Comparative Characterization of Growth and Recombinant Protein Production among Three Insect Cell Lines with Four Kinds of Serum Free Media

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Abstract Three insect cell lines, Sf9, Sf21 and Tn5B1-4, and four different kinds of serum free media (SFM), Sf 900 II, EX-CELL 420, EX-CELL 405 and Express Five, were used to compare the nutrient consumption, byproduct formation, production of recombinant protein and protease activity in suspension cultures. The Sf 900 II SFM was appropriate for the cell growth and protein production of the Sf9 and Sf21 cell lines. When the Tn5B1-4 cell line was grown in the Express Five SFM, the specific growth rate was 1.6 fold higher than those of either the Sf9 or Sf21 cell lines. The glucose and glutamine consumption rates per cells, were 4 and 2.3 times higher than those of the Sf9 cell line, respectively. The overall yield coefficients of the lactate and ammonium ion were 2.8 and 1.5 times higher compared to those of the Sf9 cell line, respectively. The maximum specific β-galactosidase production rate was 4.5 fold that of the Sf9 cell line, a 3 times higher protease activity per cell.

Keywords: baculovirus expression vector system, serum free medium, insect cell, β-galactosidase, protease

INTRODUCTION

Insect cells are an attractive host for the production of recombinant proteins, via their infection with a genetically modified baculovirus expression vector system (BEVS). They allow for a high level of protein production that are functionally similar to the native form [1]. Moreover, their ease of genetic manipulation means the insect cells can be utilized in high eukaryotic hosts, which are capable of performing many posttranslational modifications present in human proteins [2]. Since the introduction of the BEVS in the early 1980s, heterogeneous recombinant proteins have been expressed using this system [3], recently has been widely used for the quick production of significant amounts of protein expression [4].

The success of the BEVS for the production of recombinant proteins in insect cells has significantly increased interest in the methods involving their cultures, particularly aimed at inexpensive serum free medium (SFM), easy to preparation from individual components, suitable for both growth and infection efficiency and capable of supporting high cell densities.

Various SFMs have recently been developed and used for insect cell cultivation, but the results obtained have varied with the cell lines or culture media utilized [5,6].

In this study, the production of recombinant proteins, and their growth characteristics were investigated using a combination of three insect cell lines and four different SFM in culture.

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MATERIALS AND METHODS

Cell lines, Media, and Virus

The cell lines, *Spodoptera frugiperda* Sf9, Sf21 and *Trichoplusia ni* BTI-TN 5B1-4 (Tn5B1-4), purchased from Invitrogen Co (San Diego, CA, USA), were used throughout these experiments. The four SFMs, Sf 900 II, Express Five (Gibco BRL, Rockville, MD, USA), EX-CELL 420 and EX-CELL 405 (JRH Biosciences, Lenexa, KS, USA) were used in the suspension cultures.

Autographa californica nuclear polyhedrosis virus (AcNPV) was used, which contained the *Escherichia coli* β-galactosidase gene inserted downstream of the polyhedral promoter (Invitrogen, San Diego, CA, USA).

Culture and Infection Methods

The cells were seeded at a density of 5×10^5 cells/mL, at their mid-exponential growth phase, in 100 mL flat bottom flasks, with a 20 mL working volume. All cultures

were carried out in a rotary shaker at an agitation rate of 100 rpm and a temperature of 27°C.

The recombinant baculovirus infections were carried out as follows; 1×10^6 cells/mL were added to 10 mL of fresh medium with 12% FBS, in a 50 mL conical tube. Lfter the suspension of the, the virus was added at a multiplicity of infection (MOI) of 20, and the conical tube was mildly shaken in a rotary shaker for 1 h, to allowed infection of the cells. After infection the medium was exchanged for 20 mL of fresh SFM, and cultivated at 27 °C in a rotary shaker at 100 rpm.

Analytical Methods

The cells were counted using hemacytometer, and their viability was determined by trypan blue exclusion. Virus titers were carried out by modified endpoint dilution; the virus stock sample was diluted serially from 10^4 to 10^7 times. 120 μL of each virus dilution was added to 1.2 mL of Sf9 cell suspension (1×10^5 cells/mL), and then aliquots of 110 μL from each mixture seeded into 12 wells of 96 well plates. After incubating at 27 °C for 8 d, 53 μL of Z-buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 0.001 M $MgSO_4$, and 0.05 M β-Mercaptoethanol, pH 7.0) and 21 μL of *O*-nitrophenyl-β-galactopyranoside (ONPG, 4g/L) were added. Samples were incubated at 55°C for 15 min and then cooled on ice, following the addition 53 μL 1 M Na_2CO_3 to stop the reaction [7]. The wells of reaction mixtures containing infected cells would become yellow. The titers for the AcNPV-βgal were calculated using the standard method (50% tissue culture infectious dose: $TCID_{50}$) [8].

