J. Microbiol. Biotechnol. (2007), **17**(5), 739–744

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Production of Recombinant Polyhedra Containing Cry1Ac Fusion Protein in Insect Cell Lines

KIM, JAE SU[1,2], JAE YOUNG CHOI[3], JONG YUL ROH[1], HAN YOUNG LEE[2], SEUNG SIK JANG[2], AND YEON HO JE[1]*

[1]*School of Agricultural Biotechnology, College of Agriculture & Life Science, Seoul National University, Seoul 151-742, Korea*
[2]*Agricultural Technology Research Institute, Dongbu Hannong Chemical Co. Ltd., Daejon 305-708, Korea*
[3]*Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul 151-742, Korea*

Received: October 9, 2006
Accepted: January 2, 2007

Abstract Insect cell lines and the control of infection for obtaining the maximum amount of polyhedrin-Cry1Ac-polyhedrin fusion protein from Bactrus in monolayer and suspension culture systems were tested. Growth rates of the *Trichoplusia ni* (High-Five) cell line in both culture systems were better than the other insect cell lines, *Spodoptera frugiferda* (Sf-9, Sf-21), *Trichoplusia ni* (Tn5), and *Spodoptera exigua* (Se301). The expression of the fusion protein in a monolayer culture showed that Se301 cells were 2.3–4.8 times more productive on a per cell basis than the other cell lines. However, in suspension culture, only High-Five cells were productive. High-Five cells infected with Bactrus at a multiplicity of infection (MOI) of 5 and a cell density of 3.0×10^5 cells per ml were more productive than the other infection condition in a suspension culture suitable for a large-scale production of baculovirus. In conclusion, for the large-scale production of Bactrus *in vitro*, High-Five cells showing good growth and high productivity are suitable.

Keywords: Polyhedra, Bactrus, monolayer culture, suspension culture, multiplicity of infection

The development of fast-killing, recombinant baculoviruses has strikingly improved the field performance of viral insecticides [14, 23, 24]. However, these improvements have also resulted in a reduced productivity of the *in vivo* system, which has been reported for the production of a recombinant baculovirus expressing the LqIT gene [2]. Better crop protection provided by the recombinant baculovirus, due to faster killing, resulted in less of that baculovirus able to be economically produced *in vivo*.

*Corresponding author
Phone: 82-2-880-4706; Fax: 82-2-873-2319;
E-mail: btrus@snu.ac.kr

To overcome these difficulties, an *in vitro* production system has been suggested. Insect cells are an attractive host for recombinant protein expression via infection with a genetically modified baculovirus, as this system allows for a high level of protein production that is functionally similar to the native protein [4, 5, 12, 15]. Larger scale efforts have focused on suspension culture *in vitro*, since most insect cell lines can be maintained in suspension, which yields a higher productivity than that of a monolayer culture [3, 20, 25, 26].

In *in vitro* production, cell growth and control of viral infection are key parameters [6, 11, 17, 21]. If a high-level yield is to be achieved, cells are to be held in a healthy physiological state, free of oxygen limitation or mechanical damage, and must have a high growth rate to reduce culture time and contamination [1, 7, 8, 10, 13]. To control viral infection, all viral seed stock must demonstrate genetic stability during propagation without detectable genetic rearrangements, loss of productivity, or loss of infectivity [18]. In addition, optimum multiplicity of infection (MOI) and cell density at the time of infection should be established [19]. The effect of MOI on infectivity and yield is as important as the condition of the cells and the time of infection. Production of recombinant protein has been reported at different MOIs ranging from high to low [7, 19]. The use of a high MOI rather than a low MOI has been preferred for two main reasons: easier harvest time definition, and the elimination of the "passage-effect" from the recombinant baculovirus inoculum [19]. This phenomenon is similar to that observed in wild-type baculovirus, where the virus passaged at a high MOI generates few polyhedra (FP) phenotypes [18]. Although the use of a high MOI will result in an easier optimal harvest time definition, the use of a low MOI on a large scale is preferential, since an intermediate step of virus inoculum production is avoided [9, 27].

