Fed-batch cultivation of recombinant *Escherichia coli* producing human interferon-γ under controlled specific growth rate

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Abstract

A simple fed-batch process with pre-determined exponential feeding strategy for high-cell-density cultivation of recombinant E. coli BL21 (DE3) in defined medium was developed. In this feeding method glucose and glycerol were used as the sole sources of carbon and energy to increase the cell density exponentially at controlled specific growth rates, which do not cause the accumulation of acetate. Thus, sophisticated feedback control or extra equipment to prevent the accumulation of toxic level of acetate is not necessary. The final cell densities of 100 and 118 gl^{-1} of dry cell mass for recombinant E. coli producing human interferon-γ (hIFN-γ) were obtained by using glucose and glycerol, respectively. The concentration of acetate was always maintained below toxic level. The specific yield of hIFN-γ with glucose and glycerol was 93 and 92 mgg^{-1} of dry cell mass, and the overall productivity of hIFN-γ was 0.16 and 0.14 $gl^{-1}h^{-1}$ for these two carbon sources, respectively.

Keywords: *Human interferon-gamma, fed-batch cultivation, recombinant E. coli, specific growth rate, glucose, glycerol*

INTRODUCTION

The interferons (IFNs) were discovered in 1957 as biological agents interfering with virus replication (Isaacs and Lindenmann, 1957). IFN-γ is secreted by lymphocytes stimulated by mitogen and it is involved in differentiation, maturation and proliferation of hematopoietic cells and enhances non-specific immunity to tumors, as well as to microbial, viral and parasitic pathogens (Sen and Lengyel, 1992 and Mamane *et al.,* 1999). Natural human interferon-γ (hIFN-γ) is a glycosylated protein and composed of 143 amino acid residues with a total molecular weight of approximately 20-25 kDa. (Farrar and Schreiber, 1993 and Rinderknecht *et al.,* 1984). In 1982, its cDNA has been successfully cloned and expressed in *E. coli* (Gray *et al.,* 1982 and Devos *et al.,* 1982), which made possible the production of recombinant hIFN-γ (rhIFN-γ) in a relativley large amount (Zhang *et al.,* 1992). The non-glycosylated form of rhIFN-γ (produced in *E. coli*) with a total molecular weight of approximately 17 kDa proved to be biologically active (Zhang *et al.,* 1992).

E. coli is the most commonly used host for heterologous protein production because it is classified as a generally recognized safe organism for production of various therapeutic recombinant proteins, and it is a well-characterized organism in the genetics, physiology and cultivation condition (Riesenberg and Guthke, 1999; Lee, 1996 and Lim *et al.,* 2000). Many proteins such as interferons, interleukins, colony-stimulating factors, growth hormones, insulin-like growth factors and human serum albumin have been successfully produced by using various recombinant *E. coli* strains (Lee, 1996; Zhang *et al.,* 1998 and Lim *et al.,* 2000). Many of these proteins accumulated in the form of insoluble biologically inactive inclusion bodies. In the case of intracellularly accumulated proteins, volumetric productivity ($gl^{-1}h^{-1}$) is dependent on the final cell-density (i.e. the amount of dry cell weight per liter), specific yield (i.e. the amount of product formed per

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Table 1. Medium and feeding solution.

Components	Batch medium (per liter)	Feeding solution (per liter)
Glucose (glycerol)	10 g (10 g)	700 g (750 g)
$Na_2HPO_4.7H_2O$	12.8 g	-
KH_2PO_4	3 g	-
NaCl	0.5 g	-
NH_4Cl	1 g	-
$MgSO_4.7H_2O$	0.5 g	20 g
Trace elements solution [a]	1 ml	5 ml

[a] The stock solution of trace elements contained (gl^{-1} in 1 M HCl): $FeSO_4.7H_2O$, 2.78; $MnCl_2.4H_2O$, 1.98; $CoSO_4.7H_2O$, 2.81; $CaCl_2.2H_2O$, 1.47; $CuCl_2.2H_2O$, 0.17; and $ZnSO_4.7H_2O$, 0.29 (Rothen *et al*., 1998). This solution was sterilized by filtration.

unit dry cell mass) and the time of process.

The high-cell-density cultivation of *E. coli* for the biosynthesis of various recombinant proteins is a method of choice for increasing the cell-density, which will result in maximizing the volumetric productivity. Moreover, high-cell-density cultivation techniques have several additional advantages such as: decreased culture volume, enhanced downstream processing, decreased fermentation effluents, lower production costs and decreased investment in equipment (Lee, 1996; Riesenberg and Guthke, 1999).

