Biotechnol. Bioprocess Eng. **1997**, 2, 27-32

Optimization of Fusion Proinsulin Production by High Cell-density Fermentation of Recombinant *E. coli*

Cheon-Soon Bae[1], Min-Sun Hong[1], Seung-Gu Chang[2], Dae-Young Kim[2], and Hang-Cheol Shin[2]*

Laboratories of [1]Bioprocess Engineering and [2]Protein Engineering
Hanhyo Institute of Technology, 461-6 Jeonmin-dong, Yusong-ku, Taejon #305-390, Republic of Korea

The optimum conditions for mass production of fusion proinsulin were studied in recombinant *Escherichia coli* strain BL21 (DE3) [pT7-PI] using fed-batch culture employing pH-stat method. Yeast extract was found to enhance both the growth rate of recombinant *E. coli* strain BL21 (DE3) [pT7-PI] and its cell mass yield. When the glucose concentration was 10 g/L in the initial medium, 10 g/L concentration of yeast extract was found to be optimal to control the acetate production and to augment both the cell mass yield and the growth rate. Optimum ratio of glucose to yeast extract to minimize the cost of the feeding medium in the fed-batch culture was calculated to be 1.225 and verified by the subsequent experiments. The appropriate inducer concentration and induction time were examined with isopropyl-β-D-thiogalactopyranoside (IPTG). Irrespective of the induction time, IPTG induction resulted in the reduction of growth rate, but the expression level of the fusion protein was maintained at the level of about 20% of the total proteins. Since the volumetric productivity was well maintained in the range between 0.15 and 0.18 g/L.hr at the inducer concentration of above 0.025 mM, the appropriate inducer concentration, in relation to the inducer cost, is considered to be about 0.025 mM.

Key words: high cell density, fed batch, pH-stat, fermentation, medium optimization

INTRODUCTION

Heterologous expression of naturally scarce eukaryotic proteins in *Escherichia coli* has provided a more abundant source of these proteins for clinical use. The efficient production of these proteins necessitates to optimize each step of host selection, gene cloning, high cell-density culture, protein purification, etc. Optimization of the high cell-density culture is one of the major requirements in obtaining high productivity in a fermentation process. Up to now, fed-batch culture technique has been mainly used for mass culture of recombinant *E. coli*. To supply nutrients to the fermentor in fed-batch culture, various feeding system have been developed, such as predetermined feeding system [1], DO-stat system [2-5], pH-stat system [6-7], and system using on-line glucose analyzer [8] etc. In this study, pH-stat method where nutrient addition is triggered by the pH change was used.

Among the various factors to be considered for high productivity in a fermentation process, lowering acetate concentration is one of the problems to be solved. It has been reported by many researchers that under excessive glucose concentration even in the aerobic condition, *E. coli* produces acetate (*E. coli* Crabtree effect), which inhibits cell growth [1, 2, 9, 10] and product synthesis [10]. Ammonia is another substance to be considered since *E. coli* growth was reported to be rapidly inhibited when ammonia concentration is above 170 mM [11]. To maintain protein expression level (specific protein productivity) at high cell-density culture, plasmid stability should be maintained for overall period of culture. It was known that several factors affect plasmid stability, and Hopkins *et al.* [12] reported that DO limitation is one of the environmental stresses that gave rise to plasmid segregation of recombinant *E. coli*. Therefore, considering all previous results for high density culture of recombinant *E. coli* by fed-batch culture technique, it is desirable to maintain DO concentration at least above 10% of that saturated by air, glucose concentration at low level to inhibit acetate accumulation, and ammonia concentration below 170 mM.

In addition to the several factors described above, medium composition is also important. Usually, one or several complex nitrogen sources are added to the medium to increase cell growth rate and to enhance cell mass yield [8]. Yeast extract has been used to increase cell growth rate and cell mass yield [8, 16, 17]. The optimum concentration of yeast extract, however, in relation to the carbon source has not been well studied.

In this study, we aimed at the medium optimization for high cell-density culture of *E. coli*, in consideration of the minimum medium cost and the optimum concentration ratio of glucose to yeast extract. Also, we have determined the induction time and the optimum inducer concentration to maximize the volumetric productivity of target protein.

