Physiological and morphological changes of recombinant *E. coli* during over-expression of human interferon-γ in HCDC

Seyedeh Marjan Varedi Koolaee[1], Seyed Abbas Shojaosadati[2*], Valliollah Babaeipour[2], Nasser Ghaemi[3]

[1]Department of Biotechnology, University College of Science, University of Tehran, Tehran, P.O. Box 14155-6455, I.R. Iran [2]Biotechnology Group, Department of Chemical Engineering, Faculty of Engineering, Tarbiat Modares University, Tehran, P.O. Box 14155-143, I.R. Iran [3]School of Chemistry, Faculty of Science, University of Tehran, P.O. Box 14155-6455, I.R. Iran

Abstract

The objective of this research was to investigate the influence of the over-expression of recombinant interferon-γ during high cell density cultivation on cellular characteristics of recombinant *E. coli.* Batch and fed-batch culture techniques were employed to grow *Escherichia coli* BL21 for production of human gamma-interferon in pET expression system. Final cell densities in batch and fed-batch cultivations were approximately 7 and 127 g cell dry weight (CDW) l^{-1}, respectively. In both systems, specific growth rate decreased and reached zero, 4 hours after the induction. It was found that high cell density and over-expression of interferon-γ had no substantial effects on cell lysis and plasmid stability. Plasmid content of the cells was nearly similar and remained constant during the post-induction period in both batch and fed-batch cultures (60 mg plasmid per g^{-1} CDW). In both systems, time profiles of acetate and lactate production were similar, lactate concentration was lower than that of acetate and the concentrations of both were lower than the inhibitory level. Maximum extracellular cAMP concentration occurred at the start of induction in fed-batch culture and was higher than the amount produced during the batch process. The size of *E. coli* cells reduced significantly as cell density increased and the morphology of the cells in high cell density changed from the usual rod shape to spherical, while the expression of interferon-γ remained almost constant.

[*]*Correspondence to:* **Seyed Abbas Shojaosadati, Ph.D.**
Tel: +98 21 88011001, Ext: 4431; Fax: +98 21 88005040
E-mail: shoja_sa@modares.ac.ir

INTRODUCTION

High cell density cultivation (HCDC) is a widely used method for the production of recombinant proteins (Lee, 1996; Shiloach and Fass, 2005; Choi *et al*., 2006). In spite of the numerous reports on high cell density cultivation of *E. coli* for the production of heterologous proteins, there is little information on the effects of high cell density on morphology and physiology of cells (Ling and Enfors, 2002; Choi *et al*., 2006). Recombinant protein production is stressful to the cells, not only because of the additional metabolic burden mounted on the microorganism but also because of the high density of cells which exposes them to a medium with high osmolarity, viscosity, high evolution rates of CO_2 and heat, etc. (Lee, 1996; Riesenberg and Guthke, 1999). In these conditions, cells encounter a particular situation which may lead to the formation and secretion of organic acids, plasmid instability and cell lysis. Studying these changes can be beneficial for detection and selection as an indicator of monitoring state of the cell during fermentation. Moreover, combination of this information with data derived from genomics, proteomics and metabolic flux analysis (Yoon *et al*., 2003; Haddadian and Harcum,

230

2005; Phue *et al*., 2005) can lead to a more clarified insight into the conditions of cells in HCDC and thus may result in better expression and cultivation systems for recombinant protein production in *E. coli.*

Lin (1999) comprehensively investigated the physiology of *E. coli* in response to over-expression of yeast $\alpha-$ glucosidase, CRIMI (creatinine imino hydrolase) and ZZ (a modified domain B of staphylococcal protein A) in fed-batch cultivation. There are also some other reports on accumulation of by-products such as acetate, lactate and formate (Van de Walle and Shiloach, 1998; Vidal *et al*., 2005) in high cell density cultures. However, there is no report on the influence of the over-expression of recombinant interferon-γ proteins in HCDC on cellular characteristics of recombinant *E. coli*, especially at cell concentrations above 100 g CDW l^{-1}.

In this study we employed fed-batch and batch culture techniques to grow *Escherichia coli* BL21 (DE3) for the intracellular production of human gamma-interferon in the pET3a expression system. The fed-batch fermentation was a typical high cell density cultivation, which was used as a model system to examine the changes of certain parameters with cell density increment (up to 120 g CDW l^{-1}). Batch culture was performed to distinguish the effects of high cell density from those of over-expression of recombinant protein on physiological and morphological changes of recombinant *E. coli*. We investigated cell growth and lysis, total protein status recombinant protein production, plasmid stability and amplification, acetate and lactate formation, extracellular cAMP concentration and morphological changes in both systems.

MATERIALS AND METHODS

Bacterial strain and plasmid: *Escherichia coli* strain BL21 (DE3) (Novagen, Inc.) was used as the host for human gamma-interferon (hIFN-γ*)* expression. This strain was transformed with the pET3a inducible expression vector (Novagen, Inc), in which hIFN-γ gene (Noor Research & Educational Institute, Tehran, Iran) was inserted into the *Not*I and *Nde*I sites (Khalilzadeh *et al.,* 2003).