Fed-batch processes most often used to obtain high-cell-density. Different strategies have been reviewed to grow *E. coli* in high-cell-densities using fed-batch cultures (Riesenberg, 1991; Yee and Blanch, 1992; Lee, 1996; Riesenberg and Guthke, 1999). In the many cases reported in the literature, have been used sophisticated control schemes or methods for removal of toxic by-products (mainly acetate) to obtain cell density of non-recombinant or recombinant *E. coli* more than 100 gl^{-1} of dry cell mass (Riesenberg and Guthke, 1999). The feeding rate of carbon source is critical factor in the control of various fed-batch processes (Lee, 1996). Moreover, specific growth rate is an important parameter in the fed-batch fermentation, because it affects the production of growth-inhibitory metabolites and the production of recombinant protein (Riesenberg *et al.,* 1991 and Yoon *et al.,* 1994).

In this study, we mainly focused to develop a simple fed-batch technique for high-cell-density cultivation of recombinant *E. coli* producing hIFN-γ on defined medium by controlling the specific growth rate, and avoiding the formation of acetate. Thus, using this simple technique, sophisticated feedback controls are not necessary to prevent the accumulation of high levels of acetate. The effect of *hifn-γ* gene and types of carbon source (glucose or glycerol) on the cell growth and plasmid stability, as well as the effect of induction on the cell growth and production of hIFN-γ were studied during high-cell-density culture of recombinant *E. coli* BL21 (DE3).

MATERIALS AND METHODS

Microorganism: The *Escherichia coli* strain BL21 (DE3) (Novagen, Inc.) was used as the host for hIFN-γ production. Transformation of this strain was performed using commercially available plasmid, pET3a inducible expression vector (Novagen, Inc.), in which *hifn-γ* gene (Noor Research & Educational Institute, Tehran, I.R. Iran) was inserted into the *Not*I and *Nde*I sites (Khalilzadeh *et al.,* 2003). Recombinant *E. coli* strain was stored at –70°C using 20% (w/w) glycerol stock solution for long-term storage and at 4°C solidified Luria-Bertani (LB) medium between experiments.

Media and inoculum preparation: A defined medium (M9 modified medium) was used for preparation of seed culture and batch fermentation. The composition of the batch medium and feeding solution are given in table 1. Stock solutions of carbon source (glucose or glycerol) and $MgSO_4$ were sterilized separately for 30 min at 121°C. These solutions were mixed aseptically to make the feeding solution.

For cultivation of the recombinant *E. coli,* it was first removed from –70°C freezer and grown at 37°C for 24h on LB plates supplemented with ampicillin (100 mgl^{-1}). Then one colony was transferred into 100 ml defined medium containing of 100 mgl^{-1} ampicillin in the 500-ml flask and incubated overnight at 37°C under shaking condition (200 rpm).

Bioreactor system and fed-batch fermentation: The fed-batch fermentation was carried out in a 2-L bench top bioreactor (INFORS AG, Switzerland). The initial batch culture was started by inoculation of 100 ml of overnight-incubated seed culture (0.4-0.6 gl^{-1} of dry cell mass) to 1000 ml of defined medium supplemented with ampicillin (100 mgl^{-1}) in the bioreactor. Cultivation condition was controlled at 37±0.5°C, airflow rate of 1 vvm, pH 7.0±0.05, and 400 rpm. Foam formation was suppressed by manually addition of silicon-antifoaming reagent when necessary. Dissolved oxygen was measured using a polarographic electrode (Ingold, Mettler Toledo, Germany) and adjusted to 20-30% of air saturation by controlling both air flow and agitation speed. During the fed-batch operation, pure oxygen was used for enrichment of entering air. The pH was adjusted at 7.0±0.05 and ammonium was maintained between 0.1 and 1.5 gl^{-1}. The KH_2PO_4 solution was added to fermentation broth to avoid phosphate limitation in high cell density according to the biomass/phosphate yield coefficient.

After depletion of the initial carbon source (glucose or glycerol) in the batch medium, as indicated by rapid increase in the dissolved oxygen concentration, the feeding was initiated and the flow rate was increased stepwise based on exponential feeding strategy. The exponential feeding rate was determined by a simple mass balance equation of the cell and substrate according to Yee and Blanch (1992).

$$(d/d\ t)\ (VX) = \mu VX \text{ (for biomass)} \quad (1)$$

$$(d/d\ t)\ (VS) = FS_0 + (\mu VX)/Y_{x/s} \text{ (for substrate)} \quad (2)$$

where V is the medium volume in the bioreactor (l), X is the biomass concentration in the bioreactor (gl^{-1} of dry cell mass), t is the time (h), μ is the specific growth rate (h^{-1}), S is the carbon source concentration in the bioreactor (gl^{-1}), S_0 is the carbon source concentration in the feeding solution (gl^{-1}), F is the feeding rate ($l\ h^{-1}$), and $Y_{x/s}$ is the biomass/substrate yield coefficient (gg^{-1}). If assume specific growth rate (μ) is constant, equation 1 can be integrated as:

$$XV = X_0V_0 \exp.(\mu t) \quad (3)$$

where X_0 (gl^{-1} of dry cell mass) is the biomass concenration when the feeding is started, V_0 (l) is the medium volume in the bioreactor at the start of feeding. Assuming a quasi-steady state exists for the substrate concentration, and constant volume fed-batch fermentation (d/d t) (VS) = 0, also if assume $Y_{x/s}$ is constant, then by substituting equation 3 into 2, equation 4 will be:

$$M_s(t) = F_s(t)\ S_0 = (\mu X_0V_0/Y_{x/s}) \exp(\mu t) \quad (4)$$

where $M_s(t)$ is the mass flow rate of carbon source (g h^{-1}).

