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Improved Recombinant β-Galactosidase Production Using Medium Additives at AcNPV Infection of Insect Cells in Batch and Continuous Two-Stage Bioreactors

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회분식과 연속식 2단계 생물반응기에서 AcNPV의 곤충세포에의 감염시 배지 첨가물을 이용한 재조합 β-Galactosidase 생산의 증진

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ABSTRACT

The medium additives such as $CaCl_2$, glucose, frutose, glutamine, glutamate and lipids were examined to enhance recombinant β-galactosidase(β-gal) production in batch and continuous two-stage bioreactor systems. The presence of each medium additive such as $CaCl_2$, fructose, glutamate, cholesterol and tocopherol at AcNPV infection of Sf 21 cells had an effect on improved β-gal production. The recombinant β-gal production using the infection media supplemented with a mixture of 30mM $CaCl_2$, 2.2mM fructose, 4.1mM glutamate and 0.34mM cholesterol was increased by about 40%.

INTRODUCTION

The baculovirus vector has been extensively developed and improved for the efficient expression of heterologous genes(1-4). However, maximal expression potential of the various gene employed in the *Autographa californica* nuclear polyhedrosis virus(AcNPV) vector system still remain undetermined. There are many identified factors that affect foreign gene expression in insect cell culture. Several factors such as multiplicity of infection, cell density at infection, cell culture conditions, choice of media and operating mode of bioreactors have been investigated by several researchers(5-11). The medium components or additives at viral infection also have an effect on recombinant protein production. It is important that these factors be examined for insect cell culture in order to maximize protein production. In this study, we evaluated the medium additives

such as $CaCl_2$, glucose, frutose, glutamine, glutamate and lipids at AcNPV infection of *Spodoptera frugiperda* 21(Sf 21) cells in batch and two-stage bioreactor systems.

MATERIALS AND METHODS

Cell Line, Virus and Medium

The insect cell line used in this study was *Spodoptera frugiperda* IPLB-Sf-21(Sf 21). The cells were maintained in 25㎠ and 75㎠ tissue culture flask(Corning), to provide cells for batch and two-stage bioreactors. A recombinant AcNPV expressing *E. coli* β-galactosidase was propagated and amplified on monolayer Sf 21 cells and kept at 4℃ in the form of culture supernatant(12). The medium used in batch experiments was Grace's insect medium(Gibco), which was supplemented with 50μg/ml gentamycin sulfate(Sigma), 2.5μg/ml fungizone(Gibco), 0.35g/ℓ sodium bicarbonate(Sigma) and 10% fetal bovine serum(Sigma). In the continuous two-stage bioreactor experiments the same medium was used, but with 5% fetal bovine serum.

Batch Culture Conditions

Viable Sf 21 cells($1.5-2\times10^6$ cells/plate) were allowed to attach for 1hr. Viruses were added at an MOI of 0.5 and T-flasks rocked for 1hr. The medium containing virus was then removed, replaced with fresh medium containing medium additive and incubated at 28℃ for 4 days.

Continuous Two-stage Bioreactor Culture Conditions

A continuous two-stage bioreactor system was established using spinner flasks. The first-stage bioreactor was designed for the cultivation of insect cells and the second-stage bioreactor was intended for the viral infection of the cells. The working volume of the first and second stage bioreactors was maintained at 100ml and 250ml, respectively. Two-stage bioreactor culture was operated under the conditions of the initial pH 6.2, 28℃ and 80rpm of agitation. The cells of the second stage bioreactor was infected with virus at an MOI of 10. The air supply to the bioreactor was done by a silicone tubing device.

Analytical Methods

Viable cell concentration was determined using the trypan-blue dye exclusion test. Viral titers were measured by plaque assay as described elsewhere(12). The recombinant β-galactosidase activity was determined by the procedure described as elsewhere(13).

RESULTS AND DISCUSSION

Batch Culture

Divalent cations were reported to be important for the initiation of virus infection(14). A set of experiments was carried out to determine the effect of calcium ion on β-gal production. As shown in Fig. 1, recombinant β-gal production increased upto the $CaCl_2$ concentration of 30mM and then decreased at higher concentration of $CaCl_2$. Apparently, the level of $CaCl_2$ present in the medium at AcNPV infection was found to affect recombinant β-gal production. Also glucose, fructose, glutamine and glutamate were reported to be the most rapidly consumed components as carbon or energy sources(15). To find out the

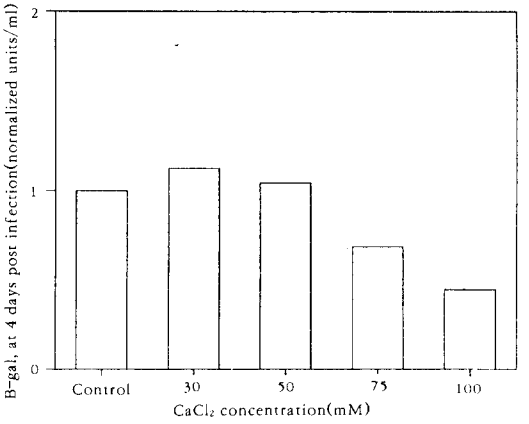

Fig. 1. Effect of $CaCl_2$ concentration on recombinant β-galactosidase production. Initial cell density, 1.5×10^6 cells/plate. MOI, 0.5.