Media and solutions: LB (Luria-Bertani) medium was used for subculturing *E. coli* and preparing recombinant *E. coli*. Defined media (Modified M9) was used for inoculation, batch and fed-batch cultivations. The

media for inoculation and batch process consisted of 10 g glucose, 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl, 0.12 g $MgSO_4$, 100 mg Ampicillin and 1 ml trace element solution, per liter. The medium used for fed-batch cultivation contained 10 g glucose, 15 g K_2HPO_4, 7.5 g KH_2PO_4, 2.5 g $(NH_4)_2SO_4$, 2 g citric acid, 2 g $MgSO_4$, 100 mg Ampicillin, and 1 ml trace element solution, per liter. The feeding solution used in fed-batch cultivation contained 700 g glucose, 20 g $MgSO_4 \cdot 7H_2O$ and 5 ml trace element solution, per liter. The trace element solution consisted of 2.8 g $FeSO_4 \cdot 7H_2O$, 2 g $MnCl_2 \cdot 4H_2O$, 2.8 g $CoSO_4 \cdot 7H_2O$, 1.5 g $CaCl_2 \cdot 2H_2O$, 0.2 g $CuCl_2 \cdot 2H_2O$, and 0.3 g $ZnSO_4 \cdot 7H_2O$, per liter of 1 M HCl.

Cultivation method: Batch fermentation was carried out in a 2 liter bench-top bioreactor (INFORS, Switzerland) with a working volume of 1 liter. Fed-batch fermentation was carried out in a 1 liter bench-top bioreactor (INFORS, Switzerland) with a working volume of 550 ml. The batch and fed-batch cultures were started by the addition of 100 ml of an overnight-incubated seed culture (0.4-0.6 g CDW l^{-1}).

Temperature of fermentation was maintained at 37ºC. The pH was controlled at 7 ± 0.1 by the addition of 25% (w/v) NH_4OH or 1M H_3PO_4. Dissolved O_2 was maintained above 30% saturation by controlling both airflow and agitation speed. To ensure the stability of the plasmid, the medium was supplemented with 100 mgl^{-1} ampicillin. After the depletion of initial glucose in the medium, as indicated by a rapid increase in the dissolved oxygen concentration, feeding was initiated. Feeding rate was increased in a stepwise manner based on the exponential feeding strategy in order to have a maximum attainable specific growth rate during fed-batch cultivation. Exponential feeding was carried out using the equation involving a simple mass balance of the cell concentration and substrate, and by assuming that a quasi-steady state exists for the substrate concentration and that fed-batch cultivation is carried out at constant volume (Babaeipour *et al*., 2007).

Induction was performed after 6.5 h (CDW of 2.2 gl^{-1}) and 13h (CDW of 65 gl^{-1}) in batch and fed-batch fermentations, respectively].

Analytical methods: Cell growth was monitored by measuring culture turbidity and cell dry weight (CDW). Turbidity was determined by measuring Optical density (OD) at 600 nm. Samples were diluted with NaCl solution (9 gl^{-1}) to obtain an OD_{600} between

0.2 and 0.7. In order to determine cell dry weight, 5 ml of culture broth was centrifuged at 10000 $\times g$ for 10 min, washed twice with deionized water and dried at 105ºC.

Total produced protein was analyzed by the Bradford method (Bradford, 1976). Expression of hIFN-γ was determined by SDS-PAGE on 12.5% (w/v) polyacrylamide gels stained with 0.1% (w/v) Coomassie brilliant blue R250 and quantified by densitometry (Khalilzadeh et al., 2004).

Samples from the cultivation were directly chilled on ice with further centrifugation (3 min, 12000 $\times g$, 4ºC) and the resulting supernatants were collected. Afterwards the samples were stored at -20ºC for further analysis. Glucose, ammonia and lactate were analyzed enzymatically with glucose, ammonia, and lactate kits (ChemEnzyme, Iran). Acetate was also assayed using an enzymatic analysis kit (Boehringer Mannheim/R-Biopharm).

Plasmid stability was tested by aseptically sampling from the bioreactor at different cell dry weights followed by, serial dilutions using a sterile solution of 9 g NaCl l^{-1} and plating onto LB agar plates without ampicillin. All of the colonies were transferred onto LB plates supplemented with 100 mg (of ampicillin) l^{-1} by the replica plating method (Khalilzadeh *et al.*, 2003). Plasmids were exctracted from cells by plasmid DNA extraction kit (Roche, Germany) and were quantified by measuring absorption at 260 nm.

Quantitative analysis of extracellular cAMP was performed using an isocratic high-performance liquid chromatography (HPLC; Younglin) (Neubauer *et al.,* 1995) by C_{18} RP column (4.6×150 mm, 3 µm particle size) with a suitable guard column (Supelcosil LC18 T and SupelGuard, Supelco). Isocratic separation was performed with a mobile phase (pH 6.0) containing 125 mmol l^{-1} KH_2PO_4, 10 mmol l^{-1} tetrabutylammonium dihydrogen phosphate (Aldrich), 60 ml l^{-1} methanol and 3.0 g l^{-1} KOH (Lin *et al*., 2004) at a flow rate of 1 ml min^{-1} at room temperature. Solutions of pure cAMP standard (Boehringer) were used for calibration.

Samples were prepared for scanning electron microscopy, by fixation in glutaraldehyde in phosphate buffer, dehydration in an ethanol solution seri (10, 25, 50, 75, 95, and 100% v/v) and finally drying using a freeze-dryer (Ilmvac, England). Samples were coated with gold by a sputter coater (Blazers/SCD004, Germany) and viewed with SEM (Zeiss/DSM960A, Germany) (Ling *et al*., 2002).