Analytical procedures: The optical density (OD) was measured at 600 nm and converted to the dry cell weight by an appropriate calibration curve (Seeger *et al.,* 1995). Dry cell weight was determined with a 5 ml culture sample, which was centrifuged at 4000×g for 5 min, the pellet was collected and washed twice with de-ionized water and dried at 105°C to constant weight (Seeger *et al.,* 1995). Glucose and ammonia were analyzed by enzymatic kits (ChemEnzyme Co., I.R. Iran) according to the procedure suggested by the supplier. Enzymatic kits (Boehringer Mannheim/R-Biopharm, Germany) were used to measure acetate and glycerol according to the procedure suggested by the supplier. Expression level of rhIFN-γ was determined by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% (w/v) polyacrylamide gels stained with 0.1% (w/v) coomassie brillient blue R250 and quantified by densitometry. Total soluble protein was determined by Bradford method (Bradford, 1976) and rhIFN-γ measured by ELISA as described before (Maghsoudi *et al.,* 2001).

The stability of plasmid in the recombinant *E. coli* strain was measured by aseptically sampling of the bioreactor at different dry cell weight. The sample was diluted with sterile solution of NaCl (9 gl^{-1}) to yield 100-300 colonies per plate on LB-agar medium and incubated at 37°C for 16 h. All colonies on three plates were transferred on selective LB-agar plates (supplemented with 100 mgl^{-1} of ampicillin) by replica plating method. Plasmid stability was calculated by taking the ratio of the average number of colonies from three selective LB-agar plates to the average from three non-selective LB-agar plates (Panda *et al.,* 1999).

RESULTS

Fed-batch culture of recombinant *E. coli* BL21 (DE3) [pET3a]: The results of the high-cell-density cultivation of *E. coli* BL21 (DE3) [pET3a], non-producing hIFN-γ, using glucose and glycerol as carbon sources are shown in figures 1 and 2, respectively. After depletion of initial carbon source (glucose and glycerol) in the batch medium, feeding was initiated by concentrated feed solution without ampicillin at

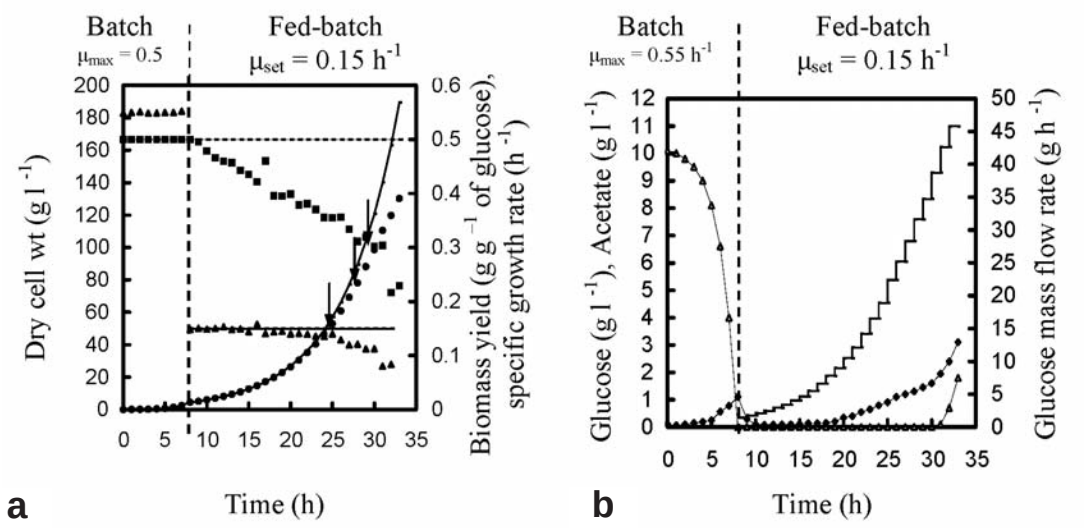

Figure 1. High cell density fed-batch cultivation of recombinant *E. coli* BL21 (DE3)[pET3a] non-expressing hIFN-γ using glucose as carbon and energy source in defined medium. After unlimited growth during the batch mode (μ_{max} = 0.55 h^{-1}), glucose-limited growth at a desired low growth rate (μ_{set} = 0.15 h^{-1}) was started by feeding of concentrated feed solution without ampicillin at constant biomass/glucose yield coefficient ($Y_{x/s}$=0.5 gg^{-1}). (●) cell density, (►)specific growth rate, (■) glucose yield coefficient, (▷) glucose concentration, (◆) acetate concentration, (▬) glucose mass flow rate. The solid lines for cell density, biomass/glucose yield coefficient and specific growth rate are the calculated values or set points. *Arrows* indicate the addition of 2 gl^{-1} KH_2PO_4 to avoid phosphate limitation in high cell density.

