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GROWTH KINETICS AND HUMAN GROWTH HORMONE PRODUCTION OF A HEAT- INDUCIBLE RECOMBINANTE *CHERICHIA COLI* DURING BATCH FERMENTATION*

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Abstract – The batch fermentation production of human growth hormone (hGH) was studied in a new recombinant *Escherichia coli*. The effect of different levels of dissolved oxygen and glucose concentration on cell density and substrate utilization were evaluated. The results indicated that the optimum level of dissolved oxygen was 25% of air saturation. The specific growth rate reached a maximum for an optimal glucose concentration of 5 gl^{-1}, the maximum yield was 0.58 gg^{-1} and glucose was completely consumed after 10 hrs. The growth of recombinant *E. coli* on semi-defined and complex media was compared. The results demonstrated that dry cell weight and maximum specific growth rate were 2.35 gl^{-1} and 0.4 h^{-1} on a complex medium, respectively, but they were 3.3 gl^{-1} and 0.24 h^{-1} on semi-defined medium. The plasmid stability and hGH production during batch fermentation were investigated. The results suggested that the specific growth rate is the most important factor on hGH production and should be investigated in fed batch cultivation to get high cell density.

Keywords – Batch fermentation, recombinant, *escherichia coli*, human growth hormone, microbial kinetics

1. INTRODUCTION

Escherichia coli is one of the most widely used hosts for the production of heterologous protein because of its ability to rapidly grow high density cultures [1] on inexpensive substrates [2], and its well-characterized genetics and physiology [3]. During production in *Escherichia coli*, recombinant proteins are placed in different parts of cells. They aggregate and form inclusion bodies in cytoplasm [4], transfer to periplasmic space [5], inner or outer membranes [6]. The strong, inducible promoter systems such as λP_L [7], λP_R [8], trp [9], T7 [10], commonly used in recombinant *E. coli*, are advantageous for overproduction of recombinant proteins. They express recombinant proteins by heat shock or chemicals [11].

Recombinant human proteins such as insulin, human growth hormone and interferon have become important as biological pharmaceuticals. The average annual market growth of human therapeutic and diagnostic biotechnology products is expected to increase at a rate of 13 and 9 percent, respectively [12]. Human growth hormone is an anterior pituitary hormone, a non-glycosilated protein consisting of 191 amino acids (21.5 KDa) and two intermolecular disulfide linkages. Because of a wide variety of biological activities, the hGH has been extensively used for treating dwarfism, bone fractures, skin burns, bleeding ulcers, and enhancing the physiological

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functions of middle-aged or elderly persons as a youth hormone [13]. The DNA encoding hGH was cloned for the first time in bacterial plasmid in 1979 [14]. Today, hGH has been successfully produced using recombinant *E. coli* in small and large scales [15-17], but it has not been produced under a heat-inducible λP_L promoter. Although maximum biomass concentration is usually reached by the addition of complex components such as yeast extract, tryptone, etc., more recently considerable effort has been spent on a defined medium [18].

In previous work, a semi-defined medium was designed for the recombinant *E. coli* strain A6-5 harboring two plasmids and producing hGH by heat shock induction. The medium, named M6, was optimized using the Taguchi experimental design method [19]. The goal of this study was to investigate the growth kinetics, plasmid stability and hGH production of the novel recombinant *E. coli* on a semi-defined medium at different conditions during batch fermentation.

2. MATERIALS AND METHODS

a) E. coli strain and recombinant plasmids construction

The host-plasmids system (provided by NRCGEB) used for the production of recombinant hGH in this study consisted of the structural gene for hGH, carried on the recombinant T7-based pET expression plasmid (amp^+, hGH^+) and another plasmid pGP1-2 [20] with a heat inducible λP_L promoter (km^+, T7 RNA $polymarase^+$) in *E. coli* strain DH5-α as the host. The hGH gene was expressed by heat induction and accumulated as inclusion body in cytoplasm.