The glucose and lactate concentrations were measured using an YSI analyzer (Model 2700, Yellow Springs Instrument, Ohio, USA). Glutamine and ammonium ion concentration were measured using amino acid analyzer (L-8500A, Hitachi, Tokyo, Japan). The β-galactosidase activity was assayed according to Miller's method [7].

The extracellular protease activity was measured according to the method described by Slack *et al.* [9]. One unit of protease activity was defined as the activity that increases the absorbance, at 405 nm, by a value of one, within 1 h, at 405 nm under the defined assay conditions.

RESULTS

Growth Characteristics of Sf9, Sf21, Tn5B1-4 cells in Suspension Culture

The Sf9, Sf21, and Tn5B1-4 cells, at 5.0×10^5 cells/mL, were cultured in the four different media, EX-CELL 420 and 405, and Sf 900 II and Express Five. The results are obtained with the various cultures are shown in Fig. 1. The Sf9 cells grew well in the Sf 900 II SFM and EX-CELL 420 media, and reached densities of 7.60×10^6 cells/mL after 6 days of culturing (Fig. 1A). The cell densities in the EX-CELL 405 and Express Five media were lower than those in the Sf 900 II and EX-CELL 420 media. For the case with the Sf 21 cells, in each of the media,

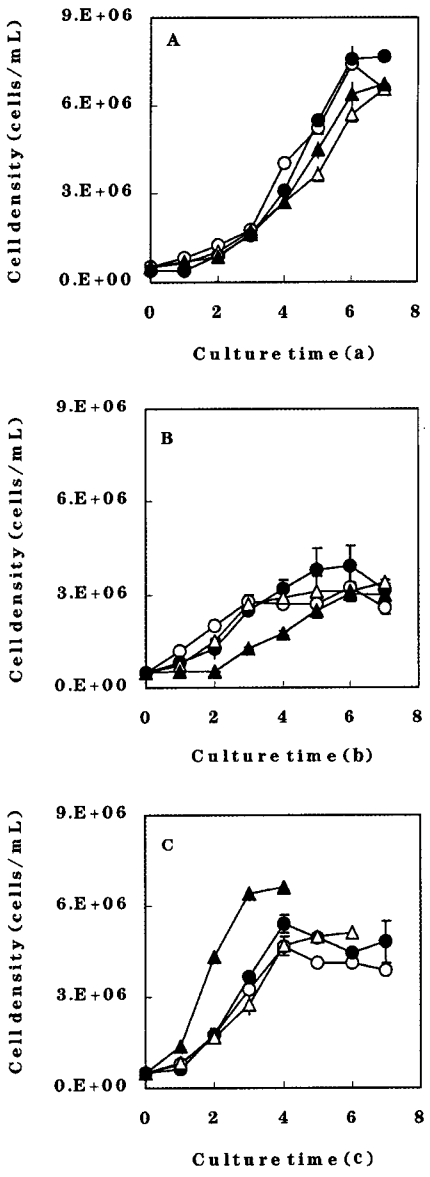

Fig. 1. Growth of Sf9 (A), Sf21 (B), and Tn5B1-4 (C) cells in suspension culture in EX-CELL 420 (○), Sf900 II (●), EX-CELL 405 (△), and Express Five (▲). Cultures were carried out in 100 mL flat bottom flasks, with a 20 mL working volumes, at 100 rpm.

the cell densities were half those of the Sf9 cell line (Fig. 1B). However, for the case of Tn5B1-4 cell line, the Express Five medium gave the highest cell growth rate, with the cell density reaching 6.43×10^6 cells/mL in a culture time of only 3 days (Fig. 1C). The other three media gave similar cell growth rates. The specific growth rates for the Sf9 and Sf21 cell lines, in the Sf 900 II medium, were 0.55 and 0.43 day^{-1}, which was the highest of all the other media. The combination of the Tn5B1-4 cell line with the Express Five medium showed a maximum specific growth rate of 0.88 day^{-1}, which was 1.6 and 2.0