MATERIALS AND METHODS

Bacterial Strain and Plasmid System

*Corresponding author
Tel: 042-866-9123 Fax: 042-866-9129
e-mail: hcshin@donald.hanhyo.co.kr

The *E. coli* host strain used in this study was BL21 (DE3) [F⁻ ompT r_B^- m_B^-] whose chromosome carries the gene for T7 RNA polymerase, lacI gene and lacUV5 promoter gene. pT7-7 plasmid, originally developed by Studier *et al.* [15], was used as an expression vector system. Proinsulin was fused at the amino terminus to an N-terminal 57 residue sequence of human tumor necrosis factor-α (TNF-α) for the ease of down stream processing. The gene for the fusion proinsulin was inserted into the restriction sites of the pT7-7 plasmid to construct recombinant expression vector system pT7-PI. When expressed in recombinant *E. coli* strain, the fusion protein was accumulated as inclusion bodies.

Cultivation and Media

Controlled fermentations were performed in a BioFlo III 5L fermentor (New Brunswick Scientific Co.), equipped with an embedded multiloop controller which regulates agitation, temperature, dissolved oxygen, pH and nutrient addition. Nutrient addition by pH-stat method was controlled with the program AFS (Advanced Fermentation Software), provided by the manufacturer. During fermentation, air and/or pure oxygen were supplied at a rate of 1-1.5 vvm to maintain the dissolved oxygen concentration greater than 50% of that saturated by air. The fermentation temperature was controlled at 37°C and the pH at 7.0 by adding 3N NaOH during batch culture period. In a fed-batch culture, medium was added automatically by AFS whenever the culture pH becomes higher than 7.04. An overnight culture in LB medium was used as an inoculum for all experiments. Ampicillin was added to inhibit the growth of plasmid-free cells and contaminants. Antifoam A (Sigma Chemical) was used to suppress the foam formation. Glucose, $MgSO_4 \cdot 7H_2O$ and trace metals were sterilized separately. Thiamine-HCl, ampicillin and IPTG were filter-sterilized with a 0.22 μM filter (Millipore) before use. The medium composition for fed-batch cultures is listed in Table 1.

Analytical Procedures

The optical density (OD) of each sample was measured at 600 nm with a UV-visible spectrophotometer (UltraspecIII, Pharmacia). One optical density unit was experimentally determined to be 0.424g dry cell weight/L. Glucose and acetate concentrations were determined using a clinical glucose analyzer (YSI model 2300 plus) and an enzyme kit (Bohringer mannheim Co.), respectively. The ammonia concentration was measured with a pH/ion meter (Mettler delta 350) and an ammonia combination electrode (Corning). Expression level of the fusion proinsulin was examined with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and quantified by scanning the stained bands with densitometer. Plasmid stability was determined by counting colonies on agar plates (LB medium) with and without 100 mg/L ampicillin. Five serially diluted samples were used for cell counting. In all fed-batch cultures performed in this study, plasmid stability was well maintained by nearly 100%.

RESULTS AND DISCUSSION

Effects of Yeast Extract on the Growth of Recombinant *E. coli*

Table 1. The medium composition for fed-batch cultures

Components	Initial medium (g/L)	Feeding medium (g/L)
KH_2PO_4	5.00	0
K_2HPO_4	3.00	0
$(NH_4)_2SO_4$	1.67	1.50
$FeSO_4 \cdot 7H_2O$	0.10	0
$CaCl_2 \cdot 2H_2O$	0.02	0
$MgSO_4 \cdot 7H_2O$	2.00	1.50
Trace metal	80 ml/L	0
Thiamine-HCl	0.10	0
Ampicillin	0.20	1
Glucose	10.00	variable
Yeast Extract	variable	variable

It has been reported that yeast extract promotes both cell growth of recombinant *Bacillus subtilis* 1A96 and production of the target protein [8]. Similar effects were investigated on the growth of recombinant *E. coli* BL21 (DE3) [pT7-PI], by performing two batch cultures using the initial media (Table 1) containing 0 g/L and 5 g/L yeast extract, respectively. 10 g/L concentration of glucose as a carbon source was used in the initial medium. Specific growth rate (μ) and cell mass yield ($Y_{x/s}$: cell optical density produced/glucose mass consumed) in the medium without yeast extract were 0.164 hr^{-1} and 0.22 O.D./g glucose, respectively. On the other hand, specific growth rate and cell mass yield in the medium with 5 g/L yeast extract were nearly doubled, with the values of 0.30 hr^{-1} and 0.42 O.D./g, respectively. These results indicate that the addition of yeast extract increases both the growth rate of recombinant *E. coli* BL21 (DE3) [pT7-PI] and the cell mass yield.

