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Metabolic changes of recombinant *Escherichia coli* BL21 (DE3) during overexpression of recombinant human interferon beta in HCDC

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Abstract

The influence of the overexpression of recombinant human interferon beta (rhINF-β) during high cell density cultivation (HCDC) on metabolic characteristics of recombinant *E. coli* BL21 (DE3) was studied. Fed-batch culture technique was employed to grow the host cell for the production of human interferon beta in pET expression system in a 5L bench-top bioreactor. The final cell density of 42.15 g L^{-1} and cell growth (OD_{600nm}) of 103.5 were obtained respectively in fed-batch culture experiment. Four hours after the induction, the specific growth rate was decreased. The initial concentration of the glucose, ammonium and phosphate in the TB culture medium were 5.0, 12.0 and 3.12 g L^{-1} respectively. The maximum amount of the produced rhINF-β was 2.8 g L^{-1} after applying the fed-batch cultivation technique. Besides, time profiles of the acetate and lactate production were similar, the acetate concentration (4.91 g L^{-1}) was lower than that of lactate (10.08 g L^{-1}) and the concentrations of both were lower than the inhibitory levels. Moreover, it was shown that the HCDC and overexpression of rhINF-β did not hamper the plasmid stability.

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Introduction

Cost-effective production of a recombinant protein is one of the major goals in an expression platform. Hence, achieving a high productivity (i. e. the amount of produced product per unit volume per unit time) technique is one the main concerns to establish a successful production system. On the other hand, the productivity depends on the specific productivity (i. e. the amount of produced product per unit cell mass per unit time) and the final cell density of the recombinant host cells. To improve the productivity, different high cell density culture (HCDC) techniques have been developed. Furthermore, this strategy provides more benefits including enhanced downstream processing, lowering the production costs, reduced culture volume, less investment in equipments and wastewater production (Lee, 1996). In a pharmaceutical biotechnology process, generally, the fed-batch cultivation method has been selected as the method of choice to improve the volumetric productivity of the requested recombinant protein. Using this cultivation technique, the rate of carbon (glucose) supply is simply controlled to avoid the oxygen limitation and acetate overflow as well. Moreover, it offers extension of the working time (especially significant in production of growth-dependent products), and provides more control on the catabolite repression effects or production of different by-products (Shojaosadati *et al,*. 2008).

Nowadays, various recombinant proteins including penicillin G acylase (Liu *et al.,* 2000), poly(3-hydroxybutyrate) (PHB) (Kim *et al.,* 2004), human glucagon-like peptide-1 (GLP-1) (Zhou *et al.,* 2012) different recombinant human interferons such as alfa (Babu *et al.,* 2000), beta (Singh and Mukherjee, 2012; Singh *et al.,* 2012) and gamma interferon (Babaeipour *et al.,* 2007) have been produced using high cell density fed-batch cultivation of *E. coli*. Although, there is no appropriate model available to predict the metabolic changes of HCDC cultivation in *E. coli* during production of heterologous recombinant proteins.

Enhancing the production of recombinant proteins in *E .coli* generates a metabolic stress response which causes the significant reduction of host cell growth and product formation rates. Also, it triggers the plasmid instability (Silva *et al.,* 2012) which causes different metabolic alterations such as increased carbon transport and TCA cycle, increased/decreased glycolysis cycle and ATP production, and decreased gluconeogenesis and pentose phosphate pathway in the host cells (Wang *et al.,* 2006), on its own. Evaluating the effects of bioprocess parameters and consequent metabolic changes occurs inside the host cells will shed light on better understanding of the cellular dynamics of prokaryotic host. Besides, this data could be used for rational design of an improved expression system for enhancement of different recombinant proteins.