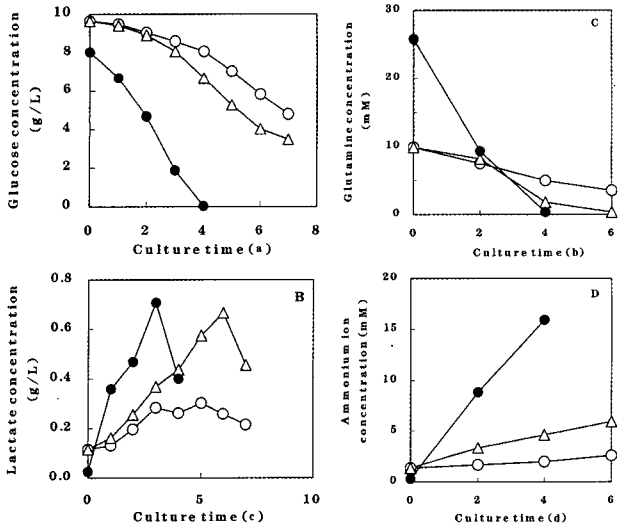

Fig. 2. The consumptions of glucose (A) and glutamine (B), and the productions of lactate (C) and ammonium ions (D) in cultures of Sf9 (○), Sf21 (△), and Tn5B1-4 (●) cells. The Sf9 and Sf21 cell lines were cultured in the Sf900 II SFM, while the Tn5B1-4 cell line was cultured in the Express Five SFM.

folds as higher than those of the Sf9 and Sf21 cell lines, respectively.

The Comparison of Metabolites in Three Cell Lines

The consumptions of glucose and glutamine, and the formations of lactate and ammonium ions, as byproducts are shown in Fig. 2. The glucose consumptions of the Sf9 and Sf21 cell lines were similar, but the Tn5B1-4 cell line completely consumed the glucose within 4 days of culturing (Fig. 2A). The lactate formations the Sf21 and Tn5B1-4 cell lines were 2.2 and 2.5 fold higher than that of the Sf9 (Fig. 2B). Tn5B1-4 cell line produced lactate 0.71 g/L of lactate in 3 days.

Even though the consumptions of twenty different amino acids were investigated, the only change in an amino acid concentration was observed with the glutamine. The Sf9 and Sf21 cell lines, in the Sf 900 II medium, slowly consumed 9.86 mM of glutamine over the entire culture period, whereas, the Tn5B1-4 cell line, in the Express Five medium, consumed 25.80 mm of glutamine by day 4 of culturing (Fig. 2C). For ammonium ion accumulation in the Sf9 and Sf21 cell lines cultures were 2.57 mm and 5.94 mm, respectively, which accumulated after 6 days, but the Tn5B1-4 cell line rapidly accumulated 15.93 mm of ammonium ions after 4 days (Fig. 2D).

Cell viability of Tn5B1-4 cells decreased significantly due to depletion of glucose and glutamine in medium, and moreover cell death rate was accelerated by the formation of byproducts (Fig. 3). However, in the case of Sf9 and Sf21 cells 80% of cell were viable in 7 days. This indicates that the viability of Sf9 and Sf21 cells may remain high level due to the low assimilation rate of nutrients in comparison to Tn5B1-4 cells.

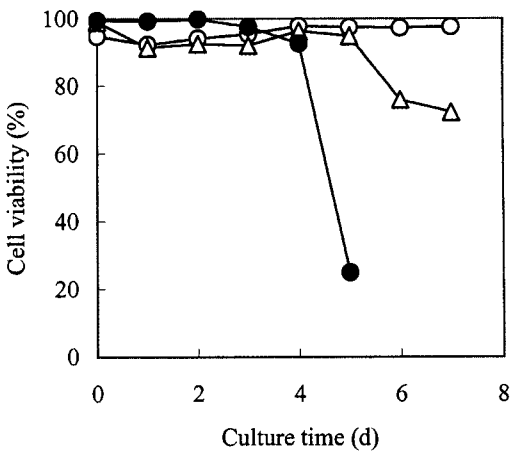

Fig. 3. Cell viabilities of Sf9 (○), Sf21 (△), and Tn5B1-4 (●) cell lines. The Sf9 and Sf21 cell lines were cultured in the Sf 900 II SFM, while the Tn5B1-4 cell line was cultured in the Express Five SFM.