In this study, the productivity of a recombinant baculovirus, Bactrus, expressing 130 kDa of the polyhedrin-Cry1Ac-polyhedrin recombinant protein and 30 kDa of an intact polyhedrin resulting from a homologous recombination between two polyhedrin genes [16], was compared in five different insect cell lines with monolayer and suspension culture systems.

MATERIALS AND METHODS

Insect Cell Lines and Virus

Spodoptera frugiferda cell lines (Sf-9, Sf-21) and the *Trichoplusia ni* cell line (Tn5) were maintained continuously in TC-100 medium (Gibco, U.S.A.) supplemented with 10% fetal bovine serum (FBS) (Gibco, U.S.A.). The *Spodoptera exigua* cell line (Se301) was maintained in IPL-41 medium (Gibco, U.S.A.) with 10% FBS, and another *T. ni* cell line (High-Five) was maintained in Express Five serum-free medium (Gibco, U.S.A.) with L-glutamine. All insect cell lines were maintained in T-25 tissue culture flasks (Falcon, U.S.A.) with 5 ml of culture volume at 27°C. The recombinant baculovirus Bactrus, [16] at passage number 3 (P3), was used for viral infection.

Cell Growth

For monolayer cultures, 2.0×10^5 cells were seeded in a 60-mm culture dish with 3 ml of fresh medium. The cell number was determined every 24 h by detaching the cells from the dish and counting the viable cells using a hemacytometer (Brant, Germany) with 0.4% trypan blue (Merk, Germany) in phosphate-buffered saline (PBS; 140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3).

For suspension cultures, insect cells were inoculated into 25-ml spinner flasks (Bellco, U.S.A.) at 2.0×10^5 cells/ml of initial cell density with a working volume of 20 ml. The impeller was rotated at 40 rpm. Cell densities were determined as above.

Viral Infection

For monolayer cultures, 5.0×10^5 cells in 6-well plates (Bellco, U.S.A.) were inoculated with 0.5 ml of the virus at a MOI of 10. The plates were rocked for 1–1.5 h to allow the virus to infect. The infection medium was then removed and the cells were refreshed with 3 ml of fresh medium.

For suspension cultures, cells were inoculated in 25-ml spinner flasks at an initial density of 5.0×10^5 cells/ml with fresh medium in a final volume of 20 ml and infected with the virus at 5 and 10 MOIs.

After 6–7 days post-infection (p.i.), infected cells were sonicated with a membrane sonicator (ARTEK, U.S.A.) 3 times for 30 sec, and polyhedra were counted using a hemacytometer.

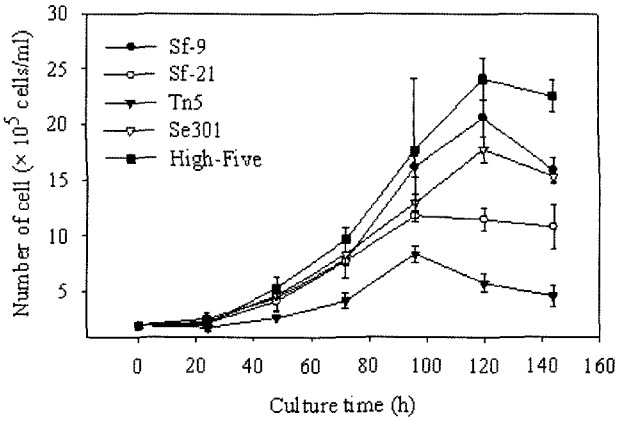

Fig. 1. Growth of insect cells in a monolayer culture.
Sf-9, Sf-21, Tn5, Se301, and High-Five cells were inoculated into 60-mm culture dishes with 3 ml of fresh media. Initial cell concentration was 2.0×10^5 cells/dish. The standard deviation is marked on the growth curve.