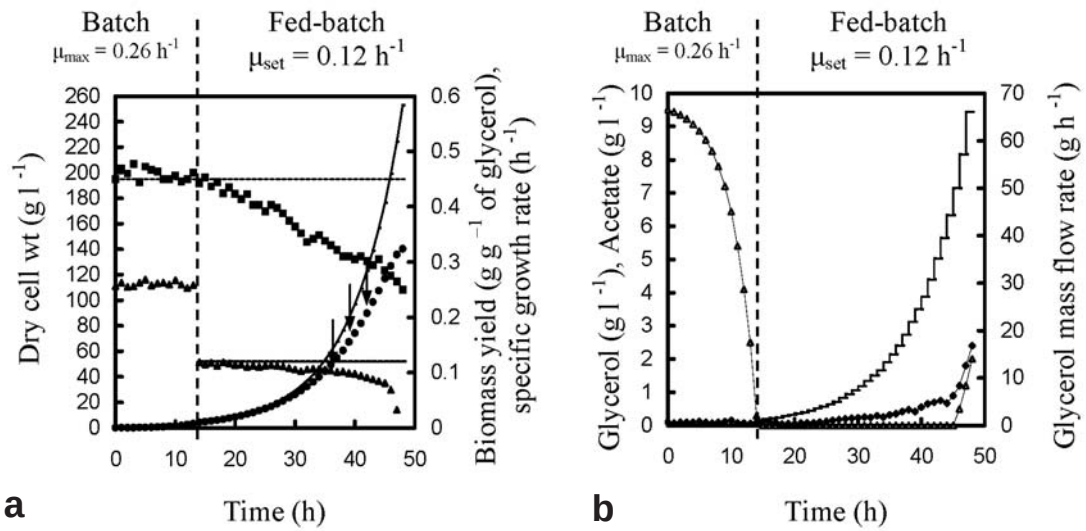

Figure 2. High cell density fed-batch cultivation of recombinant *E. coli* BL21 (DE3)[pET3a] non-producing hIFN-γ using glycerol as carbon and energy source in defined medium. After unlimited growth during the batch mode (μ_{max} = 0.26 h^{-1}), glycerol-limited growth at a desired growth rate (μ_{set} = 0.12 h^{-1}) was started by feeding of concentrated feed solution without ampicillin at constant biomass / glycerol yield coefficient ($Y_{x/s}$= 0.45 gg^{-1}). (●) cell density, (►) specific growth rate, (■) glycerol yield coefficient, (▷) glycerol concentration, (◆) acetate concentration, (▬) glycerol mass flow rate. The solid lines for cell density, biomass/glycerol yield coefficient and specific growth rate are the calculated values or set points. *Arrows* indicate the addition of 2 gl^{-1} KH_2PO_4 to avoid phosphate limitation in high cell density.

low growth rates (μ_{set} = constant < $\mu_{critical}$). The feeding flow rate was described by equation 4. The set point of specific growth rates (μ_{set}) were 0.15 h^{-1} and 0.12 h^{-1}, as well as the biomass/substrate yield coefficient ($Y_{x/s}$) was assumed to be 0.5 and 0.45 gg^{-1} for glucose and glycerol, respectively. The assumed $Y_{x/s}$ was obtained from the total amount of cell produced per consumed glucose or glycerol in previous batch experiments. Changes in the feeding rate were carried out in one-hour intervals to adequate control of the specific growth rate at selected level.

The cell mass yield coefficient during feeding of fed-batch culture varied between 0.5 to 0.23 gg^{-1} of glucose and 0.45 to 0.33 gg^{-1} of glycerol. Moreover specific growth rate decreased slightly from set points in the feeding stage. The difference between the assumed $Y_{x/s}$ and real value during feeding stage causes the experimental values of cell density to deviate from the calculated values. The equations 5 and 6 express the decreasing rates of the glucose and glycerol yield in the feeding stage, respectively. These equations were substituted in the equation 4 to determine feeding flow rate for next experiments.

$$Y_{x/s}=0.5 \exp. [-0.021 (t - t_0)] \quad (5)$$
$$Y_{x/s} =0.45 \exp. [-0.014 (t - t_0)] \quad (6)$$

where t_0 is the start time of feeding.