b) culture media

Frozen stock culture of *E. coli* A6-5 was maintained at –20°C and –70°C in glycerol 15 and 30 percent, respectively. *E. coli* cells were routinely grown in LB (Luria-Bertani) medium, which contained yeast extract, 5 gl^{-1}, tryptone, 10 $gl^{-1,}$ inoculum broth and NaCl, 5 gl^{-1} as a complex medium. In addition, the LB plates contained 1.5 % agar, 100 mgl^{-1} ampicillin and 30 mgl^{-1} kanamycin.The M6 semi-defined medium [19] contained KH_2PO_4, 4.5 gl^{-1}; $K_2HPO_4.3H_2O$, 4.5 gl^{-1}; NaH_2PO_4, 2 gl^1; NH_4Cl, 0.5 gl^{-1}; $(NH_4)_2SO_4$, 0.5 gl^{-1}; $MgCl_2.6H_2O$, 0.25 gl^{-1}; yeast extract, 5 gl^{-1} and glucose in different amounts. The trace metal solution contained the following per liter of 5 M HCl: $Na_2B_4O_7.10H_2O$, 0.02 g; $FeSO_4.10H_2O$, 10 g; $CaCl_2.2H_2O$, 2 g; $ZnSO_4.7H_2O$, 2.2 g; $MnSO_4.4H_2O$, 0.5 g; $CuSO_4.4H_2O$, 1 g; $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.1 g.

c) Batch fermentation

The inoculum was prepared in 1 l flasks containing 250 ml LB medium with antibiotics. A single colony from LB agar plates was added to each flask and incubated at 30°C and 200 rpm overnight. The batch cultures were performed in a 5 l fermentor with 3.5 l working volume (Bioflo III, New Brunswick Scientific Co., USA) containing M6 medium. The medium was autoclaved at 121°C for 20 min. Glucose and $MgCl_2.6H_2O$ were autoclaved separately. pH was adjusted to 6.9. After inoculation, the temperature and pH were automatically maintained at 30°C and 6.9, respectively. The dissolved oxygen tension was maintained at a desired level by controlling the agitation speed. The induction strategy of the recombinant cells was heating the cultures to 42°C for 20 min and then incubating them at 30°C for 4 hrs.

d) Analytical method

Biomass was determined by filtering the culture samples through 0.45 μm pore size membrane filters and drying them at 105°C to constant weight. Biomass was also measured by optical density using a spectrophotometer (Milton Roy Spectronic 20D) at 550 nm and a standard curve plotted. Glucose concentration was determined by an enzymatic test kit from Chemenzyme Co. and spectrophotometer (Beckman DU-70) at 500 nm. The hGH expression was measured by gel electrophoresis. Cell pellets were collected by centrifugation at 5000 rpm for 10 min. They were lysed in a protein sample buffer (100 mM tris-HCl, 2 % SDS, 0.1 % Bromophenol blue, 10 % glycerol, pH=8) and boiled for 10 min. The total protein pattern was visualized using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) followed by coomassie blue staining. Plasmid stability was determined by counting the colonies grown on both LB plates containing ampicillin and kanamycin using the toothpicking method [7].

3. RESULTS

a) Effect of dissolved oxygen on growth

When the dissolved oxygen was below 25 %, cell concentration increased with a rise in dissolved oxygen, but when the dissolved oxygen was above 25 %, no significant increase in cell density was achieved, i.e. about 25 % of dissolved oxygen is the optimum level for respiration of *Escherichia coli* A6-5 (Fig. 1). The following equation is used to fit the relation between optical density (OD) and dissolved oxygen (d, %):

$$OD = -0.0036d^2 + 0.2295d + 4.0571,\ R^2 = 0.9852$$

Although no significant differences between the optical density at 25% and 30% was obtained in practice, according to this equation, the maximum optical density, OD_{max}=7.715 can be achieved at dissolved oxygen d=31.875%.