In this study, the rhINF-β, as a clinically important cytokine used in the treatment of multiple sclerosis was selected as the model protein for fed-batch culture experiment in a 5L bench-top bioreactor. Many studies have been shown that the rhINF-β has antiviral, anticancer and immunomodulatory activities (Borden *et al.,* 2007; George *et al.,* 2012). Besides, it plays a critical role in treatment of multiple sclerosis (Weber *et al.,* 2012) and its worldwide demand has been increased in recent years (Aggarwal, 2014). In spite of its biological and clinical importance, there is no robust information from rhINF-β in bioreactor experiments and the most of the current available data are extracted from shake flask culture experiments because of its less cost, time and required resources. On the other hand, the obtained results from conventional laboratory scale studies are not reproducible, either not directly transferable to the pilot or large scale experiments. As a matter of fact, the effects of scale-up in a fermentation process including difficulties in oxygen transfer, limited heat dissipation and impaired mixing which leads to consequent limitation in critical substrates and formation of growth-inhibitory by-products are still missing. So, we decided to investigate the impacts of rhINF-β overexpression on metabolic characteristics of *E. coli* during HCDC experiment in a pilot scale study. Besides, the results of this study could be used for optimization of a fed-

batch cultivation technique using *E. coli* to provide a robust platform for overexpression of various recombinant proteins.

Materials and methods

Bacterial strain and vector system

In this study, *E. coli* BL21 F^- *ompT hsd*S_B ($r_B^-m_B^-$) *gal dcm* (DE3) obtained from Invitrogen, CA, USA was used as the expression host for the periplasmic overexpression of rhINF-β.

The host strain was transformed with a previously designed vector (Morowvat *et al.,* 2014) based upon pET-25b(+) with a T7 promoter as an expression vector. This expression vector contains an ampicillin resistance gene and unique restriction sites for *Xho*I and *Msc*I. After transformation, the plasmid harboring *E. coli* cells were grown on LB agar plates containing ampicillin (100 µg L^{-1}). The recombinant strain was preserved in a (1:1) glycerol: LB growth medium solution at −70 °C.

Media and culture condition

Luria Broth (LB) culture medium consisted of 10 g L^{-1} tryptone peptone, 5 g L^{-1} yeast extract and 10 g L^{-1} sodium chloride was used for preservation and preliminary studies on the strain. Solid media for plates contained 10 g L^{-1} of agar. The terrific broth (TB) medium was used for overexpression of the requested recombinant protein.

The recombinant strain was initially grown at 37 °C in a 500 mL Erlenmeyer flask containing 50 mL of LB culture medium with 100 µg L^{-1} of ampicillin in a shaking incubator (N-BIOTEK, Bucheon-si, South Korea) with 160 rpm for 16 h.

Bioreactor experiment

For rhINF-β production, the recombinant strain was first grown in a 500 mL shake flask containing 100 mL of TB culture medium with 100 µg mL^{-1} of ampicillin. After 4 h the contents were used to inoculate a 5.0 L bioreactor (Infors HT, Basel, Switzerland) containing 1.0 L of medium with 5 g L^{-1} glucose. During an initial growth phase of about 6 h the agitation was maintained at 500 rpm and air was sparged at a 1.0 vvm to maintain the dissolved oxygen above 30% of saturation until the OD reached 3.0. IPTG (2 mM) was added and the temperature was maintained at 32 °C for rhINF-β accumulation in periplasmic space of the host cells.

After entering to the stationary phase, the feeding was started using a 20 g L^{-1} of glucose and mineral salts. A fed-batch regime with an exponential substrate feed was used to provide a constant growth rate. The substrate feed was controlled by increasing pump speed according to a predicted exponential growth algorithm.

The pH was controlled at 7.0 by automatically feeding 50% (v/v) NH_3 and 1.0 M HCl. Ampicillin (100 µg mL^{-1}), as a selective pressure to maintain the plasmid in recombinant cells was added to the culture medium. The foam was controlled by the addition of a silicone-based anti-foaming reagent.