RESULTS

Cell growth and hIFN-γ production: Figure 1 shows the kinetics of the growth of recombinant *E. coli* during batch and fed-batch processes. Using exponential the fed-batch procedure with a maximum attainable specific growth rate, maximum cell density and rhIFN-γ concentration were 127 g (CDW) l^{-1} after 17h. The maximum productivity of biomass was 7.47 g (CDW) $l^{-1}h^{-1}$.

Figures 2 and 3 indicate the kinetics of rhIFN-γ production during batch and fed-batch cultures. These figures show that maximum rhIFN-γ concentration in
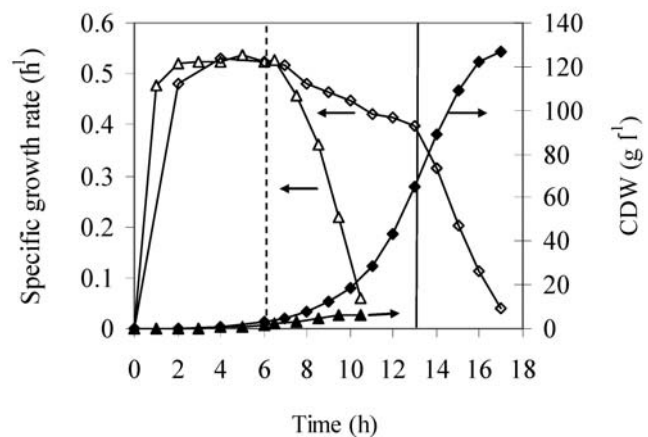

Figure 1. Kinetic of cell growth in batch and fed-batch culture of *E. coli* BL21 (DE3) [pET3a-*ifnγ*]. Specific growth rate (△) and cell density (g CDW l⁻¹) (▲) in batch process. Specific growth rate (◇) and cell density (g CDW l⁻¹) (◆) in fed-batch culture. Dotted and black lines show induction time in batch and fed-batch culture, respectively.

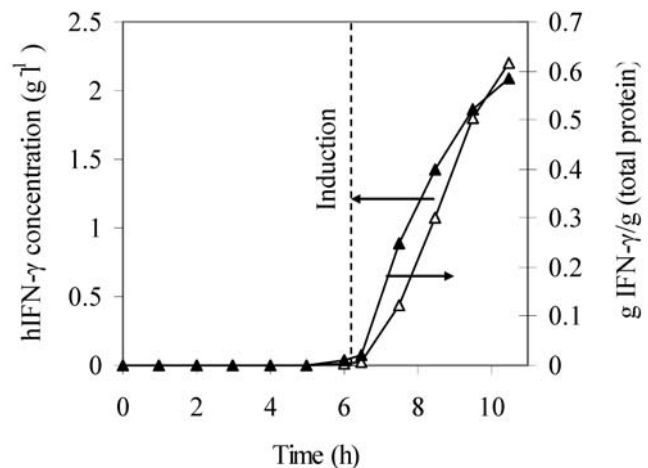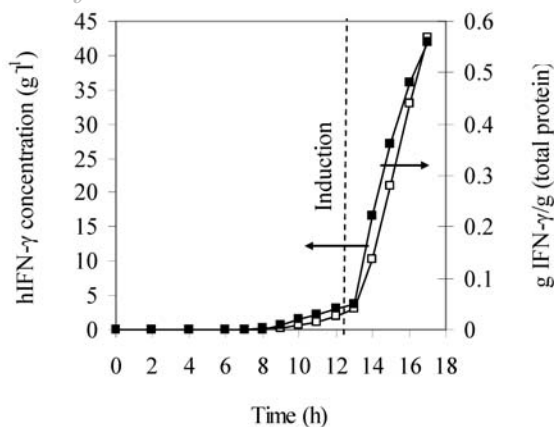**Figure 2.** Kinetic of rhIFN-γ production in batch culture of *E. coli* BL21 (DE3) [pET3a-*ifnγ*]. Concentration of rhIFN-γ (g l⁻¹) (△), ratio of rhIFN-γ value to total protein (g rhIFN-γ/g total protein) (▲).
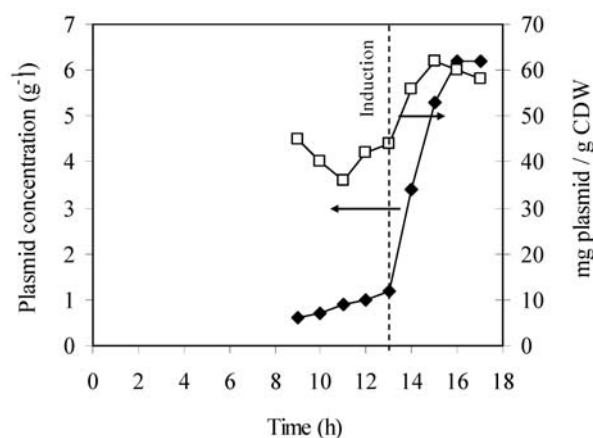
Figure 3. Kinetic of rhIFN-γ production in fed-batch culture of *E. coli* BL21 (DE3) [pET3a-*ifnγ*]. Concentration of rhIFN-γ (g l-1) ($\square$), ratio of rhIFN-γ value to total protein (g rhIFN-γ/g total protein) ($\blacksquare$).