SDS-PAGE

Polyhedra were released from infected insect cells lysed with cell lysis buffer (50 mM Tris-HCl, pH 8.0, 0.4% SDS,

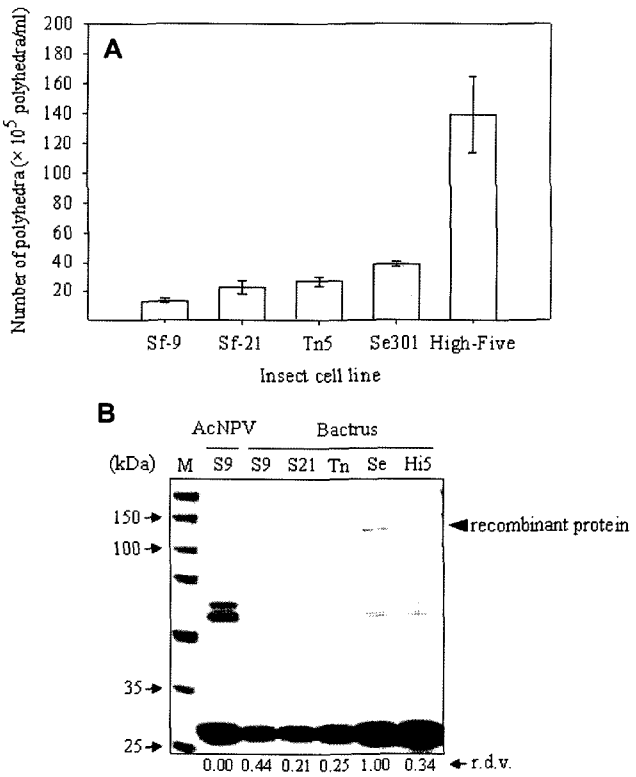

Fig. 2. Production of recombinant polyhedra (**A**) and expression of the fusion protein (**B**) in insect cells with a monolayer culture. 5×10^5 cells were infected with the Bactrus (3rd passage) at a MOI of 10.0 in a 6-well plate.
A. The number of polyhedra was counted with a hemacytometer at 7 days post-infection. The standard deviation is marked on the bar. **B.** SDS-PAGE of the recombinant polyhedra was performed using a 12% polyacrylamide gel. The relative density value (r.d.v.) of the recombinant proteins at the bottom of the lanes was determined by densitometry scanning of the gel.

10 mM EDTA, 5% β-mercaptoethanol), washed with an excess amount of phosphate-buffered saline (PBS; 140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3), and analyzed in a 12% polyacrylamide gel.

RESULTS

Production of Recombinant Polyhedra Using Monolayer Culture

To compare the productivity of recombinant polyhedra in five different insect cell lines by means of monolayer culture, the growth and recombinant polyhedra productivity of these cells were examined.

High-Five cells showed the highest growth rate of about 1.8×10^4 cells/h, and reached a peak of 2.4×10^6 cells/ml at 120 h, followed by Sf-9 cells and Se301 cells. Overall, after 120 h, most cell lines died (Fig. 1). The largest number of polyhedra was also produced in High-Five cells, followed by Se301 cells (Fig. 2A). However, in the SDS-PAGE analysis of purified polyhedra, the highest amount of fusion protein was expressed in Se301 cells (Fig. 2B).

Production of Recombinant Polyhedra Using Suspension Culture

To determine the optimum cell line suitable for the mass production of recombinant polyhedra in suspension culture, the cell growth and productivity of recombinant polyhedra in a suspension culture system were examined.

High-Five cells showed the highest growth rate of about 6.7×10^3 cells/h, and reached a maximum of 8.4×10^5 cells/ml at 96 h, followed by Sf-9 cells (Fig. 3). In contrast, Sf-21 cells grew little and Se301 cells did not grow at all, but simply retained cell viability for about 120 h. Tn5 cells died just after inoculation.

