

**GROWTH LIMITING FACTORS  
INFLUENCING HIGH DENSITY CULTURE  
OF INSECT CELLS IN GRACE'S MEDIUM**

**Sung Hwan Lee and Tai Hyun Park\***

Department of Genetic Engineering, Sung Kyun Kwan University  
300 Chunchun-dong, Suwon, 440-746, Korea

**SUMMARY**

Growth limiting factors influencing the high density cultivation of insect cells were studied. A stationary phase in Grace's medium was found to be due to the deprivation of the active form of FBS components. Various compounds were added in the early stationary phase to observe the recovery of cell growth. With the addition of yeastolate, final cell densities were 4-fold and 3.5-fold higher in monolayer and suspension cultures, respectively. Pluronic F-68 increases the specific growth rate and the growth yield of the cell as well as protects the cell from the shear damage.

**INTRODUCTION**

Insect cell-baculovirus system has wide applicability as an alternative to classical bacterial or yeast systems for the production of recombinant proteins. Insect cells are capable of most of the posttranslational modifications performed by mammalian cells including correct signal peptide cleavage, proteolytic processing, glycosylation, phosphorylation, palmitylation, and myristylation (Shuler, 1992). The posttranslational modification processes result in the expression of proteins that are functionally and immunogenetically similar to the authentic protein. Insect cell-baculovirus system is increasingly used for the production of recombinant proteins and insect pathogenic viruses. Many biotechnology companies are in the process of evaluating or are actually using this system for the synthesis of bioproducts of medical and agricultural significance (Agathos *et al.*, 1990). The high density culture of insect cells is essential to the maximization of productivity. To increase cell density, various techniques such as periodic replacement of medium, perfusion culture (Klopping *et al.*, 1990) and immobilization (King *et al.*, 1988) have been used. Immobilized insect cell cultures may improve productivity in comparison to suspension cultures; however, there appear to be biocompatibility problems (Wu *et al.*, 1989). The process developments for large-scale suspension culture of insect cells have been reported by Maiorella *et al.* (1988) and Murhammer and Goochee (1988).

This work is concerned with growth limiting factors influencing the high cell density culture in Grace's medium. The effects of seeding density, medium compounds,

and Pluronic F-68 on cell growth were investigated in monolayer and suspension cultures.

## MATERIALS AND METHODS

### Cell Line and Medium

*Spodoptera frugiperda* (Sf9) cell line was obtained from Dr. J.M. Yang, Department of Biology, Sogang University, Seoul, Korea. Cells were grown in Grace's medium (Gibco) supplemented with 0.35 g/L NaHCO<sub>3</sub> (Sigma), 5% fetal bovine serum (FBS, Hyclone) and antibiotic-antimycotic (Gibco, 600-5240AG).

### Culture Condition and Assay

Cells were grown in 25-cm<sup>2</sup> tissue culture flasks (Falcon) containing 6mL of medium for the monolayer culture. Temperature was maintained at 28°C. For the suspension culture, 100mL spinner flasks (Bellco) were used and the medium was supplemented with 3% Pluronic F-68 (Gibco). The agitation rate was set at 80 rpm (Lindsay and Betenbaugh, 1992). The cell concentration was measured using a hemocytometer and cell viability was determined by trypan blue exclusion test (Summers and Smith, 1987). Since dead cells absorb trypan blue (Sigma), they could be identified under light microscopy.

## RESULTS AND DISCUSSION

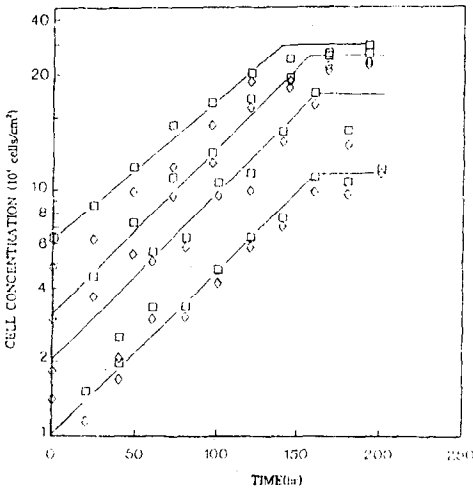
### Cell Growth in Tissue Culture Flask

Sf9 cells grow as a monolayer in a tissue culture flask. The effect of seeding density on cell growth is shown in Fig. 1. The logarithmic value of cell concentration is linear to culture time. The lines have similar slopes, which means that seeding density does not affect the specific growth rate. However, higher final cell concentration can be obtained up to a certain value with higher seeding density. About 160 h after inoculation, the cultures reach a stationary phase. Although cultures with different seeding densities reach the stationary phase at a similar time, the final cell concentration for the culture with lower seeding density is significantly lower. It seems unlikely that this phenomenon is due to the accumulation of growth-inhibitory compound, since it appears to be a time dependent phenomenon, independent of the final cell concentration. One explanation for this phenomenon may be the inactivation of a critical nutrient or growth factor over time under the culture conditions. Therefore, various compounds were added in the early stationary phase to observe the recovery of cell growth.