Optimization of Yeast Extract Concentration in Initial Medium of Fed-batch Culture

The optimum initial concentration of yeast extract was tested by varying the concentration of yeast extract from 0 to 60 g/L. The glucose concentration was fixed at 10 g/L. From each batch culture, kinetic parameters were calculated and plotted in Fig. 1 and 2. Fig. 1 shows the effect of yeast extract on cell mass yield ($Y_{x/s}$) and acetate yield ($Y_{p/s}$: acetate concentration

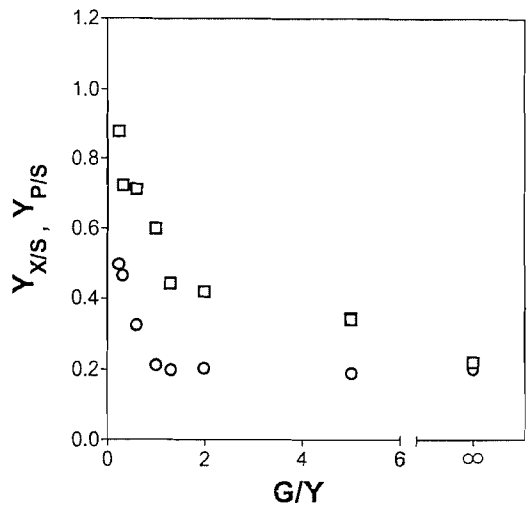

Fig. 1. Effects of G/Y on kinetic parameters (initial glucose concentration: 10 g/L, □ : $Y_{X/S}$; ○ : $Y_{P/S}$).

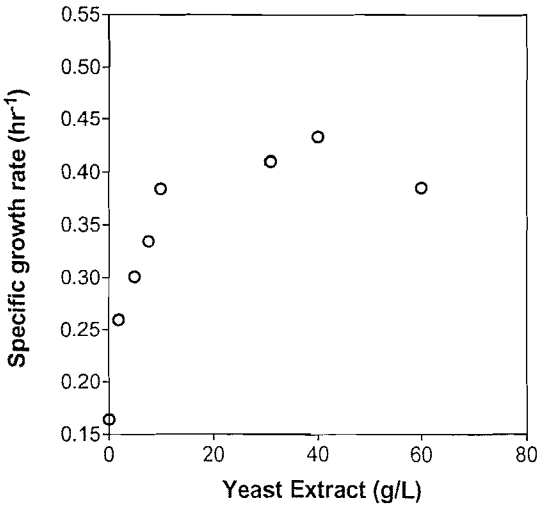

Fig. 2. Effect of yeast extract concentration on specific growth rate.

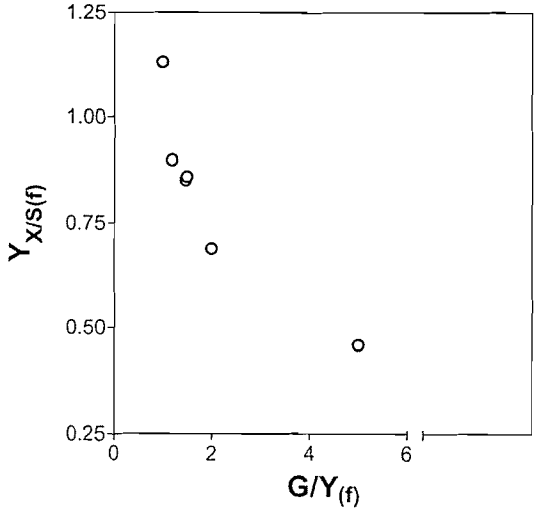

Fig. 3. Effect of $G/Y_{(f)}$ (glucose/yeast extract ratio in feeding medium) on $Y_{X/S(f)}$ (cell optical density produced/glucose mass consumed in a fed-batch period).

produced/glucose concentration consumed). $Y_{p/s}$ remains constant when the concentration ratio (G/Y) of glucose (10 g/L) to yeast extract is above 1, but $Y_{p/s}$ was inversely proportional to G/Y when the G/Y ratio is below 1. This indicates that excess nitrogen against carbon has an enhancing effect on acetate production pathway. In subsequent experiments with various G/Y ratios, it was shown that the cell mass yield ($Y_{x/s}$) increases as the G/Y ratio decreases. Fig. 2 exhibits the effect of yeast extract on specific growth rate. The specific growth rate reached a maximum value (-0.40 hr^{-1}) when the level of yeast extract is above 10 g/L. Since acetate is a major inhibitor of cell growth [1, 2, 9, 10], its excessive accumulation at the batch period in a fed-batch culture is not desirable. By considering the conditions where low $Y_{p/s}$ and high $Y_{x/s}$ and m are required, the optimum concentration of yeast extract was found to be about 10 g/L at 10 g/L of initial glucose concentration.