Table 1 summarizes the maximum specific consumption rates of the metabolites. The Sf9 cell density and production rate was twice that of the Sf21 cell line, while the cell production rate of the Tn5B1-4 cell line, in Express Five SFM, was 1.7 fold higher than that of the Sf9 a similar cell density. The glucose and glutamine consumption rates of the Sf21 were about 1.5 fold higher than those of the Sf9 cell line, but the glucose and glutamine consumption rates of the Tn5B1-4 cell line were 4 and 2.3 fold higher, respectively, than those of the Sf9 cell line. The formation of byproducts, such as lactate and ammonium ions, was noticeable higher in the Tn5B1-4 cell line than those of the Sf9, with specific production rates of 6.6 and 10.8 fold higher, respectively. The Sf21 cell line also showed around a 5 fold higher formation rates of lactate and ammonium ions than that of Sf9 cell line. The overall cell yield of the Sf21 and Tn5B1-4 cell lines produced less cells with the use of glucose than the Sf9 cell line. The Tn5B1-4 cell line especially, yielded of lactate and ammonium ions 2.8 and 1.5 fold higher, respectively, than the Sf9 cell line.

β-Galactosidase Production in Different Combination of Cell Line and Medium

When the recombinant baculovirus was used to infect in different cell line and media combinations, the expressed β-galactosidase activity was measured, and the results are shown in Fig. 4A. The difference in the β-galactosidase production between the Sf9 and Sf21 cell lines was negligible, but that of the Tn5B1-4 cell line in the Express Five medium had the maximum observed activity, 2.15×10^4 IU/mL, which was about 4 times higher than those of the Sf9 and Sf21 cell lines.

The recombinant protein sometimes produced in the BEVS was decomposed by extracellular protease. Fig. 4B

Table 1. Maximum specific rates of metabolites [formation or production] during growth of Sf9, Sf21 cells in Sf 900 II, and Tn5B1-4 cells in express five SFM

Cell lines	Sf9	Sf21	Tn5B1-4
Used serum free medium	Sf 900 II	Sf 900 II	Express five
Maximum cell production			
Specific growth rate (day^{-1})	0.549 (1)	0.427 (0.78)	0.880 (1.60)
Cell density (10^6 cells/mL)	7.69 (1)	3.92 (0.51)	6.63 (0.86)
Cell production (10^6 cells/mL/day)	1.20 (1)	0.67 (0.53)	1.98 (1.65)
Maximum specific consumption rates			
Glucose (μg/10^6 cells/day)	87.60 (1)	123.12 (1.40)	347.95 (3.97)
Glutamine (μmol/10^6 cells/day)	0.45 (1)	0.74 (1.64)	1.04 (2.31)
Maximum specific formation rates of byproducts			
Lactate (μg/10^6 cells/day)	6.01 (1)	34.23 (5.70)	39.91 (6.64)
Ammonium ion (μmol/10^6 cells/day)	0.06 (1)	0.29 (4.82)	0.65 (10.83)
Overall yield coefficient			
$Y_{cell/glucose}$ (10^3 cells/μg)	1.90 (1)	0.60 (0.3)	0.95 (0.5)
$Y_{lactate/glucose}$ (μg/μg)	0.12 (1)	0.12 (1.0)	0.33 (2.8)
$Y_{ammonium\ ion/glutamine}$ (μmol/μmol)	0.41 (1)	0.62 (1.5)	0.63 (1.5)

Parentheses indicate the ratio of the specific growth rate to that in Sf9 cell line and different kinds of media.

Table 2. Comparison of maximum β-galactosidase production rate and protease production of infected Sf9, Sf21, and Tn5B1-4 cells in different media

Cell lines	Sf9	Sf21	Tn5B1-4
Medium	Sf 900 II	Sf 900 II	Express five
Maximum specific β- galactosidase production rate (IU/10^6 cells/day)	1361.60 (1)	1305.31 (0.96)	6107.65 (4.49)
Protease production			
Protease yield (U/10^6 cells)	1.96 (1)	1.94 (1)	3.11 (1.58)
Maximum specific protease production rate (U/10^6 cells/day)	0.95 (1)	1.12 (1.18)	2.76 (2.91)

Parentheses indicate the ratio of the specific growth rate to that in Sf9 cell line and different kinds of media.