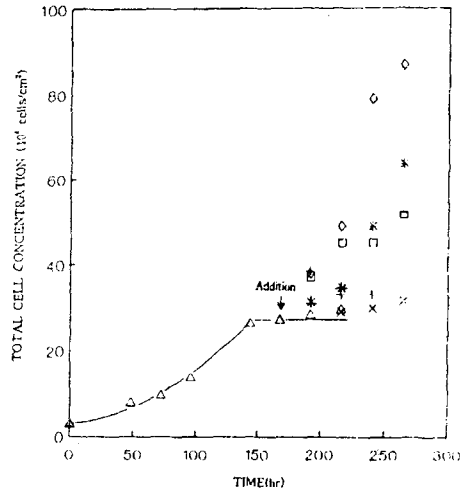
Seven days after inoculation, glutamine, FBS, or Grace's medium components were added to make the concentrations same as fresh medium. Or the waste medium was replaced with fresh medium supplemented with FBS. The results are shown in Fig. 2. The addition of glutamine or Grace's medium components did not stimulate the cell growth. The addition of vitamins was also not helpful. The glucose concentration was measured and it was not limiting. The addition of FBS and the medium replacement with fresh Grace's medium containing 5% FBS did stimulate cell growth, however. Thus, the stationary phase is considered to be due to the deprivation of the active form of FBS components. The growth-promoting activity of FBS was found to degrade

with time (Glacken *et al.*, 1989). Yeastolate (4 g/L), which has been used as one of the TNM-FH medium components (Hink, 1970), was added to supplement those components seven days after inoculation. It stimulated the cell growth as shown in Fig. 2.

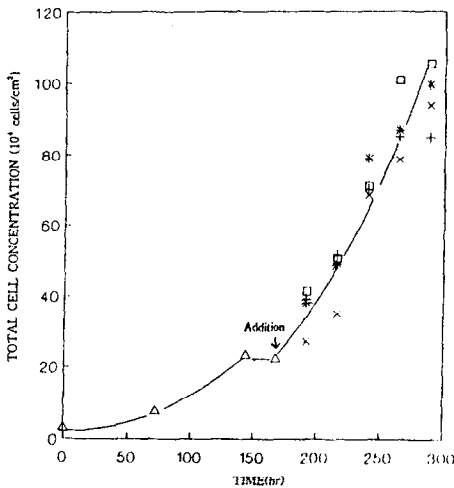
Growth behavior stimulated with the addition of FBS is similar to behavior with the addition of yeastolate until 216 h. Afterwards the cell concentration does not increase in the medium supplemented with FBS. This lack of increase implies that growth limiting factors are other than FBS components in this period. In the case of



**Fig. 1** Effect of seeding density on cell growth:  
 (  $\square$  ) total cell concentration,  
 (  $\diamond$  ) viable cell concentration



**Fig. 2** Effect of various components on cell growth:  
 (  $\Delta$  ) without addition,  
 ( + ) glutamine, (  $\square$  ) FBS,  
 (  $\times$  ) Grace's medium components without FBS, (  $\diamond$  ) yeastolate,  
 ( \* ) replacement with fresh Grace's medium containing 5% FBS



**Fig. 3** Effect of yeastolate on cell growth in monolayer culture:  
 1. instantaneous addition  
 ( + ) 2 g/L  
 ( \* ) 4 g/L  
 (  $\square$  ) 6 g/L  
 2. intermittent addition  
 (  $\times$  ) 4 g/L

the medium replacement, lower cell concentrations are observed than those in the medium supplemented with yeastolate. This could be due to the experimental procedure. Medium replacement causes some loss of the cells which are weakly attached to the surface of the tissue culture flask. However, although the cell concentration is lower, exponential growth was observed in the replaced medium until the end of the experiment.