Figure 5. Time Profile of plasmid content in fed-batch culture of *E. coli* BL21 (DE3) [pET3a-*ifnγ*]. Plasmid concentration (g l-1) ($\blacklozenge$) and plasmid content with respect to biomass (g plasmid g-1 CDW) ($\square$).
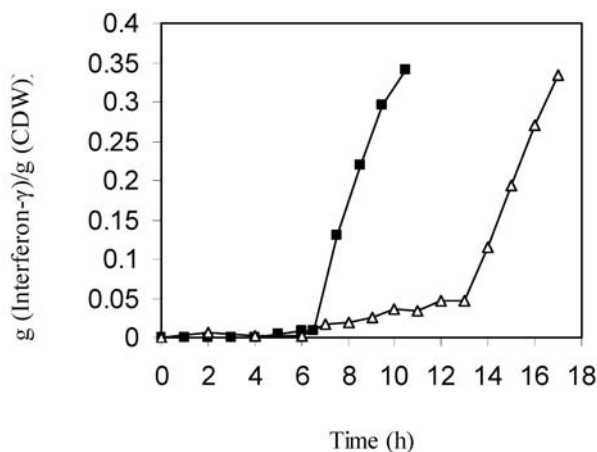
Figure 4. Specific yield of rhIFN-γ with respect to biomass (g rhIFN-γ/g CDW) in batch ($\blacksquare$) and fed-batch ($\triangle$) culture of *E. coli* BL21 (DE3) [pET3a-*ifnγ*].
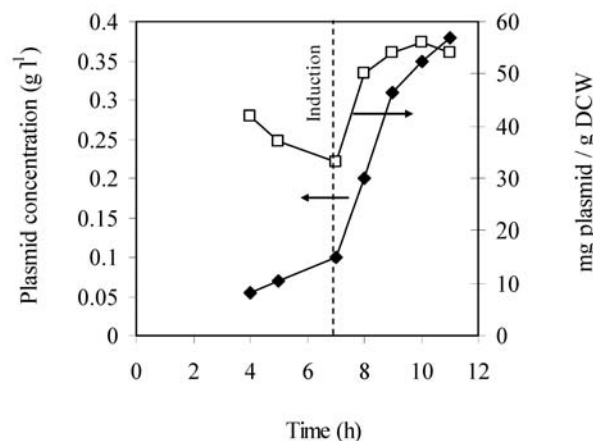

Figure 6. Profiles of plasmid content in batch culture of *E. coli* BL21 (DE3) [pET3a-*ifnγ*]. Plasmid concentration (g l-1) ($\blacklozenge$) and plasmid content with respect to biomass (g plasmid g-1 CDW) ($\square$).

both batch and fed-batch cultures is achieved 4h after induction. In batch culture, concentration and productivity of rhIFN-γ were 2.2 g (rhIFN-γ) l^{-1} and 0.2 g (rhIFN-γ) l^{-1}h^{-1}, respectively. In fed-batch culture, concentration and productivity of rhIFN-γ were 42.5 g (rhIFN-γ) l^{-1} and 2.5 g (rhIFN-γ) l^{-1}h^{-1}, respectively. The specific yield of rhIFN-γ production with respect to biomass is similar in both culture systems, which is 0.33$\pm$ 0.01 g (rhIFN-γ) g^{-1} (CDW). Also Figures 2 and 3 show that the expression levels of rhIFN-γ (g rhIFN-γ g^{-1} total protein) in both batch and fed-batch cultures are similar, so that after induction expression level is severely increased until the end of the process where cell growth and recombinant protein production are ceased. Figure 4 shows that the yield of rhIFN-γ with respect to biomass during the batch and fed-batch processes increases to a level of 0.33 $\pm$ 0.01 g g CDW^{-1}.

Plasmid stability and amplification: Production of recombinant protein is typically performed with antibiotic as selection pressure for plasmid maintenance. Jung *et al*. (1988) reported that ampicillin at a concentration of 100 mg l^{-1} can be degraded in less than 30 min by the secreted β-lactamase in high-cell density cultures, but discrete pulse additions of ampicillin to the reactor medium, taking into consideration cell density increments, seemed to solve this problem. It was observed that plasmid stability increased up to 100% in the fed-batch process before induction and only 5% of population lost its ampicillin resistance after induction. In batch culture, 100% of cells were ampicillin resistant. The changes in the plasmid content during batch and fed-batch processes are presented in Figures 5 and 6. In both fed-batch and batch cultures, at the beginning of cultivation the plasmid level

(mg plasmid g CDW^{-1}) increased severely and then remained approximately constant during the induction period.