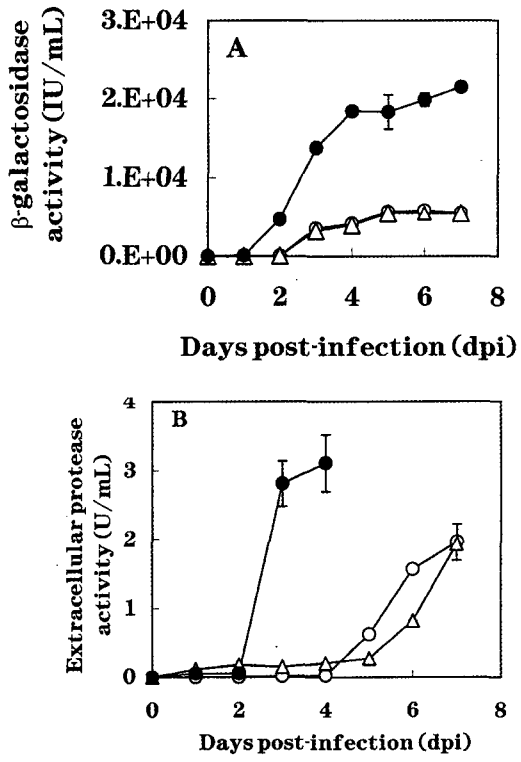

Fig. 4. β-Galactosidase production and extracellular protease activity of the infected Sf9 (○), Sf21 (△), and Tn5B1-4 (●) cell lines. The Sf9 and Sf21 cell lines were cultured in the Sf 900 II SFM, while the Tn5B1-4 cell line was cultured in the Express Five SFM.

shows the extracellular protease activity of the infected Sf9 and Sf21 cell lines, in the Sf 900 II SFM, and of the Tn5B1-4 cell line, in the Express Five SFM. The extracellular protease activity increased linearly, from 4 dpi to 7 dpi, in Sf9 and Sf21 cell lines, but rapidly, from 2 dpi to 4 dpi in the Tn5B1-4 cell line. The maximum protease activity was 1.96 U/mL for the Sf9 and 1.94 U/mL for the Sf21, while for the Tn5B1-4 cell line this was 3.11 U/mL. The increase in the protease was connected with the increase in the β-galactosidase activity (see Fig. 4A).

Table 2 summarizes the maximum specific rates of β-galactosidase production and protease formation rates. The specific β-galactosidase production rate of the Sf21 cell line was similar to that of the Sf9 cell line. For the case with the Tn5B1-4 cell line, in the Express Five SFM, in the β-galactosidase production was 4.5 fold higher than the Sf9 cell line.

The protease yields of the Sf9 and Sf21 cell lines were similar, but that of the Tn5B1-4 cell line was 1.6 fold

higher, and its formation rate 3-fold higher, than those of the Sf9 cell line.

DISCUSSION

The optimum combination of insect cell line and medium combination is a very important factor, for both the efficient cell growth and recombinant protein production in the BEVS. The Tn5B1-4 cells grew to higher cell densities in the SFM than the serum supplemented cultures, and were more productive than either the Sf9 or Sf21 cell lines. The Express Five SFM was the best medium for the cell growth and protein production with the Tn5B1-4 cell line. In the case of Sf9 and Sf21 cell lines, the Sf 900 II SFM was the most appropriate SFM for cell growth.

Nutrient consumption of the Tn5B1-4 cell lines was remarkable. The glucose and glutamine consumption rates were 4 and 2.3 fold higher, respectively, than the compared to Sf9 cell line. From these nutrients, the lactate and ammonium ions produced, were 6.6 and 10.8 fold higher, respectively, than from the Sf9 cell line. The lactate and ammonium ions may have an inhibitory effect on the cell growth, which may be resulted in a low cell density (Fig. 1C). The overall lactate yield from the consumed glucose was 2.8 fold higher than that of the Sf9 cell line, while the ammonium ion yield was 1.5 fold higher.

The Sf21 cell line showed a lower cell density, but higher formation rates of lactate and ammonium ions during the culturing than those of the Sf9 cell line. The Sf9 cell line, using the Sf 900 II SFM, showed a moderate cell growth rate, and produced less lactate or ammonium ions than the Tn5B1-4 cell line, using the Express Five SFM.

The Tn5B1-4 cells production of β-galactosidase was 4.5 fold higher than those of either the Sf9 or Sf21 cells. This increased production may be partly due to the Tn5B1-4 cells being 1.2-1.5 times larger than the Sf21 cells (data not shown). Taking into account this differences in size, the Tn5B1-4 cells were still 1.3-3 times more productive on per cell basis.

Conversely, the Tn5B1-4 cell line produced 3 times the protease of either the Sf9 or Sf21 cell line, which caused a severe problem in the production of the recombinant protein. Baculoviruses have cystein protease by which host degradation after cell death occurred [10], while insect cells have carboxyl protease [11]. Those proteases degrade recombinant proteins in virus infected cell culture. In insect cell culture, since cystein protease activity was higher than that of carboxyl protease, the degradation of recombinant protein was due to not carboxyl protease, but cystein proteas [12]. The β-galactosidase activity could be increased by 50% on the addition of a cystein protease inhibitor to the culture (data not shown). With the use of a cystein protease deficient recombinant virus, the production of protein from the Tn5B1-4 cell line could be much improved in the BEVS.

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