The same experiments were carried out using variable $Y_{x/s}$ expressed in equations 5 and 6. Results are shown in figures 3 and 4. The solid lines for cell density, biomass/substrate yield coefficient and specific growth rate are the calculated values. Under this condition, the cell-densities reached to maximum of 132 and 145 gl^{-1} of dry cell mass at the end of culture. The specific growth rate was well controlled and the calculated cell density was agreed well with the experimental data. At the end of the fed-batch phase, the actual specific growth rate decreased and cells growth stopped completely after 31 and 44 h using glucose and glycerol, respectively. The glucose and glycerol were not accumulated in medium throughout the fermentation, but an increase in the glucose and glycerol concentration was observed toward the end of the fermentation.

In the presence of glucose in the batch mode, acetate concentration reached to 1.1 gl^{-1} and it was consumed in the beginning of the fed-batch phase, but it was not produced in the presence of glycerol during batch mode. This may be due to the lower maximum specific growth rate of 0.26 h^{-1} supported by glycerol as carbon source as compared to that of the glucose, 0.55 h^{-1}. The acetate formation gradually increased during fed-batch phase, up to 3 g l^{-1} at the end of fermentation. The plasmids were stable during fed-batch fermentation in both cases up to 99.5%. A summary of the results are shown in table 2.

Fed-batch culture of recombinant *E. coli* BL21 (DE3) [pET3a-*hifn-γ*]: In order to determine the effect of the *hifn-γ* gene on the cell growth during high-cell-density culture, recombinant *E. coli* BL21 (DE3) harboring plasmid pET3a-*hifn-γ* was used in fed batch fermentation. The feeding was carried out using variable biomass/substrate yield coefficient ($Y_{x/s}$) expressed by equations 5 and 6 for glucose and glycerol, respectively. Results of high-cell-density cultivation in defined medium using glucose or glycerol are shown in table 3.

The concentration of hIFN-γ was slightly increased during feeding period and reached to 1.3 and 1.8 gl^{-1} in the presence of glucose and glycerol at the end of fermentation, respectively. Moreover, plasmid stability was continuously decreased throughout the fermenta-

Table 2. Results of high-cell-density cultivation of *E. coli* BL21 (DE3)[pET3a].

Parameters	Carbon source	
	Glucose	Glycerol
Initial batch concentration (g l^{-1})	10	10
Maximum specific growth rate, μ_{max} (h^{-1})	0.55	0.26
Batch mode time (h)	8	14
Desired specific growth rate, μ_{set} (h^{-1})	0.15	0.12
Total fermentation time (h)	32	46
Overall biomass/carbon source yield coefficient, $Y_{x/s}$ (g g^{-1})	0.32	0.25
Acetate concentration (g l^{-1})	3	2.4
Final dry cell mass (g l^{-1})	132	145

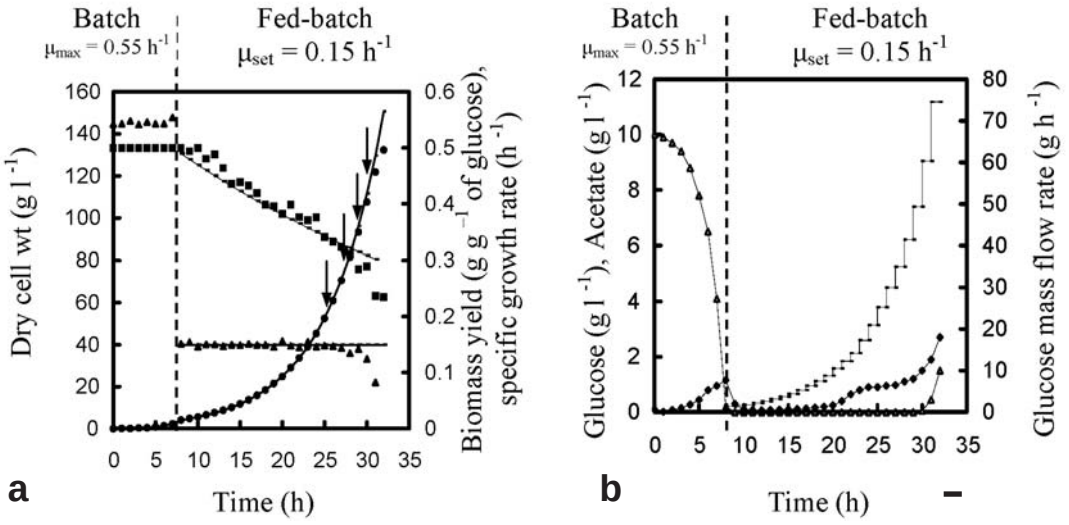

Figure 3. High cell density fed-batch cultivation of recombinant *E. coli* BL21 (DE3) [pET3a-*hifn-γ*] producing hIFN-γ using glucose as carbon and energy source in defined medium. After unlimited growth during the batch mode (μ_{max} = 0.52 h^{-1}), glucose-limited growth at a desired low growth rate (μ_{set} = 0.15 h^{-1}) was started by feeding of concentrated feed solution without ampicillin using variable biomass/glucose yield coefficient determined by equation 5. (●) cell density, (▶) specific growth rate, (■) glucose yield coefficient, (▷) glucose concentration, (◆) acetate concentration, (▬) glucose mass flow rate. The solid lines for cell density, biomass/glucose yield coefficient and specific growth rate are the calculated values or set points. *Arrows* indicate the addition of 2 gl^{-1} KH_2PO_4 to avoid phosphate limitation in high cell density.