Acetate and lactate production: In the both batch and fed-batch cultures, excretion of acetic and lactic acids commenced nearly simultaneously. In batch culture, lactate production was negligible (0.0018 mgl^{-1}), while acetate concentration was approximately 0.9 gl^{-1} (Fig. 7). In fed-batch culture, acetate and lactate increased continuously and reached 3 gl^{-1} and 0.045 gl^{-1}, respectively (Fig. 8). In both systems acetate and lactate concentrations were lower than the inhibitory level.
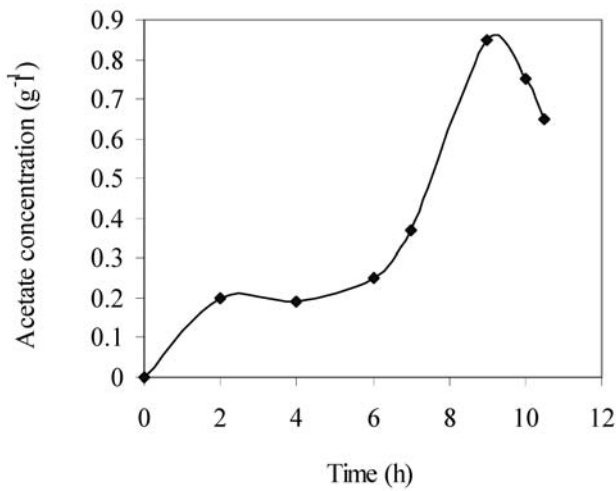

Figure 9. Time profile of extracellular cAMP concentration during batch and fed-batch culture of *E. coli* BL21 (DE3) [pET3a-*ifnγ*]. extracellular cAMP concentration in batch (◇) and fed-batch (■).

Figure 7. Acetate concentration during batch culture of *E. coli* BL21 (DE3) [pET3a-*ifnγ*].
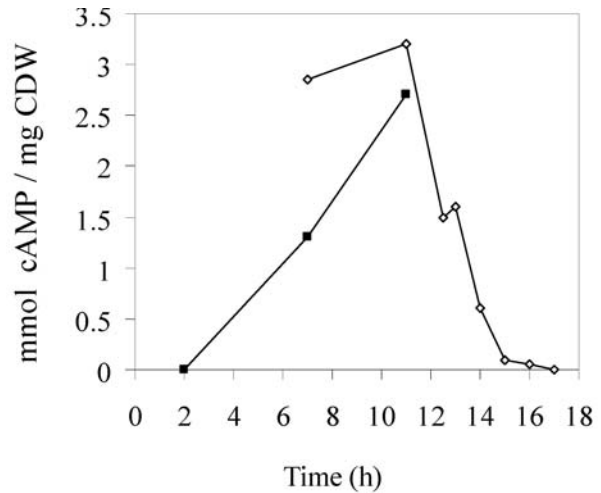
Figure 10. Time profiles for the ratio of concentrations of extracellular cAMP to biomass during batch and fed-batch culture of *E. coli* BL21 (DE3) [pET3a-*ifnγ*]. Ratio concentrations in batch (◇) and fed-batch (■).

Extracellar cAMP concentration: Figures 9 and 10 show concentrations of cAMP in batch and fed-batch cultures. The maximum concentration of extracellular cAMP occurs at the start of induction in fed-batch culture which is higher than that produced during batch process, this value reduces drastically until it reaches zero at the end of the process. In batch culture, concentration of extracellular cAMP increases gradually with time until the end of the process.

Morphological Changes: The morphological changes of cells are depicted in Figure 11. The size of *E. coli* cells reduce significantly with increasing cell density,
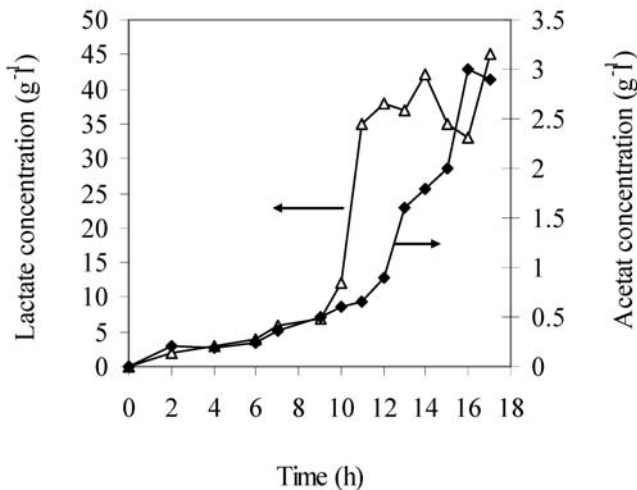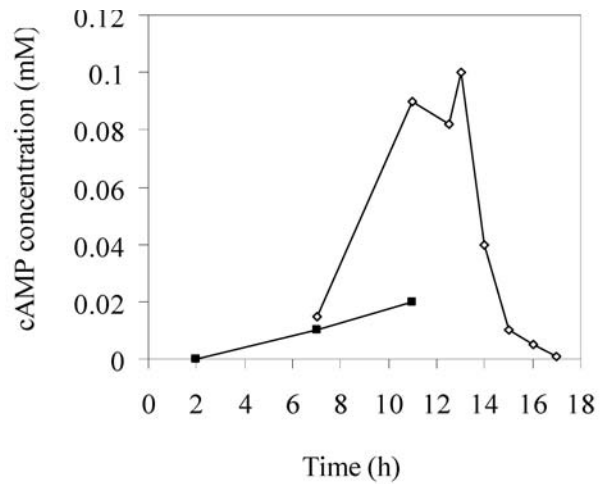