Optimization of Glucose/yeast Extract Ratio ($G/Y_{(f)}$) in the Feeding Medium of Fed-batch Culture

Optimization of feeding medium composition is important to achieve high growth rate and high cell-density cost-effectively. As shown in Table 1, cost determinants of feeding medium are glucose and yeast extract. To find out the effect of $G/Y_{(f)}$ (glucose/yeast extract ratio in the feeding medium) on $Y_{x/s(f)}$ (cell optical density produced/glucose mass consumed in a fed-batch period), experiments were carried out at several different concentration ratios ($G/Y_{(f)}$), 150/30, 150/75, 150/100, 150/150, 180/150 and 220/150, respectively. The result was plotted in Fig. 3 and from the experimental data, $Y_{x/s(f)}$ is expressed by power equation as follows:

$$Y_{x/s(f)} = 1.069\,(G/Y_{(f)})^{-0.547} \quad (1)$$

Eq. (1) is useful in the culture conditions in which the glucose concentration during fed-batch period is maintained at the very low level (it was maintained below 100 mg/L in our experiments), enough to inhibit the acetate production. Comparing Fig. 3 with Fig. 1 (batch result with acetate production), it appears that acetate production reduces the cell mass yield to carbon source considerably and that fed-batch culture without Crabtree effect shows higher yield.

Calculation of Optimum Feeding Medium Composition ($G/Y_{(f)}$)

In the fed-batch culture to obtain high cell-density, it is preferable to optimize feeding medium composition as follows: 1) minimization of medium cost, 2) use of small volume of feeding medium to prevent excessive increase of working volume, and 3) maintenance of high growth rate.

Maximum solubilities of glucose and yeast extract were experimentally determined to be 600 and 350 g/L, respectively. Also, maximum solubilities of glucose and yeast extracts in a mixture were obtained with stepwise increase of glucose from 150 g/L to 500 g/L with 50 g/L interval. The results are displayed in Table 2. These maximum solubilities of glucose and yeast extract in media were used to calulate the feeding volume and the cost of the feeding medium. The feeding volume of medium (V_t) is calculated from the following equations:

$$C_G \times V_t = M_G \quad (2)$$

$$M_G \times Y_{x/s(f)} = OD_f \times V_f - OD_i \times V_i \quad (3)$$

$$V_f = V_i + V_t \quad (4)$$

From Eqs. (2), (3) and (4)

$$V_t = (OD_f - OD_i) \times V_i / (Y_{x/s(f)} \times C_G - OD_f) \quad \text{when } Y_{x/s(f)} \times C_G > OD_f \quad (5)$$

where C_G is the glucose concentration (g/L) of the feeding medium, V_t is the feeding volume of medium (L) and M_G is the total glucose mass (g). OD_i and OD_f designate the initial OD of 6.0 and the desired final OD of 100 and V_i and V_f denote the initial working volume of 2.415 L and the final working volume (L). As shown in Table 2, the volume of the feeding medium becomes minimum when $G/Y_{(f)}$ is between 1.225 and 1.714, and the medium cost becomes minimum when $G/Y_{(f)}$ is in the vicinity of 2.397. Therefore, in consideration of fermenter size to be used, optimum $G/Y_{(f)}$ is thought to be located between 1.225 and 2.397.