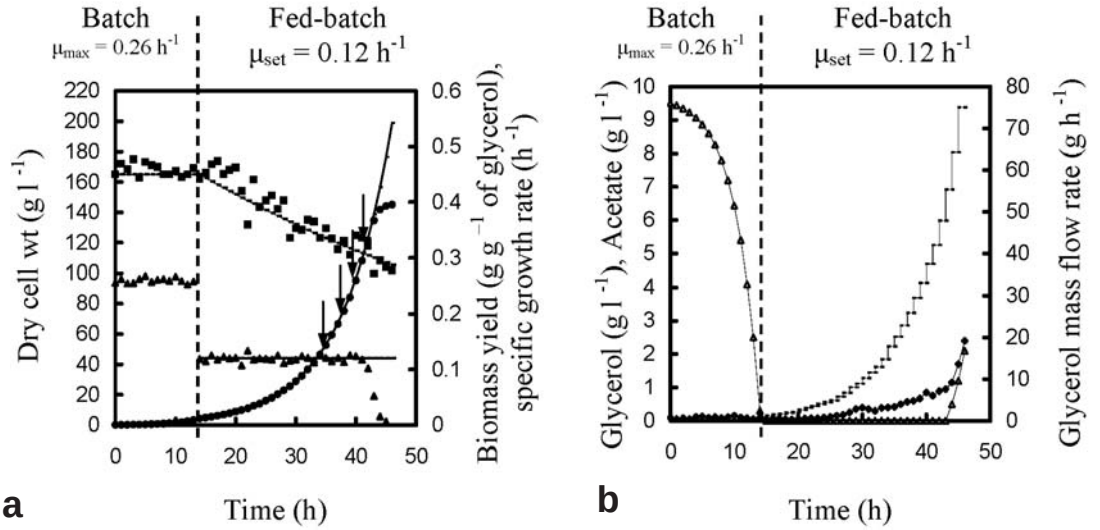

Figure 4. High cell density fed-batch cultivation of recombinant *E. coli* BL21 (DE3) [pET3a] non-producing hIFN-γ using glycerol as carbon and energy source in defined medium. After unlimited growth during the batch mode (μ_{max} = 0.26 h^{-1}), glycerol-limited growth at a desired reduced growth rate ((μ_{set} = 0.12 h^{-1}) was started by feeding of concentrated feed solution without ampicillin using variable biomass/glycerol yield coefficient determined by equation 6. (●) cell density, (▶) specific growth rate, (■) glycerol yield coefficient, (▷) glycerol concentration, (◆) acetate concentration, (▬) glycerol mass flow rate. The solid lines for cell density, biomass/glycerol yield coefficient and specific growth rate are the calculated values or set points. *Arrows* indicate the addition of 2 g l^{-1} KH_2PO_4 to avoid phosphate limitation in high cell density.

Table 3. Results of high-cell-density cultivation of *E. coli* BL21 (DE3)[pET3a-hifn-γ].

Parameters	Carbon source	
	Glucose	Glycerol
Initial batch concentration (g l^{-1})	10	10
Maximum specific growth rate, μ_{max} (h^{-1})	0.52	0.25
Batch mode time (h)	8	14
Desired specific growth rate, μ_{set} (h^{-1})	0.15	0.12
Total fermentation time (h)	32	43
Overall biomass/carbon source yield coefficient, $Y_{x/s}$ (g g^{-1})	0.24	0.21
Acetate concentration (g l^{-1})	2.8	2.1
Final dry cell mass (g l^{-1})	100	118

tion and reached to about 45% in both cases (Fig. 5). At the end of fed-batch phase, the actual specific growth rate was decreased rapidly and bacterial cell growth completely stopped.

The effect of ampicillin (5 gl^{-1}) in the feeding solution as a selective pressure was also studied. Results showed that the plasmid stability was not affected significantly by addition of ampicillin (Fig. 5).

Induction of *E. coli* BL21 (DE3)[pET3a-*hifn-γ*] in high-cell-density culture: In order to determine the effect of the induction on the cell growth and production of hIFN-γ in high-cell-density culture, fed-batch cultures of recombinant *E. coli* BL21 (DE3) [pET3a-*hifn-γ*] were induced with 3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when cell-density reached to about 50 gl^{-1} of dry cell mass. Results of these experiments are shown in table 4. The specific growth rate was decrease from set point of 0.12 h^{-1} before induction to about 0.04 h^{-1} three hours after induction and then increase slightly. Increased specific growth rate at the end of fermentation may be due to the faster growing of plasmid-free cells, which is in agreement with plasmid stability test (Fig. 6). The fraction of hIFN-γ rapidly increased from 4-5% before induction to approximately 10-12%, 15-17% and 19-21% of the total cell proteins at the first, second and third hour after induction, respectively. These results revealed a decreasing in the specific yield of rhIFN-γ (gg^{-1} of dry cell mass) compared to that of the simple batch culture, which the fraction of hIFN-γ was approximately 50% of the total protein (data not shown).