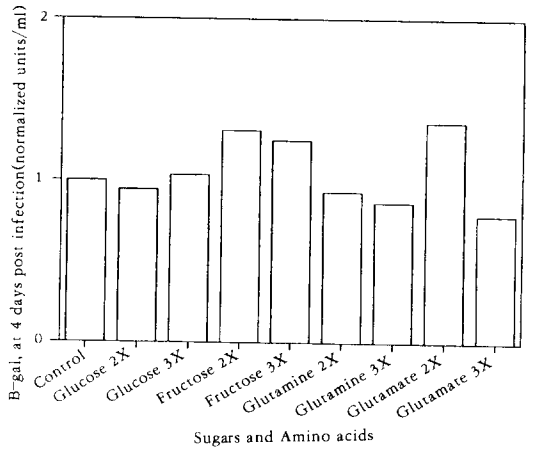

Fig. 2. Effect of glucose, fructose, glutamine and glutamate on recombinant β-galactosidase production. Initial cell density, 2×10^6 cells /plate. MOI, 0.5.

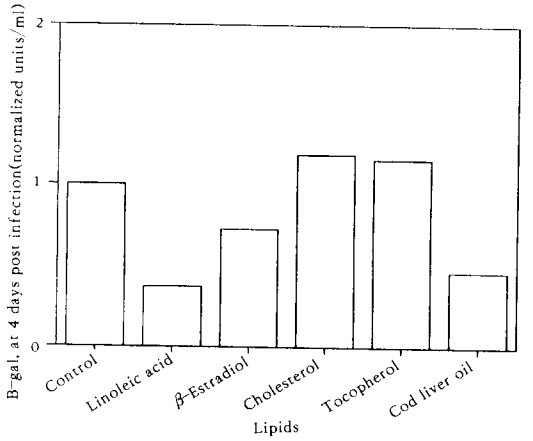

Fig. 3. Effect of lipids at AcNPV infection on recombinant β-galactosidase production. Initial cell density, 1.5×10^6 cells/plate. MOI, 0.5.

effect of these components on β-gal production several runs were made. Fig. 2 shows that the addition of 2.2mM fructose or 4.1mM glutamate to the infection medium(indicated by Fructose 2X and Glutamate 2X) yielded higher β-gal production. These components seem to be one of the important energy substrates for infected Sf 21 cells. Furthermore, commercially available lipids as another medium additive were tested to facilitate recombinant β-gal production. In a preliminary experiment, direct addition of lipids to the medium resulted in insoluble droplets. Hence these lipid components were prepared as a microemulsion using Pluronic polyol F68 and ethanol. As shown in Fig. 3, the addition of lipids such as cholesterol or tocopherol to the infection medium improved recombinant β-gal production. Evidently, the lipid component seems to play an important function in AcNPV replication and recombinant protein production, but the exact role of lipid at AcNPV infection remains to be clarified.

Continuous Two-Stage Bioreactor Culture

Previous studies demonstrated that appropriate dilution rate for a singe-stage bioreactor run was $0.015hr^{-1}$(13). Thus the first stage bioreactor was operated at this dilution rate and a steady supply of 8×10^5 cells to the second stage bioreactor was maintained. To find out optimum dilution rate of the second stage bioreactor, several runs were made by varing the dilution rate from $0.018hr^{-1}$ to $0.075hr^{-1}$. At each dilution rate steady state samples were taken from the bioreator for quantification of β-gal activity. Fig. 4 shows that the amount of β-gal synthesis in the second stage bioreactor was the highest at the dilution rate of $0.018hr^{-1}$. As the dilution rate was further increased, the reduction of β-gal synthesis was observed. These studies have revealed that recombinant β-gal production rate is sensitive to the operating condition of the second stage bioreactor.

Several two-stage bioreactor runs were made to see the effects of medium additives on contiunous β-gal production. In principle, cell suspension of the continuously operated cell growth bioreactor(Stage 1) was pumped into the infection bioreactor(Stage 2) and second feed stream of recombinant AcNPV containing concentrated medium additives was added to the infection bioreactor. The dilution rate of the second stage bioreactor was $0.018hr^{-1}$, corresponding to a residence time of 56hr. As expected, noticeable effect was observed in the evaluation of medium

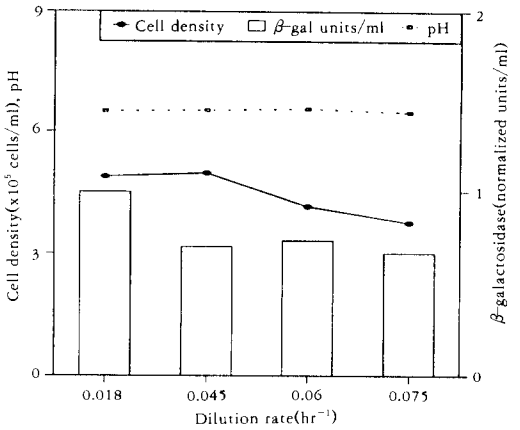

Fig. 4. Continuous two-stage bioreactor culture: Effect of dilution rate of the second stage bioreactor on recombinant β-galactosidase production.