Table 2. The estimated costs and volumes according to $G/Y_{(f)}$ (glucose/yeast extract ratio in feeding medium) at maximum solubility. The costs of glucose and yeast extract were based on the price list of Difco Co. Specific cost indicates the ratio of feeding medium cost fed to total medium volume (Vi+Vt)

Glucose (g/L)	Yeast extract (g/L)	$G/Y_{(f)}$	$Y_{X/S(f)}$	Feeding medium volume fed (L)	Feeding medium cost fed ($)	Specific cost ($/V_f$)
150	262	0.573	1.450	1.932	68.3	15.7
200	233	0.858	1.162	1.715	56.7	13.7
250	204	1.225	0.957	1.630	50.1	12.4
300	175	1.714	0.796	1.636	46.5	11.5
350	146	2.397	0.663	1.719	44.8	10.8
400	117	3.419	0.546	1.917	45.6	10.5
450	87	5.172	0.437	2.349	50.0	10.5
500	58	8.621	0.329	3.519	66.9	11.7

The Determination of Optimum Feeding Medium Composition ($G/Y_{(f)}$) by Experiment

To verify the above calculated optimum ratio of glucose/yeast extract, fed-batch fermentations were performed using four different concentrations of glucose/yeast extract, 200/233, 250/204, 300/175 and 350/146, respectively (Fig. 4). During each fed-batch period, the glucose concentration was well maintained at the minimum level below 100 mg/L, which was low enough to inhibit the acetate production. And the final ammonia concentration was below 30 mM, much smaller than 170 mM which has been reported to inhibit cell growth by Thompson *et al.* [11]. After the start of fed-batch period, acetate started to be consumed by glyoxylate bypass of *E. coli* and its level was maintained below 0.5 g/L. When the ratios of $G/Y_{(f)}$ were 300/175 and 350/146, respectively, high cell-density was not achieved due to the rapid decrease of growth rate. But in the cases of $G/Y_{(f)}$ with 250/204 and 200/233, much improved growth patterns were found. The experimental values of $Y_{x/s(f)}$, feeding medium volume, and its cost were 0.941, 1545 ml and 47.5$ for $G/Y_{(f)}$ ratio of 250/204, and 1.1786, 1640 ml and 54.2$ for $G/Y_{(f)}$ ratio of 200/233, respectively. These data were similar to calculated results shown in Table 2. From these results, it is considered that optimum $G/Y_{(f)}$ is in the vicinity of 1.225.

Determination of Induction Time Using Optimized Medium

The effect of induction time on cell growth and expression level was determined using the above optimized initial and feeding media. IPTG was added into the culture broth at three different stages of cell density (OD of 30, 50 and 67) to give a final concentration of 2.0 mM. As shown in Fig. 5, the addition of inducer causes reduction of cell growth rate, which is mainly due to the utilization of cellular machinery for the synthesis of target protein. Similar effect was also observed in other chemically inducible gene expression systems [13-14]. The expression level has reached to the maximum level of 20% in 3 hrs after induction in all cases (data not shown). This indicates that the expression level of fusion proinsulin is not affected by the induction time between OD of 30 and 67. From these results, it was decided that the optimum induction time for mass production of fusion proinsulin using recombinant BL21 (DE3) [pT7-PI] is OD of 67 at this experimental range (OD of 30 - OD of 67). These results suggest that the induction in high cell-density culture of *E. coli* BL21 (DE3) [pT7-PI] is preferable to be done in the late stage of the growth phase.

Determination of Inducer Concentration Using Optimized Medium

The optimum inducer concentration was examined with various IPTG concentrations between 0.01 and 2.0 mM. IPTG was added into the culture broth when OD reached about OD of 67. The results are shown in Fig. 6. It was found that the growth rate is inversely pro-

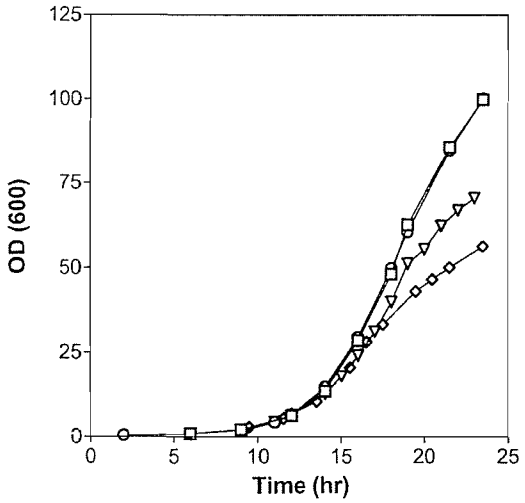

Fig. 4. The growth curves according to $G/Y_{(f)}$ (glucose/yeast extract ratio in feeding medium) (◇ : 350/146; ▽ : 300/175; ○ : 250/502; □ : 200/233).

