the cells remained alive and therefore, this method is feasible when it comes to storing the cells over long durations. The high vulnerable nature of Sf9 cells to the *Autographa californica* MNPV cell is a strong scientific foundation of its use in baculovirus-based expression systems. Thus, this research determines the best growth and storage conditions of the Sf9 cell line and gives encouraging opportunities in developing environmental safe biotechnological products, such as diagnostic devices and vaccines against Human Papillomavirus (HPV) and Hepatitis B virus (HBV).

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