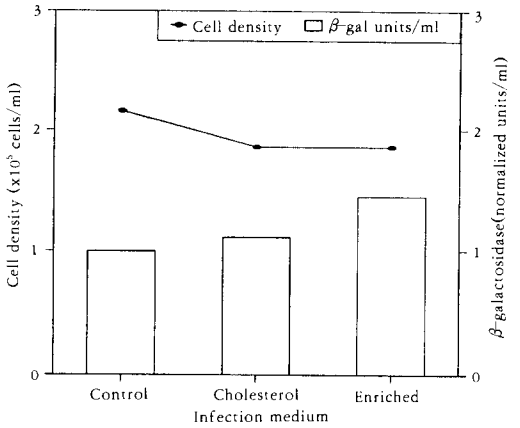

Fig. 5. Effect of infection medium supplemented with additives on β-galactosidase production in continuous two-stage bioreactor culture.

additives for recombinant β-gal production in continuous two-stage bioreactor systems. The β-gal production using the infection medium supplemented with either 0.34mM cholesterol or a mixture of 30mM $CaCl_2$, 2.2mM fructose, 4.1mM glutamate and 0.34mM cholesterol was increased by about 10% and 40%, respectively, compared to that of the control(Fig. 5). The presence of medium additives was an important improvement for recombinant β-gal production.

This result indicates that nutrient limitation is an important parameter during AcNPV infection process. It appears that further investigations are needed to identify other factors in the medium composition limiting the performance of insect cell-baculovirus systems. However, our studies reveal that when appropriate medium additives are provided at viral infection of insect cells, recombinant AcNPV utilizes more efficiently the cell machinery for the expression of foreign heterologous proteins.

요 약

$CaCl_2$, glucose, frutose, glutamine, glutamate 그리고 lipids와 같은 배지 첨가물들이 회분식 그리고 연속식 2단계 생물반응기 시스템에서 재조합 β-galactosidase(β-gal) 생산을 증진시키는가를 조사하였다. Sf 21 세포에 AcNPV의 감염시 $CaCl_2$, frutose, glutamate, cholesterol 및 tocopherol과 같은 배지 첨가물을 첨가하였을 때 β-gal 생산이 증진되었다.

30mM $CaCl_2$, 2.2mM frutose, 4.1mM glutamine, 그리고 0.34mM cholesterol이 보강된 감염 배지를 이용한 재조합 β-gal 생산은 약 40%의 증가를 보였다.

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REFERENCES

1. P. D. Friesen and L. K. Miller(1986), In : The Molecular Biology of Baculoviruses, Doerfler, W. and Boehm, P.(eds), Springer-Verlag, Berlin., 31.
2. V. A. Luckow(1991), In : Recombinant DNA Technology and Applications, Prokop, A., Bajpai, R. K. and Ho, C. S.(eds), Mcgraw-Hill, New York, 97.

3. V. A. Luckow and M. D. Summers(1988), *Bio/technology*, **6**, 47.
4. S. M. Thiem and L. K. Miller(1990), *Gene*, **91**, 89.
5. D. R. Broussarad and M. D. Summers(1989), *J. Invert. Pathol.*, **52**(2), 144.
6. A. W. Caron, J. Kamen and B. Massie(1990), *Biotechnol. and Bioeng.*, **36**, 1133.
7. A. A. Kamen, R. L. Thon, A. W. Caron, C. Chavarie, B. Massie and J. Archambault (1991), *Biotechnol. and Bioeng.*, **38**, 619.
8. J. E. Lazarte, P. F. Tosi and C. Nicdau (1992), *Biotechnol. and Bioeng.*, **40**, 214.
9. R. Neutra, B. Z. Levi and Y. Shoham(1992), *Appl. Microbiol. and Biotechnol.*, **37**, 74.
10. F. L. Van Lier, E. T. van den End, C. D. de Gooijer, J. M. Vlak and J. Tramper(1990), *Appl. Microbiol. Biotechnol.*, **33**, 43.
11. M. Y. Wang, S. Kwong and W. E. Bentley (1993), *Biotechnol. Prog.*, **9**, 355.
12. Y. M. Park(1991), M. S. Thesis, Dept. of Genetic Engineering, Kyung Hee University, Korea.
13. J. S. Kim(1994), M. S. Thesis, Dept. of Genetic Engineering, Kyung Hee University, Korea.
14. X. Wang and D. C. Kelly(1985), *J. Gen. Virol.*, **66**, 541.
15. C. Bedard, R. Tom, A. Kamen and G. Andre (1993), In : Proceedings of the Baculovirus and Recombinant Protein Production Workshop, Vlak, J. M., Schlaeger, E. J. and Bernard, A. R.(eds), Switzerland, 339.