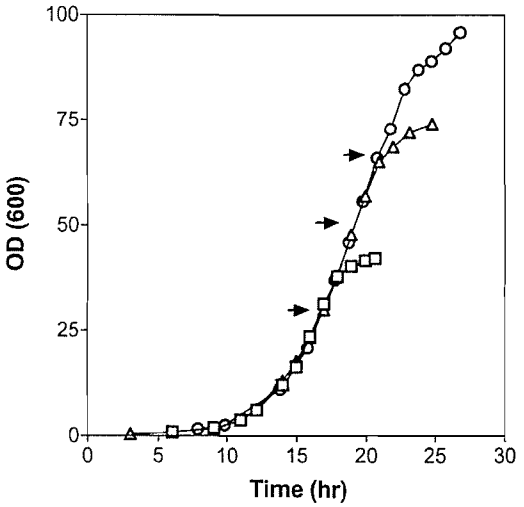

Fig. 5. The effects of inducer concentration on the cell growth (□ : 30; △ : 50; ○ : 67 of induction OD).

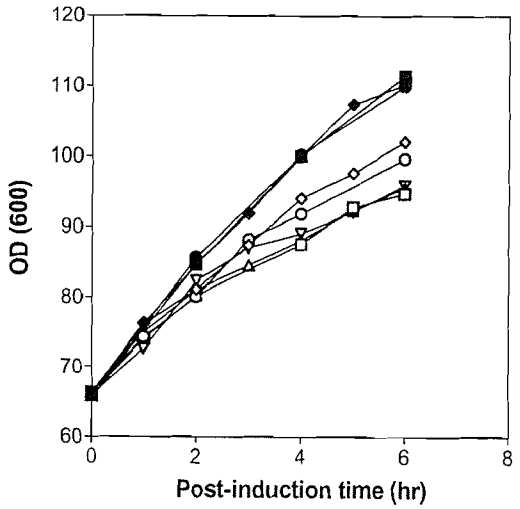

Fig. 6. The effects of inducer concentration on the cell growth (■ : No IPTG; ◆ : 0.01; ● : 0.025; ◇ : 0.05; ○ : 0.25; □ : 0.5; △ : 1.0; ▽ : 2.0 mM).

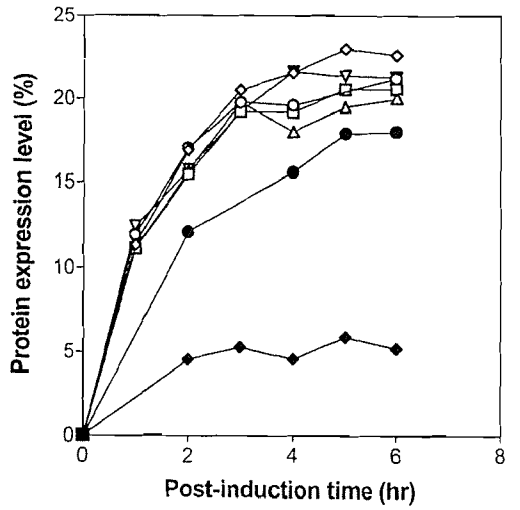

Fig. 7. The effects of inducer concentration on the fusion proinsulin expression rate and level (◆ : 0.01; ● : 0.025; ◇ : 0.05; ○ : 0.25; □ : 0.5; △ : 1.0; ▽ : 2.0 mM).

portional to the inducer concentration of between 0.025 and 0.5 mM. On the other hand, the growth rate was not affected by the variation of inducer concentration below 0.025 mM or above 0.5 mM. Fig. 7 shows fusion proinsulin synthesis rate and maximum expression level at various inducer concentrations. Above 0.05 mM of IPTG, fusion proinsulin was expressed to a maximum level between 20 and 23% in 3 hrs after induction and their synthesis rates were similar irrespective of inducer concentration. Below 0.05 mM of IPTG, however, the synthesis rate of the fusion proinsulin and the maximum expression level appeared to reduce as the inducer concentration decreased. At 0.025 mM of IPTG, for example, the time required to reach a maximum expression level of 18%, was 5 hrs after induction and fusion proinsulin synthesis rate reduced slightly. At 0.01 mM of IPTG, the maximum expression level was only 5.9% and the synthesis rate reduced remarkably. Final volumetric fusion proinsulin productivities were calculated with respect to the inducer concentration. When the inducer concentrations were 0.01, 0.025, 0.05, 0.25, 0.5, 1.0, and 2.0 mM, the corresponding final volumetric fusion proinsulin productivities were calculated to be 0.052, 0.162, 0.187, 0.170, 0.156, 0.152, and 0.170 g/L.hr, respectively. Since the volumetric productivity was well maintained between 0.15 and 0.18 g/L.hr when IPTG level was above 0.025 mM, it was considered that about 0.025 mM of the inducer concentration, in relation to the inducer cost, is appropriate.