Figure 8. Acetate and lactate concentrations during fed-batch culture of *E. coli* BL21 (DE3) [pET3a-*ifnγ*]. Lactate concentration (△) and Acetate concentration (g l^{-1}) (◆).
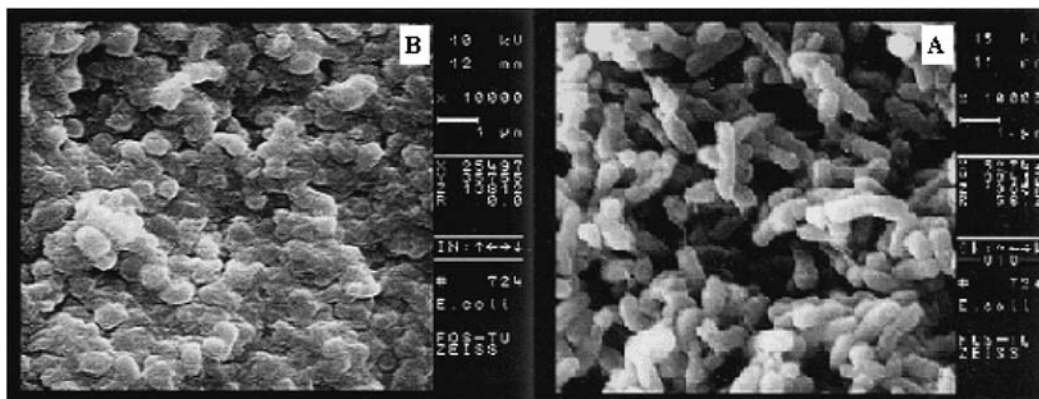
Figure 11. SEM of *E. coli* BL21 (pET3a) at the end of (A) batch process (6.5 g CDW l⁻¹) (B) fed-batch process (127 g CDW l⁻¹).

so that cell morphology at high cell densities changes from the usual rod-shape to spherical, but in batch cultures the morphology of cells do not change.

DISCUSSION

Cell growth and hIFN-γ production: Correlation between absorption at 600 nm and cell dry weight was linear in the batch process, while in the fed-batch process with increasing cell density this correlation deviated from the linear relationship and a significant decrease of the ratio g(CDW) /$OD_{600\,nm}$ (from 0.48 to 0.34) was observed. The variation of the relationship between biomass and $OD_{600\,nm}$ can not be attributed to changes in cell composition due to the accumulation of hIFN-γ, because the g (CDW) /$OD_{600\,nm}$ ratio did not change after induction in the batch process. Apparent changes of *E. coli* BL21 morphology, observed with scanning electronic microscopy, caused the decrease in the ratio g (CDW) /$OD_{600\,nm}$ by increasing cell density.

In both systems, the specific growth rate decreased continuously to zero, 4 h after induction. The overproduction of recombinant proteins results in inhibition of cellular growth. Growth inhibition in both systems during 4 h after induction should be considered as the result of the metabolic burden caused by over-expression of hIFN-γ not high cell density culture. Because in batch culture, in which the cell density is low and over-expression exists, growth inhibition is also observed too. The metabolic burden is attributable to inhibition of cellular reaction, transcription and translation (lin, 1999).

The level of hIFN-γ at the time of the induction in batch culture was negligible while in fed-batch culture

was approximately 3 g l⁻¹. This pre-induction expression can be explained by cAMP/CRP control. 3′, 5′-monophosphate (cAMP) together with the cAMP receptor protein (CAP or CRP) regulate the transcription of numerous operons including the *lac* operon, which regulates the production of hIFN-γ in the pET expression systems (Primrose *et al.*, 2001), as a part of the cellular response to glucose starvation (lewin, 2000). cAMP is synthesized by adenylate cyclase, which is strongly activated by phosphorylated IIA protein (IIAglc-his-P). IIAglc-his-P accumulates during the phosphotransferase (PTS)-mediated uptake of sugars including glucose, when the availability of the PTS sugar substrate is limited (Saier *et al.*, 1996). Therefore, the intracellular concentration of cAMP increases when the glucose concentration becomes growth limiting in the fed-batch process. Accumulated cAMP in the cytoplasm activates the CRP. Active CRP causes the expression of genes of the *lac* promoter, therefore leading to pre-induction expression. In this study, there was no glucose limitation before induction in the batch process hence, no pre-induction expression was observed.

After induction, the yield of rhIFN-γ with respect to biomass during batch and fed-batch processes increased to a level of 0.33 ± 0.01 g g CDW⁻¹. This may indicate that high cell density has no negative effect on yield in this HCD fed-batch technique.

Cell lysis: Extracellular protein was measured in order to estimate the amount of cell lysis. The amount of extracellular protein in all samples was less than 1 gl⁻¹. The highest protein content in medium was equal to 1.4% of the total protein and occurred at the end of the fed-batch process. This quantity was not significantly

higher than the protein content in the batch fermentation medium (data are not shown). Therefore, it can be concluded that high cell density in the employed fed-batch technique does not affect cell lysis significantly.

Plasmid stability and amplification: The results of plasmid content comply with the results of hIFN-γ production (constant yield after induction). This shows that in the employed fed-batch technique, high cell density has no negative effect on plasmid stability and amplification. Although gene dosage has a direct relationship with plasmid content, plasmid existence is considered as a metabolic burden for the cell and causes growth inhibition. Therefore, by considering the fact that plasmid level is approximately constant after induction, optimization of plasmid quantities may lead to increases in productivity.