The samples were collected every one hour and analyzed for dry cell weight, glucose, nitrogen and phosphate consumption, acetate and lactate excretion, plasmid stability and rhINF-β production. The culturing was continued for 8 additional hours. The cell pellet was then collected using centrifugation at 5000 g for 10 min at 4 °C and frozen. All fermentations were run in duplicate.

Analytical procedures

The cell growth profile was monitored by measuring the optical density at 600 nm in a spectrophotometer (E-Chrome Tech, Taipei, Taiwan). The cultures were diluted to the linear range using a 9% (w/v) NaCl isotonic solution, before measuring the absorbance. For measuring the cell dry weight, 5 mL of the culture medium was centrifuged at 9000 rpm for 10 min, washed twice and dried at 105 °C until constant weight.

Glucose, ammonia, phosphate, lactate and acetate were analyzed enzymatically using the appropriate kits (ZiestChemie® Diagnostics, Tehran, Iran; and

Megazyme, Wicklow, Ireland), according to the suggested protocols.

Total soluble protein was determined using the Bradford method with bovine serum albumin (BSA) as the standard. The expression level of rhIFN-β was analyzed by SDS-PAGE using 12.5% (w/v) poly-acrylamide gels. Gels were then stained sing Coomassie Brilliant Blue R-250, and then quantified by a gel scanner using densitometry.

Results and discussion

Cell growth and rhINF-β production

The cell growth profile of the prokaryotic host cell was monitored using spectroscopy and dry cell weight methods. A mixture of mineral salts together with glucose (20 g L^{-1}), sufficient for the synthesis of cellular and recombinant proteins, was fed in fed-batch culture of *E. coli* BL21 (DE3) to improve the yield of biomass and the recombinant protein production.

To amount of rhINF-β production was determined using densitometry method and reported as grams per liter. The maximum values for the observed cell growth (OD_{600nm}=103.5), dry cell weight (42.15 g L^{-1}) and rhINF-β production (2.8 g L^{-1}) were comply in an agreement (Fig. 1a-c).

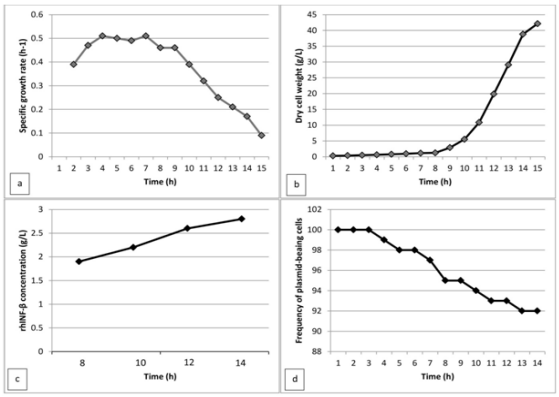

Fig. 1. Fed-batch fermentation o the recombinant E. coli BL21 (DE3) cells in a laboratory scale bioreactor for the overexpression of rhINF-β. Plots showing **a)** specific growth rate (h^{-1}), **b)** cell dry weight (g L^{-1}), **c)** amount of rhIFN-β production (g L^{-1}), and **d)** time profile of the plasmid stability in fed-batch culture. The culture was induced by 2 mM IPTG when the system reached at an OD_{600nm} of 3.0.

The specific growth rate has an upper limit which is recognized by the onset of glucose overflow metabolism (Valgepea *et al.*, 2010) and acetate secretion (Wolfe, 2005) which is harmful for heterologous protein production (Eiteman and Altman, 2006). Hence, providing an optimal condition for enhancement of recombinant proteins seems crucial.

The observed decline in specific growth rate of the prokaryotic host after 9 h relates either on inhibitory effects of acetate accumulation or limitation in precursors required for biomass synthesis. More studies on the intra- and extracellular concentration of different metabolites such as acetate are required to shed light on the proposed hypotheses.