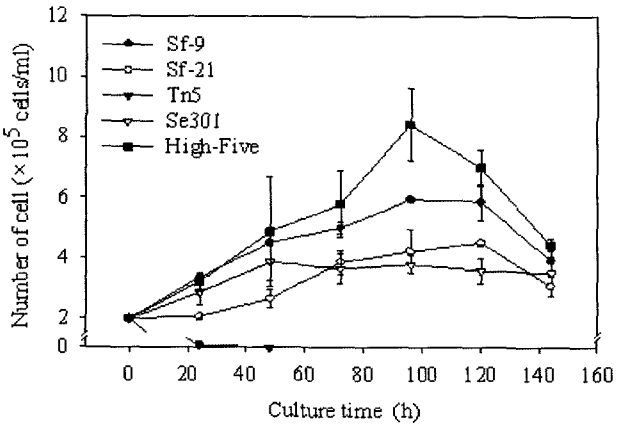

Fig. 3. Growth of insect cells in suspension culture.
Sf-9, Sf-21, Tn5, Se301, and High-Five cells were inoculated into 25-ml spinner flasks at a density of 2.0×10^5 cells/ml with a 20 ml working volume. The standard deviation is marked on the growth curve.

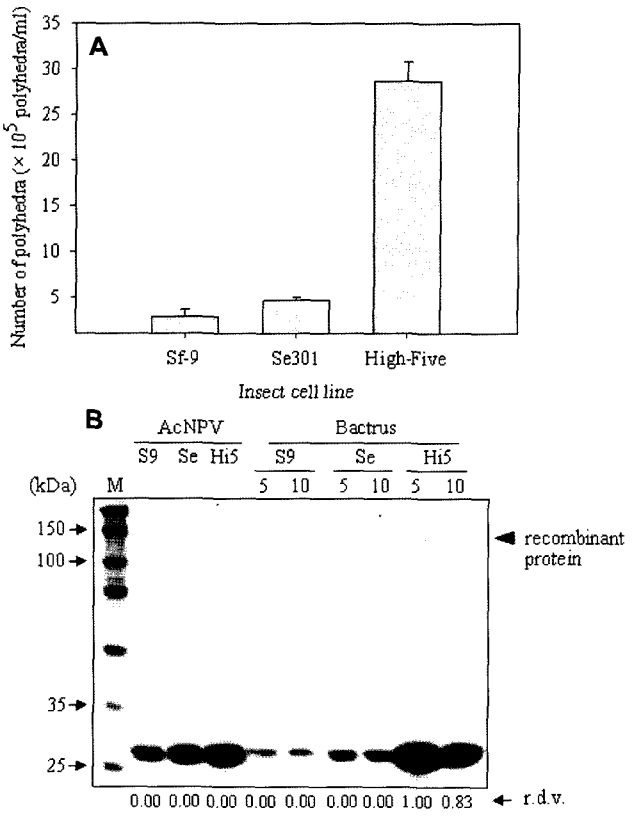

Fig. 4. Production of recombinant polyhedra (**A**) and expression of fusion protein (**B**) in insect cells in suspension culture. Sf-9, Se301, and High-Five cells were inoculated into 25-ml spinner flasks at a density of 5×10^5 cells/ml with a 20 ml working volume. Insect cells were infected with the Bactrus (3rd passage) at a MOI of 10.0.
A. The number of polyhedra was counted with a hemacytometer at 6 days post-infection. The standard deviation is marked on the bar. **B**. SDS-PAGE of the recombinant polyhedra produced in Sf-9, Se301, and High-Five cells, which were infected at MOIs of 5.0 and 10.0, was performed using a 12% polyacrylamide gel. The relative density value (r.d.v.) of the recombinant proteins at the bottom of the lanes was determined by densitometry scanning of the gel.