Acknowledgment We thank Dr. Baik-Lin Seong and Dr. Jeewon Lee for helpful discussions. This work was supported by Hanil Synthetic Fiber Co., Ltd.

REFERENCES

[1] Yee, L. and H. W. Blanch (1992) Recombinant trypsin production in high cell density fed-batch cultures in *Escherichia coli*. *Biotechnol. Bioeng.* 41: 781-790.

[2] Konstantinov, K., M. Kishimoto, T. Seki, and T. Yoshida (1990) A Balanced DO-stat and its application to the control of acetic acid excretion by recombinant *E. coli*. *Biotechnol. Bioeng.* 36: 750-758.

[3] Mori, H., T. Yano, T. Kobayashi, and S. Shimizu (1979) High density cultivation of biomass in fed-batch system with DO-stat. *J. Chem. Eng. Japan* 12: 313-319.

[4] Mao, W., R. Pan, and D. Freedman (1992) High production of alkaline protease by *Bacillus lichenifoem* is in a fed-batch fermentation using a synthetic medium. *J. Ind. Microbiol.* 11: 1-6.

[5] Ohta, K., T. Shibui, Y. Morimoto, S. Iijima, and T. Kobayashi (1993) High level production of human proapo A-I by fed-batch culture of recombinant *E. coli*. *J. Ferment. Bioeng.* 75: 155-157.

[6] Robbins, J. W. and K. B. Taylor (1989) Optimization of *E. coli* growth by controlled addition of glucose. *Biotechnol. Bioeng.* 34: 1289-1294.

[7] Frude, M. J., A. Read, and L. Kennedy (1993) Induction of recombinant protein production by pH stress: A novel glucose feeding strategy. *Bietechnol. Lett.* 15: 797-802.

[8] Cayuela, C., K. Kai, Y. S. Park, S. Iijima, and T. Kobayashi (1993) Insecticide production by recombinant *B. subtilis* 1A96 in fed-batch culture with control of glucose concentration. *J. Ferment. Bioeng.* 75: 383-386.

[9] Park, Y. S., K. Kai, S. Iijima, and T. Kobayashi (1992) Enhanced-galactosidase production by high cell-density culture of recombinant *B. subtilis* with glucose concentration control. *Biotechnol. Bioeng.* 40: 686-696.

[10] Vila, P. and A. Villaverde (1993) Inhibition of CI 857-controlled recombinant gene expression in *E. coli* at very low concentration of glucose. *Biotechnol. Lett.* 15: 549-552.

[11] Thompson, B. G., M. Kole, and D. F. Gerson (1985) Control of ammonium concentration in *E. coli* fermentations. *Biotechnol. Bioeng.* 17: 818-824.

[12] Hopkins, D. J., M. J. Betenbaugh, and P. Dhurjati (1987) Effects of dissolved oxygen shock on the stability of recombinant *E. coli* containing plasmid pKN401. *Biotechnol. Bioeng.* 29: 85-91.

[13] Bentley, W. E. and D. S. Kompala (1991) Dynamics of induced CAT expression in *E. coli*. *Biotechnol. Bioeng.* 38: 749-760

[14] Miao, F. and D. S. Kompala (1992) Overexpression of cloned genes using recombinant *E.coli*

regulated by a T7 promoter. 1. Batch cultures and kinetic modeling. *Biotechnol. Bioeng.* 40: 787-796.
[15] Studier, F. W. and B. A. Moffatt (1986) Using of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* 189: 113-130.
[16] Yoon, S. K., W. K. Kang, and T. H. Park (1994) Fed-batch operation of recombinant *E. coli* containing trp promoter with controlled specific growth rate. *Biotechnol. Bioeng.* 43: 995-999.
[17] Yoon, S. K., S. H. Kwon, M. G. Park, W. K. Kang, and T. H. Park (1994) Optimization of recombinant *Escherichia coli* fed-batch fermentation for bovine somatotropin. *Biotechnol. Lett.* 16: 1119-1124.