Acetate and lactate production: Acetic and lactic acids are by-products of glucose metabolism, and they can affect the productivity of the fermentation process by slowing bacterial growth and/or inhibiting recombinant protein biosynthesis (Van de Walle and Shiloach, 1998). Production of these metabolites depends on the bacterial strain, growth conditions, glucose supply strategy and growth media, for example strain BL21 which was used in this research has a reputation for producing less acetate than other *E. coli* strains (Van de Walle and Shiloach, 1998). In addition, due to the special feeding strategy employed (Babaeipour *et al.*, 2006), glucose did not accumulate in the media during the fed-batch process, and therefore the capacity of tricarboxylic acid (TCA) cycle was not full. It is assumed that overloading the TCA cycle via fast oxidation through glycolysis is the main reason for acetate accumulation (Majewski and Domach, 1990). Therefore, little acetic and lactic acids were produced during the fed-batch culture. Slight increases in concentration of acetic and lactic acids during the process can be explained by reduced capacity of the TCA cycle due to the down-regulation of tTCA s' enzymes by increasing cell density. This has been confirmed through transcriptome and proteome analysis done by Yoon *et al.* (2003) and Haddadian and Harcum (2005). The decrease of acetate concentration in the batch process can be explained by consumption acetate by cells because of the shortage of glucose at the end of the process. This decrease was not observed in the fed-batch process because of glucose feeding.

Extracellar cAMP concentration: cAMP was first identified in *E. coli* by Makman and Sutherland (1965). It has been known that the intracellular concentration of cAMP varies with growth conditions, which reflects the differential rates of cAMP synthesis, degradation, and excretion (Lin *et al.*, 2004). Furthermore, synthesis of cAMP is dependent on a high-energy status, as it is synthesized by dephosphorylation of adenosine triphosphate (ATP). Therefore, the intracellular concentration of cAMP transiently increases when the concentration of glucose becomes growth limiting (e.g., at the end of a batch culture), but the cAMP concentration decreases under persisting glucose exhaustion. The net rate of cAMP synthesis increases with increasing growth rate. If the membrane potential is intact, however, cAMP is released into the cultivation medium and the intracellular level is kept low (Matin and Matin, 1982). During steady-state growth in a glucose-limited chemostat, more than 99.9% of the synthesized cAMP is released into the medium (Matin and Matin, 1982). Extracellular cAMP is degraded by a periplasmic 2′,3′-cyclic phosphodiesterase, which is under the control of cAMP (Liu *et al.*, 1986; Liu and Beacham, 1990]), however, extracellular cAMP is also taken up by the cell. This fact has been utilized in a number of studies in which exogenous cAMP was added to cultures to evaluate the dependency of gene regulation by cAMP (e.g., Lange and Hengge-Aronis, 1991; Potamitou *et al.*, 2002).

In fed-batch cultivation, extracellular cAMP concentration increased up to 0.1 mM at the time of induction and thereafter decreased (Figure 9). This result approves the aforementioned reason for pre-induction expression of hIFN-γ . The decrease after the induction may be caused by the effects of over-expression. e.g., over-expression of hIFN-γ changes the permeability of the membrane toward absorbing extracellular cAMP, or induces the release of periplasmic 2′, 3′-cyclic phosphodiesterase into the medium. It seems that more molecular research is needed in this area.

Morphological changes: Cells were observed with a scanning electron microscope inorder to examine morphological changes. As it is shown in Figure 10, cells significantly became smaller and more globular at the end of the fed-batch process. Since these changes were not observed at the end of the batch process, they can not be caused by over-expression of recombinant protein , but are caused by high cell density.

CONCLUSION

In this investigation, hIFN-γ produced in *E. coli* was used as model system to obtain a more comprehensive knowledge about the influence of recombinant protein over-expression in high cell density on the physiology and morphology of cells. It was found that 1) high cell density and over-expression of recombinant proteins do not have considerable effects on cell lysis and plasmid stability, 2) there is not a straightforward relationship between cell density or expression of recombinant proteins and extracellular cAMP concentration. Extracellular cAMP concentration is more affected by concentration of glucose in the medium, 3) morphology of cells is affected by high cell density, not by recombinant protein over-expression and as a consequence in high cell density the shape of cells changed from rod-shape to spherical, while the expression of interferon-γ remained almost the same, 4) high cell density and over-expression of recombinant proteins do not affect the plasmid level of recombinant cells, so that plasmid level of cells after induction in fed-batch is found to be similar to that in batch culture and is almost constant during induction (60 mg plasmid per gram DCW), 5) concentrations of metabolic by-products are not affected by high cell density and over-expression of recombinant proteins but are more influenced by glucose concentrations in the medium.

It is needless to say that cells' physiology is dependent on medium composition, type of product, growth technique, and dimensions of the vessel, the results of this investigation can not simply be applied to other systems and can only be used as a tool for predicting cell behavior and responses in similar systems.

References

Babaeipour V, Robatjazi SM, Shojaosadati SA, Khalilzadeh R, Maghsoudi N (2007). Over-production of human interferon-γ in HCDC of recombinant *Escherichia coli*. *Proc Biochem.* 42:112-117.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle protein dye-binding. *Anal Biochem.* 72: 248-54.

Choi, JH, Keum KC, Lee SY (2006). Production of recombinant proteins by high cell density culture of *Escherichia coli. Chem Engin Sci.* 61: 876-885.

Haddadin FT, Harcum SW (2005). Transcriptome profiles for high-cell-density recombinant and wild-type *Escherichia coli. Biotechnol Bioeng.* 90: 127-53.