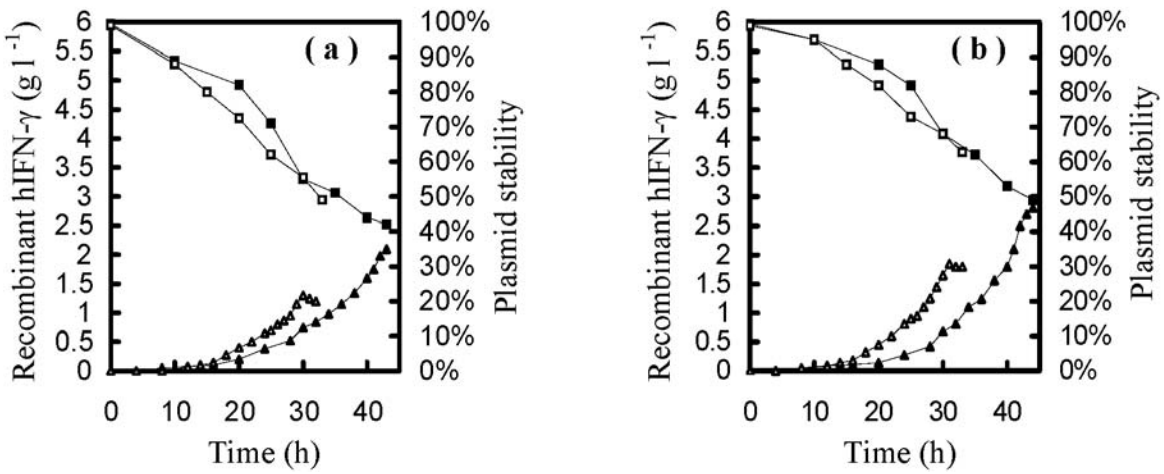

Figure 5. The effect of ampicillin addition on high-cell-density cultivation of *E. coli* BL21 (DE3) [pET3a-hifn-γ] producing *hIFN-γ* in fed-batch operation using variable biomass/carbon source yield coefficient (determined by equation 5 or 6 for glucose or glycerol as carbon source, respectively). (a) Feed solution without ampicillin and (b) feed solution supplemented with 5 g l^{-1} ampicillin. (▷) rhIFN-γ concentration and (□) plasmid stability when using glucose, (►) rhIFN-γ concentration and (■) plasmid stability when using glycerol.

Table 4. Effect of induction on the cell growth in high-cell-density cultivation of *E. coli* BL21 (DE3)[pET3a-hifn-γ].

Parameters	Carbon source	
	Glucose	Glycerol
Desired specific growth rate, μ_{set} (h^{-1})	0.12	0.12
Total fermentation time (h)	32	40
Overall biomass/carbon source yield coefficient, $Y_{x/s}$ (g g^{-1})	0.23	0.2
Acetate concentration (g l^{-1})	1.5	1.2
Final dry cell mass (g l^{-1})	56	61
rhIFN-γ concentration (g l^{-1})	5.2	5.5
Specific yield of rhIFN-γ per dry cell mass (mg g^{-1})	93	92
Overall productivity of rhIFN-γ (g l^{-1} h^{-1})	0.162	0.138

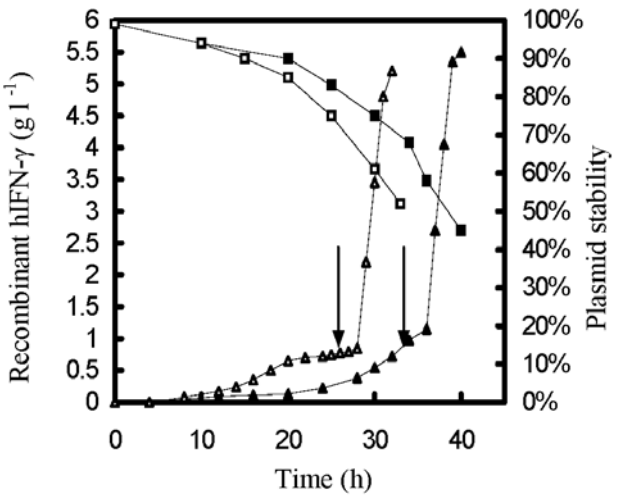

Figure 6. The profile of rhIFN-γ concentration and plasmid stability in high-cell-density cultivation of recombinant *E. coli* BL21 (DE3) [pET3a-*hifn-γ*] producing hIFN-γ in fed-batch operation after induction with 3 mM IPTG, using variable biomass/carbon source yield coefficient (determined by equation 5 or 6 for glucose or glycerol, respectively). (▷) rhIFN-γ concentration and (□) plasmid stability when using glucose, (►) rhIFN-γ concentration and (■) plasmid stability when using glycerol. The set point of the specific growth rate (μ_{set}) was 0.12 h^{-1} and *arrows* indicate induction time.