When the productivity of polyhedra was determined using Sf-9, Se301, and High-Five cells, which showed the highest productivity of polyhedra and fusion protein with monolayer cultures but have generally been used for suspension cultures, the largest number of polyhedra was produced in High-Five cells, followed by Se301 and Sf-9 cells (Fig. 4A). In SDS-PAGE analysis of the purified polyhedra, the largest quantity of fusion protein and the 30 kDa polyhedrin were expressed in High-Five cells (Fig. 4B).

Optimization of Recombinant Polyhedra Production Using High-Five Cells

For the comparison of recombinant polyhedra productivity at different MOIs with suspension cultures of High-Five cells, 5×10^5 cells/ml were infected with the recombinant

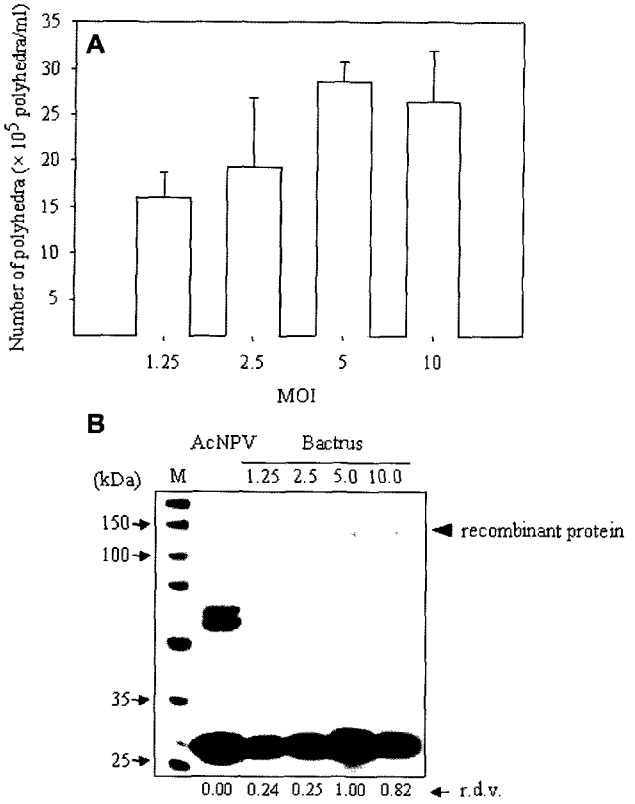

Fig. 5. Production of recombinant polyhedra (**A**) and expression of fusion protein (**B**) in suspension cultured High-Five cells infected with various MOIs.

A. High-Five cells were inoculated into 25-ml spinner flasks at a density of 5×10^5 cells/ml. The number of polyhedra was counted using a hemacytometer at 6 days post-infection. The standard deviation is marked on the bar. **B.** SDS-PAGE of the recombinant polyhedra was performed using a 12% polyacrylamide gel. The relative density value (r.d.v.) of the recombinant proteins at the bottom of the lanes was determined by densitometry scanning of the gel.

baculovirus, Bactrus, at a MOI of 1.25, 2.5, 5.0, or 10.0. The largest number of polyhedra was produced at a MOI of 5.0, with no further increase at a MOI of 10 (Fig. 5A). The largest amount of fusion protein was also expressed at a MOI of 5.0 (Fig. 5B).

To compare the productivity of recombinant polyhedra at different cell densities on the time of infection, High-Five cells at densities of 1, 3, 5, 7, and 9×10^5 cells/ml were infected with the Bactrus at a MOI of 5.0. The largest number of polyhedra was produced when the cell density at the time of infection was 3×10^5 cells/ml (Fig. 6). There was little or no production of polyhedra when the cell density at the time of infection was too low or too high.