Jung G, Denefle P, Becquart J, Mayaux JF (1988). High-cell density fermentation studies of recombinant *Escherichia coli* strains expressing human interleukin-1. *Annales de l'Institut Pasteur/ Microbiology* 139: 129-146.

Khalilzadeh R, Shojaosadati SA, Bahrami A, Maghsoudi N (2003). Over-expression of recombinant human interferon-gamma in high cell density fermentation of *Escherichia coli. Biotechnol Lett.* 25: 1989-1992.

Khalilzadeh, R, Shojaosadati SA, Maghsoudi N, Mohammadian-Mosaabadi J, Mohammadi MR, Bahrami A, Maleksabet N, Nassiri-Khalilli MA, Ebrahimi M, Naderimanesh H (2004). Process development for production of recombinant human interferon-γ- expressed in *Esherichia coli. J Ind Microbiol Biotechnol.* 31: 63-69.

Lange R, Hengge-Aronis R (1994). The cellular concentration of the σ^S subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation, and protein stability. *Gene Dev.* 8: 1600-1612.

Lee SY (1996). High cell density cultivation of *Escherichia coli Trend Biotechnol.* 14: 98-105.

Lewin B (2000). *Gene* IIV, New York: Oxford University Press.

Lin H, Hoffmann F,Rozkov A, Enfors SO, Rinas U, Neubauer P (2004). Change of extracellular cAMP concentration is a sensitive reporter for bacterial fitness in high-cell-density cultures of *Escherichia coli. Biotechnol Bioeng.* 87: 602-613.

Ling HY, Enfors SO (2002). Physiology of *Escherichia coli* in batch and fed-Batch cultures with special amino acid and glucose metabolism, Department of Biotechnology, Royal institute of Technology, Stockholm, Sweden, *PhD Thesis*, ISBN 91-7283-76-2

Lin HY (1999). Cellular responses to the induction of recombinant genes in *Escherichia coli* fed-batch cultures. *PhD Thesis*. Halle (Saale), Germany: Martin-Luther-Universität Halle-Wittenberg.

Liu J, Beacham IR (1990). Transcription and regulation of the cpdB gene in *Escherichia coli* K12 and *Salmonella typhimurium* LT2: evidence for modulation of constitutive promoters by cyclic AMP-CRP complex. *Mol Gen Genet.* 222: 161-165.

Liu J, Burns DM, Beacham IR (1986). Isolation and sequence analysis of the gene (cpdB) encoding periplasmic 2´,3´-cyclic phosphodiesterase. *J Bacteriol.* 165: 1002-1010.

Majewski RA, Domach MM)1990(. Simple constrained optimization view of acetate overflow in *E. coli. Biotechnol Bioeng.* 35: 732-738.

Makman RS, Sutherland EW (1965). Adenosine 3', 5'-phosphate in *Escherichia coli*. *J Biol Chem*. 240: 1309-1314.

Matin A, Matin MK)1982(. Cellular levels, excretion, and synthesis rates of cyclic AMP in *Escherichia coli* grown in continuous culture. *J Bacteriol*. 149: 801-807.

Neubauer P, Ahman M, Tornkvist M, Larsson G, Enfors SO (1995). Response of guanosine tetraphosphate to glucose fluctuations in fed-batch cultivations of *Escherichia coli*. *J Biotechnol.* 43: 195-204.

Phue J, Noronha SB, Hattacharyya R, Wolfe AJ, Shiloach J (2005). Glucose metabolism at high density growth of *E. coli* B and *E. coli* K: differences in metabolic pathways are responsible for efficient glucose utilization in *E. coli* B as determined by microarrays and northern blot analyses. *Biotechnol Bioeng.* 90: 805-820.

Potamitou A, Neubauer P, Holmgren A, Vlamis-Gardikas A (2002). Expression of *Escherichia coli* glutaredoxin 2 is mainly regulated by ppGpp and σ^S. *J Biol Chem.* 277: 17775-17780.

Primrose S, Twyman R, Old B (2001). *Principles of Gene Manipulation*, 6th ed., Germany: Blackwell Science.

Risenberg D, Guthker R (1999). High cell density cultivation of microorganism. *Appl Microbiol Biotechnol.* 51: 422-430.

Saier MH, Ramseier TM, Reizer J (1996). Regulation of carbon utilization. In: Neidhardt FC, Curtiss R III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbarger HE. *Escherichia coli and Salmonella cellular and molecular biology*. Washington, DC: ASM Press, 1325-1343.

Shiloach, J, Fass R (2005). Growing *E. coli* to high cell density-A historical perspective on method development. *Biotechnol Adv.* 23: 345-357.

Van de Walle M, Shiloach J (1998). Proposed mechanism of acetate accumulation in two recombinant *Escherichia coli* strains during high density fermentation. *Biotechnol Bioeng.* 57: 71-78.

Vidal L, Ferrer P, Alvaro G, Benaiges MD, Caminal G (2005). Influence of induction and operation mode on recombinant rhamnulose 1-phosphate aldolase production by *Escherichia coli* using the T5 promoter. *J Biotechnol.* 118: 75-87.

Yoon SH, Han MJ, Lee SY, Jeong KJ, Yoo JS (2003). Combined transcriptome and proteome analysis *Escherichia coli* during high cell density culture. *Biotechnol Bioeng.* 81: 753-767.