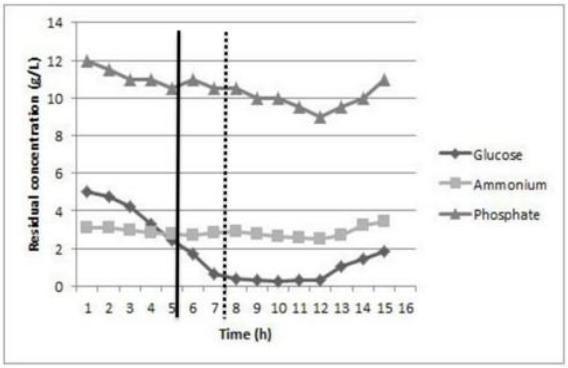

Fig. 2. The residual concentration of glucose, ammonium and phosphate (g L^{-1}), measured by enzymatic method, in HCDC of recombinant *E. coli* cells. Glucose; *diamonds*, Ammonium; *squares*, Phosphate; *triangles*. Vertical straight line and dotted line indicate the time to begin the feeding and starting induction, respectively.

Glucose, ammonium and phosphate consumption

The residual concentration of glucose, ammonium and phosphate in *E. coli* BL21 (DE3) HCDC culture during overexpression of the rhINF-β was analyzed using appropriate enzymatic kits. As shown in Fig. 2, the batch culture experiment was started using 5.0, g L^{-1} of glucose in culture medium. After finishing the initial source of glucose (during 6 h) in the culture medium, the fed-batch culture experiment was begun using a feeding solution. The feeding strategy was applied in a manner to keep the minimum required metabolites for the producing recombinant cells. The

residual glucose lately accumulated up to 1.82 g L^{-1}, a phenomenon also observed by other researchers (Lin *et al.,* 2001; Xu *et al.,* 1999). On the other hand, the required nitrogen source for biomass and rhINF-β production was provided using a 50% (v/v) solution of ammonia. The primary concentration of ammonium in the TB culture medium was 3.12 g L^{-1}.

The changes in extracellular pool of ammonium were monitored hourly (Fig. 2). After an initial reduction in ammonium concentration, some transient fluctuations in its extracellular amounts were observed. This phenomenon suggests that there is a cell density-dependent metabolic switch in the nitrogen metabolism. Since the ammonium assimilation needs the energy and intermediates from central metabolism of carbon (Reitzer, 2003), hence understanding the exact mechanism of integration of intermediary carbon metabolism with nitrogen assimilation seems important. Based upon this fact, determining the residual concentration of carbon and nitrogen might be useful.

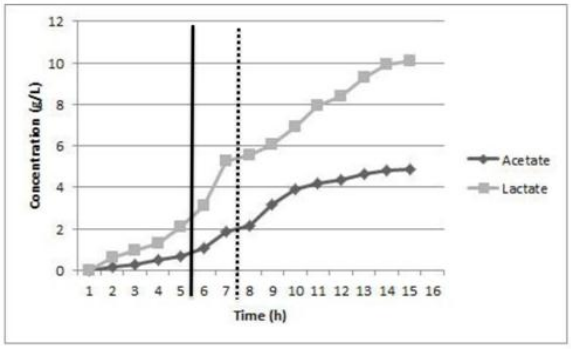

Fig. 3. Acetate and lactate concentrations (g L^{-1}) measured by enzymatic method, during the fed-batch cultivation experiment of *E. coli* BL21 (DE3). Acetate (*diamonds*) and Lactate (*squares*) concentrations. Vertical straight line and dotted line indicate the time to begin the feeding and starting induction, respectively.

Moreover, the metabolic changes in extracellular concentration of phosphate were quantified. As it has been depicted (Fig. 2) the initial concentration of phosphate was 12.0 g L^{-1}. In the first hours of the batch culture experiment, the phosphate was consumed by rapidly growing recombinant cells. After addition of the feeding solution some noticeable oscillations was observed in the phosphate consumption trend. In the last hours of fed-batch culture, the cells entered to the stationary phase and the residual phosphate concentration in the culture medium was gradually increased. Previously performed researches, have proved that there is a close relationship between the phosphate metabolism and different metabolic pathways in *E. coli* including central carbon metabolism and energy pathways (Marzan and Shimizu, 2011).