DISCUSSION

In order to select a suitable insect cell line for the *in vitro* mass production of recombinant polyhedra of the recombinant

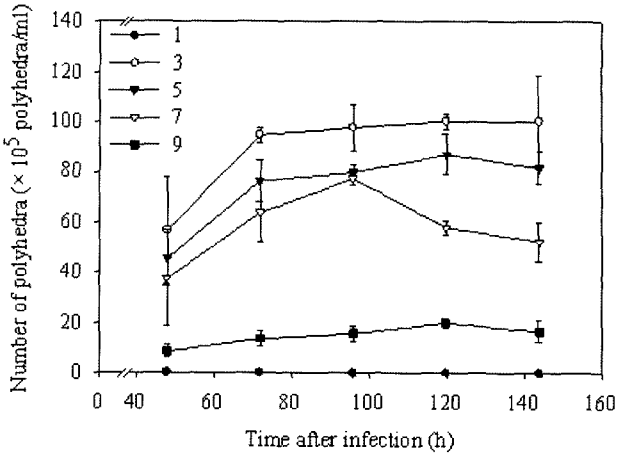

Fig. 6. Production of recombinant polyhedra in suspension cultured High-Five cells with different cell densities at the time of infection.

High-Five cells were inoculated into 25-ml spinner flasks at a density of 1, 3, 5, 7, or 9×10^5 cells/ml with a 20 ml working volume. Cells were infected with the Bactrus at a MOI of 5.0. The number of polyhedra was counted with a hemacytometer at 6 days post-infection. The standard deviation is marked on the production curve.

baculovirus Bactrus, the productivities of recombinant polyhedra in five different cell lines were compared in monolayer and suspension cultures of these cells. With monolayer cultures, the expression of the fusion protein in Se301 cells was larger than that in High-Five cells, which showed the largest productivity of recombinant polyhedra. The difference of fusion protein expression might originate from the difference in the number of viral replication times between Se301 cells and High-Five cells. Relatively fewer replication times might occur in Se301 cells, which caused the Bactrus to express more fusion protein by prohibiting the passage-effect. Moreover, although five kinds of insect cells were infected with the Bactrus at the same MOI, the real MOI in High-Five cells during viral infection was relatively smaller than that in Se301 cells because of the higher growth rate of High-Five cells compared with Se301 cells.

Despite the fact that the largest expression of fusion protein was produced in Se301 cells in a monolayer culture, there was little expression of fusion protein in this cell line with a suspension culture. The major reason for this might be due to the different growth rates of Se301 cells in the two culture systems - that is, Se301 cells grew very well in a monolayer culture but could not grow well in a suspension culture. Furthermore, it might be caused by the difference in polyhedral maturation. The size of polyhedra produced in Se301 cells was bigger than that produced in the other four cells with monolayer cultures. However, this was not the case with suspension cultures. Therefore, in terms of mass production of fusion protein, healthy cell growth could be essential for larger expression of the fusion protein.

Although the expression of the fusion protein in Se301 cells was larger than that in High-Five cells with a monolayer culture, both polyhedra productivity and fusion protein expression were the highest in High-Five cells with a suspension culture. These results suggested that the High-Five cells could be useful because scaled-up suspension cultures rather than monolayer culture might be preferred for the mass production of recombinant polyhedra.

Establishment of a suitable MOI is important for the production of recombinant protein on a large scale. Infection of the virus at a lower MOI might have several advantages for the large-scale production of recombinant proteins: 1) Very little amount of high-quality virus stock is required, avoiding the need for separate virus stock scale-up procedures; 2) the risk of defective interfering virus particles (D.I. particle) being produced through a reduction in the number of passages of the virus stock is reduced. However, infection with a lower MOI resulted in lower productivity of the fusion protein. This result could be caused by the fact that recombinant viruses experience more passages at a lower MOI than at a higher MOI, which causes more recombinant virus to return to wild-type, as previously reported [16].

In the case of infection with a higher MOI, the efficiency of recombinant virus infection and the productivity of recombinant polyhedra were higher and the passage-effect of recombinant virus could be eliminated; however, this method requires a larger volume of virus stock, which could be an obstacle to mass production of recombinant baculovirus [22, 27]. In this study, the largest amount of recombinant polyhedra and fusion protein was produced when the High-Five cells were infected at a MOI of 5.0. There was no significant difference between a MOI of 5.0 and 10.0.