Cell growth was observed at various concentrations (2 g/L, 4 g/L and 6 g/L) of yeastolate added seven days after inoculation. The effect of yeastolate concentration on cell growth is shown in Fig. 3. To compare repeated addition of a smaller amount with the instantaneous addition, the yeastolate was added five times at intervals of one day. Total amount of added yeastolate was the same as that in 4 g/L. Similar behaviors of the cell growth were observed at various concentrations and intermittent addition, as shown in Fig. 3. Final cell concentration increased more than 4-fold with the addition of yeastolate.

### Cell Growth in Spinner Flask

Suspension culture offers a number of advantages for the large-scale cultivation of insect cells. Fig. 4 shows the effect of seeding density on cell growth. Seeding density does not affect the specific growth rate and final cell concentration. It should be noted that final cell concentration is dependent on the seeding density in the tissue culture flask, as shown in Fig. 1. The longer time is needed to reach the stationary phase in the case of lower seeding density in the spinner flask. In other words, exponential growth phase can still be maintained 160 h after inoculation in the suspension culture, while cultures reached a stationary phase about 160 h after inoculation regardless of the seeding density in tissue culture flask. This is suspected to

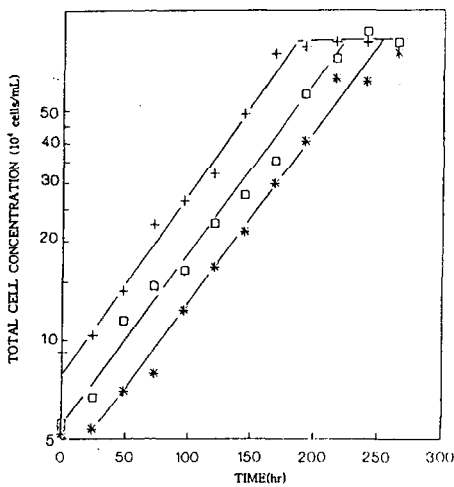


Fig. 4 Effect of seeding density on cell growth in suspension culture

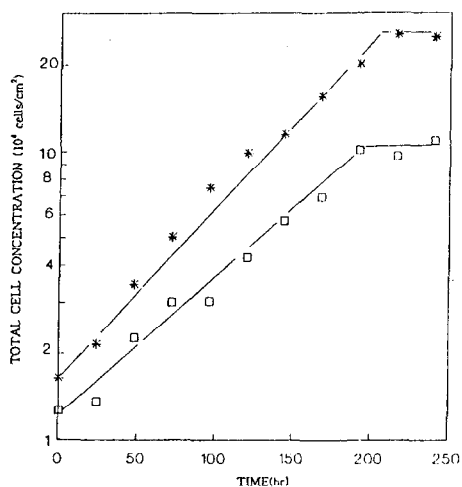


Fig. 5 Effect of Pluronic F-68 on cell growth in monolayer culture:  
( □ ) without addition  
( \* ) 3% Pluronic F-68

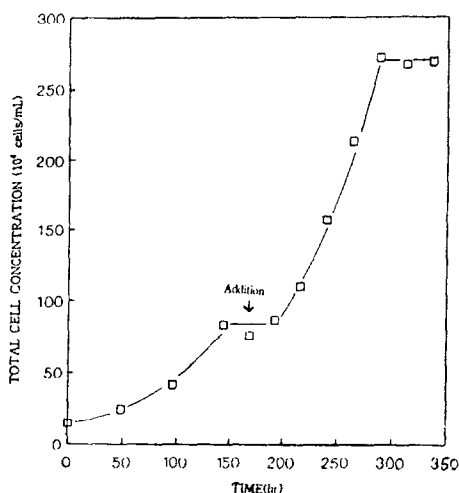


Fig. 6 Effect of yeastolate on cell growth in suspension culture

be due to Pluronic F-68 which was added to the medium in the spinner flask culture, since Pluronic F-68 is the only difference in the medium compositions of monolayer culture and suspension culture. Pluronic F-68 has been believed to have shear and bubble protective functions.

To determine whether Pluronic F-68 has additional effects on cell growth besides protection from shear damage, it was added to the monolayer culture medium. Pluronic F-68 increases the specific growth rate and final cell concentration in monolayer culture as shown in Fig. 5. The results suggest that the Pluronic F-68 may increase nutrient uptake (Bentley *et al.*, 1989) or protect the activity of FBS from degradation.

To observe the effect of yeastolate on the cell density in the spinner flask, yeastolate (4 g/L) was added 8 days after the inoculation. It stimulated the cell growth as shown in Fig. 6. Final cell concentration increased about 3.5-fold with the addition of yeastolate in the spinner flask.

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