DISCUSSION

According to the results of fed-batch cultures of recombinant *E. coli* BL21 (DE3) [pET3a] and recombinant *E. coli* BL21 (DE3) [pET3a-*hifn-γ*], the presence of the *hifn-γ* gene affected the cell growth in high-cell-density cultivation. Furthermore, the maximum specific growth rates in the batch mode and overall cell mass yield coefficients ($Y_{x/s}$) in the hIFN-γ producing *E. coli* are slightly lower than non-producing *E. coli* (Tables 2 and 3). This could be due to the increased metabolic burden placed on the cells in the presence of *hifn-γ* gene. Although pE3a is an inducible vector, hIFN-γ produced at a low level before induction. This background expression probably increased plasmid instability in the recombinant *E. coli* BL21 (DE3) harboring pET3a-*hifn-γ* plasmid compare to that pET3a harboring *E. coli*. Plasmid instability is the most important problem in high-level expression systems such as T7. Production of foreign proteins causes a significant decreasing in specific growth rate in plasmid containing cells, while the specific growth rate of plasmid free cells remains high. This will amplify the effect of plasmid instability, resulting in a rapid decrease in the fraction of recombinant cells. In the T7 expression system, which used ampicillin resistance gene as a selection marker, this instability is difficult to overcome by adding ampicillin, because the cells excrete β-lactamase, which degrades ampicillin (Miao and Compala, 1992).

It is well established that various *E. coli* strains accumulate acetate during the growth on fast metabolizing carbon sources (Shiloach *et al.,* 1996; Rothen *et al.,* 1998 and Contiero *et al.,* 2000). Acetate accumulation occurs when the carbon flux exceeds the capacity of the Krebs cycle (Crabtree effect). Likewise acetate accumulation has been shown depends on the medium composition, type of strain, growth and carbon source uptake rates (Risenberg *et al.,* 1991; Shiloach *et al.,* 1996 and Rothen *et al.,* 1998). The lower transport rate of glycerol into the cell compared to that of glucose appears to lead to a decreasing in the carbon flux through glycolysis (Korz *et al.,* 1995). This greatly decreases the formation of acetate in unlimited growth condition during the batch mode in the presence of glycerol.

Production of acetate is greater in fed-batch cultures than in batch cultures owing to the extended cul-

ture period (Lee, 1996). Decline acetate production and removal of the produced acetate from culture broth are two main approaches for decreasing the acetate accumulation in fed-batch cultures. Acetate concentration has been significantly declined by removing the culture broth *in situ* using dialysis or by recycling the culture medium. However, these processes are difficult to scale-up, and considerable amounts of nutrients can be wasted (Lee, 1996). A number of strategies have been developed to decrease acetate formation in fed-batch cultures (Riesenberg and Guthke, 1999 and Lee, 1996).

In this work, acetate formation decreased by controlling the specific growth rate by limiting carbon source. This approach is simple and efficient and does not need any special equipment, advanced computer controller, or special feedback control system. For all the experiments, acetate concentration in the culture medium was below 3 gl^{-1}. This level of acetate concentration is much lower than the reported growth inhibitory concentration of acetate (Shiloach *et al.,* 1996 and Rothen *et al.,* 1998).

Decreased levels of recombinant protein fraction in the high-cell-density cultivation compared with simple batch culture may be due to the lack of some component required for product synthesis, accumulation of inhibitory by-products, declined protein synthesis and alterations in the cell physiology at high cell density (Strandberg and Enforse, 1991; Yee and Blanch, 1993; Yoon *et al.,* 1994; Seeger *et al.,* 1995; Shin *et al.,* 1998; Panda *et al.,* 1999 and Yoon *et al.,* 2003).

CONCLUSION

High-cell-density cultivation was achieved by controlling the specific growth rate using simple pre-determined exponential feeding without any advance control schemes or excess equipment. This fed-batch technique is not affected by short-term disturbances in the dissolved oxygen concentration caused by the addition of antifoam reagent, which makes this superior to the advance feedback control strategies. Under such condition, it is sufficient to maintain the dissolved oxygen concentration around 20% of air saturation to meet the oxygen requirement of the microorganism. This can be achieved by increasing the agitation speed and/or blending air with pure oxygen. In this work, although the higher cell density was obtained with glycerol as carbon and energy source, while productivity was higher when glucose was used because of shorter fermentation time. The specific yield of rhIFN-γ was approximately similar in the presence of both carbon sources.

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