Another important factor for the mass production of recombinant polyhedra is the cell density at the time of infection. When cells with too low a density were infected with recombinant virus, the productivity was also low. In contrast, when cells with too a high density were infected, the productivity was also significantly low because the cells were dying [7, 19]. For a much higher productivity, the High-Five cells have to be viable for at least about 48 h post-infection.

In conclusion, our approach has shown that the High-Five cell line is optimal for the large-scale production of the recombinant baculovirus Bactrus, and that the productivity might be maximized in the above-mentioned infection conditions. These results would provide useful information on the development of recombinant baculovirus insecticides, especially for the mass production of viral ingredients.

Acknowledgment

This study was supported by the Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea and the Brain Korea 21 Project.

REFERENCES

1. Bedard, C., R. Tom, and A. Kamen. 1993. Growth, nutrient consumption and end-product accumulation in Sf-9 and BTI-EAA insect cell cultures: Insights into growth limitation and metabolism. *Biotechnol. Prog.* **9:** 615–624.
2. Bill, M., F. Lindsey, and H. Gary. 1996. A brief overview of biotechnology at Dupont Ag. and a case study with recombinant baculovirus. Cornell Community: Conference on biological control.
3. Caron, A. W., L. Archambault, and B. Massie. 1990. High level recombinant protein production in bioreactors using the baculovirus-insect cell expression system. *Biotechnol. Bioeng.* **36:** 1133–1140.
4. Chen, Y., Z. Z. Zhu, X. A. Lin, Y. Z. Yi, Z. F. Zhang, and G. F. Shen. 2005. Overexpression and characterization of appA phytase expressed by recombinant baculovirus-infected silkworm. *J. Microbiol. Biotechnol.* **15:** 466–471.
5. Choi, J. Y., S. D. Woo, Y. H. Je, and S. K. Kang. 1999. Development of a novel expression vector system using *Spodoptera exigua* nucleopolyhedrovirus. *Mol. Cells* **9:** 504–509.
6. Donaldson, M. S. and M. L. Shuler. 1998. Low-cost serum-free medium for the BTI-Tn5N1-4 insect cell line. *Biotechnol. Prog.* **14:** 573–579.
7. Dougherty, E. M., R. M. Weiner, J. L. Vaughn, and C. F. Reichelderfer. 1981. Physical factors that affect *in vitro Autographa californica* nuclear polyhedrosis virus production. *Appl. Environ. Microbiol.* **41:** 1166–1172.
8. Drews, M., T. Paalme, and R. Vilu. 1995. The growth and nutrient utilization of the insect cell line *Spodoptera frugiferda* Sf9 in batch and continuous culture. *J. Biotechnol.* **40:** 187–198.
9. Elias, C. B., A. Zeiser, C. Bedard, and A. A. Kamen. 2000. Enhanced growth of Sf-9 cells to a maximum density of 5.2×10^7 cells per ml and production of β-galactosidase at high cell density by fed batch culture. *Biotechnol. Bioeng.* **68:** 381–388.
10. Ferrance, J. P., A. Goel, and M. M. Ataai. 1993. Utilization of glucose and amino acid in insect cell cultures: Quantifying the metabolic flows within the primary pathways and medium development. *Biotechnol. Bioeng.* **42:** 697–707.
11. Hensler, W., V. Singh, and S. N. Agathos. 1994. Sf9 insect cell growth and β-galactosidase production in serum and serum-free media. *Ann. N. Y. Acad. Sci.* **745:** 149–166.
12. Ikonomou, L., Y. J. Schneider, and S. N. Agathos. 2003. Insect cell culture for industrial production of recombinant proteins. *Appl. Microbiol. Biotechnol.* **61:** 1–20.
13. Ismail, D. and Z. Demirbag. 2006. A productive replication of *Hyphantria cunea* nucleopolyhedrovirus in *Lymantria dispar* cell line. *J. Microbiol. Biotechnol.* **16:** 1485–1490.
14. Je, Y. H., B. R. Jin, J. Y. Roh, J. H. Chang, and S. K. Kang. 1999. Construction of a novel recombinant baculovirus