Acetate and lactate production

The extracellular concentrations of acetic and lactic acids as two major short chain organic acids which are the by-products of glucose metabolism were analyzed. Due to the inhibitory effects of this metabolites on the cell growth rate and the recombinant protein production (Eiteman and Altman, 2006), the productivity of the target protein during a fed-batch process could be influenced.

As could be seen in the Fig. 3, the acetate and lactate concentration were increased gradually till a maximum in the last hours of batch culture (5.26 and 1.87 g L^{-1} of lactate and acetate, respectively). At the late growth phase of the batch culture, the glucose shortage drives the host cells to consume acetate as the carbon source. This fact could explain lowering of the acetate concentration before beginning the fed-batch stage. After starting the fed-batch culture fermentation, their amounts were increased slowly in a continuous fashion. A slight increase in the acetate and lactate concentration was observed in the last hours of HCDC process (10.08 and 4.91 g L^{-1} of lactate and acetate, respectively). Because, in higher cell densities of the host cell, the TCA enzymes will be down-regulated and as a result, the TCA cycle capacity will be reduced.

Some studies have been proved that the acetate and lactate secretion levels in host cell is influenced by the culture medium condition, glucose supplying strategy, the host strain and the applied growth conditions. In the present study, we used the BL21 (DE3) strain of *E. coli*, an *E. coli* B derivative, which shows lower affinity for the acetate accumulation due

to its particulate genotype which takes advantage of a more active glyoxylate shunt (Van De Walle and Shiloach, 1998). Besides, we employed a special feeding strategy to prevent the glucose accumulation in the culture media during the fed-batch experiment, and therefore, less amounts of acetic and lactic acids were accumulated during recombinant protein production stage. Hence, the cellular capacity for tricarboxylic acid (TCA) cycle was not fully engaged. These data were in agreement with some previous studies which assumed the overloading of the TCA cycle via fast oxidation through glycolysis pathway as a major reason for acetate excretion (Majewski and Domach, 1990). Some researchers have been exploited the transcriptome and proteome analysis approaches to study the metabolic and physical changes effects of HCDC on *E. coli* (Haddadin and Harcum, 2005; Yoon *et al.,* 2003). Application of these newly emerging -omics technologies could be helpful to understand the exact metabolic changes during rhINF-β production as well.

Plasmid stability

The results of plasmid content assay were in agreement with the trend of rhINF-β overexpression (Fig. 1d). This facts proves that the applied HCDC in fed-batch culture has not negative influenced the plasmid stability till the end of fed-batch fermentation experiment.

Plasmid bearing leads to the loss of host fitness which is characterized by reduced biomass content, growth rate and competitiveness. The exact mechanisms of this alteration in different cellular metabolites and energy level are still remained unclear. Although, it has been suggested that these costs are depends on the expression level of plasmid-encoded proteins, replication and maintenance of the plasmid, and disruption of cellular regulatory status. This phenomenon is thought to be the main cause of the metabolic burden (Nojiri, 2013).

Quantification of metabolic fluxes is one of the most important aspects of physiological studies and Metabolic Flux Analysis (MFA) is a powerful technique for determination of the fluxes through various pathways (Chen *et al.,* 2011).

In conclusion, the influence of recombinant protein overexpression through HCDC on the metabolic characteristics of *E. coli* BL21 (DE3) recombinant cells was examined. The results show that high cell density and overexpression of the rhINF-β do not have considerable disfavored effects on the plasmid stability, because the expression of beta interferon remained almost the same during the culture experiment. 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 the cell physiology is dependent on medium composition, type of product, growth technique, and dimensions of the vessel, the results of this investigation cannot simply be applied to other systems and can only be used as a tool for predicting cell behavior and responses in similar systems

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