producing polyhedra with a *Bacillus thuringiensis* Cry1Ac crystal protein. *J. Korean Soc. Virol.* **29:** 145–153.
15. Kang, C. S., S. Y. Son, and I. S. Bang. 2006. High-level expression of T4 endonuclease 5 in insect cells as biologically active form. *J. Microbiol. Biotechnol.* **16:** 1583–1590.
16. Kim, J. S., J. Y. Choi, J. H. Chang, H. J. Shim, J, Y. Roh, B. R. Jin, and Y. H. Je. 2005. Characterization of an improved recombinant baculovirus producing polyhedra that contain *Bacillus thuringiensis* Cry1Ac crystal protein. *J. Microbiol. Biotechnol.* **15:** 710–715.
17. Kioukia, N., A. W. Nienow, A. N. Emery, and M. A. Rubeai. 1994. Physiological and environmental factors affecting the growth of insect cells and infection with baculovirus. *J. Biotechnol.* **38:** 243–251.
18. Krell. P. J. 1996. Passage effect of virus infection in insect cells. *Cytotechnology* **20:** 125–137.
19. Licari, P. and J. E. Bailey. 1991. Factors influencing recombinant protein yields in an insect cell-baculovirus system: Multiplicity of infection and intracellular protein degradation. *Biotechnol. Bioeng.* **37:** 238–246.
20. Maiorella, B., D. Inlow, A. Shauger, and D. Harano. 1988. Large-scale insect cell-culture for recombinant protein production. *Bio/Technology* **6:** 1406–1410.
21. Maranga, L., A. S. Coroadinha, and M. J. T. Carrondo. 2002. Insect cell culture medium supplementation with fetal bovine serum and bovine serum albumin: Effects on baculovirus adsorption and infection kinetics. *Biotechnol. Prog.* **18:** 855–861.
22. Maranga, L., P. Rueda, A. F. G. Antonis, C. Vela, J. P. M. Langeveld, J. I. Casal, and M. J. T. Carrondo. 2002. Large scale production and downstream processing of a recombinant porcine parvovirus vaccine. *Appl. Microbiol. Biotechnol.* **59:** 45–50.
23. Martens, J. W. M., G. Honee, D. Zuidema, J. W. M. Van Lent, B. Visser, and J. M. Vlak. 1990. Insecticidal activity of a bacterial crystal protein expressed by a recombinant baculovirus in insect cells. *Appl. Environ. Microbiol.* **56:** 2764–2770.
24. Merryweather, A. T., U. Weyer, M. P. G. Harris, M. Hirst, T. Booth, and R. D. Possee. 1990. Construction of genetically engineered baculovirus insecticides containing the *Bacillus thuringiensis* subsp. *kurstaki* HD-73 delta endotoxin. *J. Gen. Virol.* **71:** 1535–1544.
25. Rhiel, M., C. M. Mitchell-Logean, and D. W. Murhammer. 1997. Comparison of *Trichoplusia ni* BTI-Tn-5B1-4 (High-Five™) and *Spodoptera frugiferda* Sf-9 insect cell line metabolism in suspension cultures. *Biotechnol. Bioeng.* **55:** 909–920.
26. Taticek, R. A., C. Choi, S. E. Phan, L. A. Palomares, and M. L. Shuler. 2001. Comparison of growth and recombinant protein expression in two different insect cell lines in attached and suspension culture. *Biotechnol. Prog.* **17:** 676–684.
27. Wong, K. T. K., C. H. Peter, P. F. Greenfield, S. Reid, and L. K. Nielsen. 1996. Low multiplicity infection of insect cells with a recombinant baculovirus: The cell yield concept. *Biotechnol. Bioeng.* **49:** 659–666.