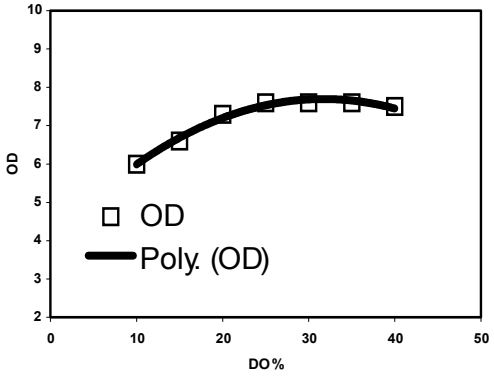

Fig.1. Effect of dissolved oxygen on final cell concentration of *E.coli* A6-5

b) Effect of glucose concentration

Growth and substrate consumption of *E. coli* A6-5 were investigated at different initial glucose concentrations (Fig. 2). The results showed that at a higher concentration of glucose, the yield coefficient ($Y_{x/s}$) was decreased and glucose was not consumed completely. $Y_{x/s}$ for 20, 10 and 5 gl^{-1} of glucose was 0.43, 0.52 and 0.58 gg^{-1}, and maximum specific growth rate (μ_{max}) was 0.22, 0.22 and 0.24 h^{-1}, respectively.

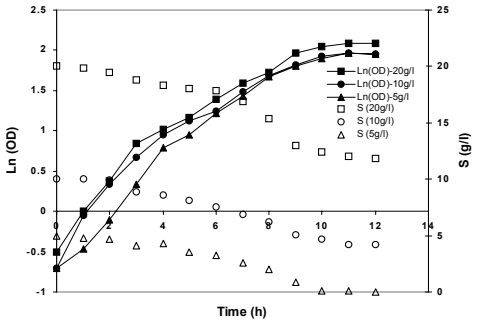

Fig.2. Growth and substrate consumption of *E.coli* A6-5 at different initial glucose concentrations

c) Growth in semi-defined and complex media

E. coli A6-5 was grown in batch fermentation on M6 and LB (Fig. 3). The growth in M6 was more than LB, but the fermentation time was longer. Dry cell weight (DCW) in Land M6 was 2.35 and 3.3 gl^{-1}, respectively. The maximum specific growth rates, μ_{max}, were 0.4 h^{-1} in LB and 0.24 h^{-1} in semi-defined medium. Glucose was completely consumed during fermentation and $Y_{x/s}$ was 0.58 gg^{-1}.

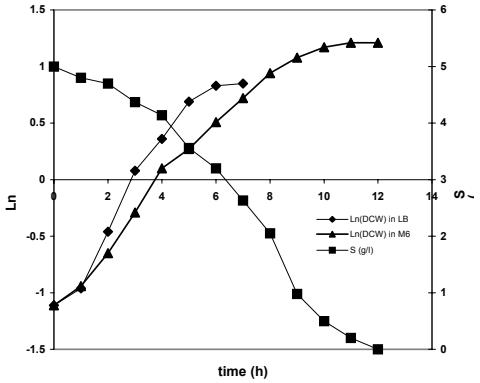

Fig.3. Comparison of *E.coli* A6-5 growth on defined (M6) and complex (LB) media

d) hGH production and plasmid stability

hGH production was investigated at different growth phases of batch fermentation. It occurred briefly in both LB and M6. Despite the stability of both plasmids, pET21 and pGP1-2, during batch fermentation (data were not shown), hGH was only produced when culture induction occurred at the early-exponential phase as shown in Fig. 4. On the other hand, μ of the mid- and late-exponential phase induced batch cultures was considerably lower than that of the batch culture induced at the early-exponential growth phase, and the decrease in specific growth rate occurred sooner as shown in Fig. 5. The results indicated that hGH production depends on the postinduction specific growth rate, so that it stops when μ is much smaller than μ_{max}.

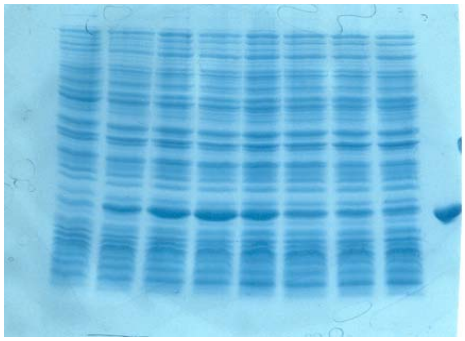

Fig. 4. SDS-PAGE analysis of total cell protein of *E.coli* A6-5 during batch fermentation in M6. The total cell protein before induction (lane 1) and after induction at 1 to 7 h of batch fermentation (lanes 2-8). The last one is hGH

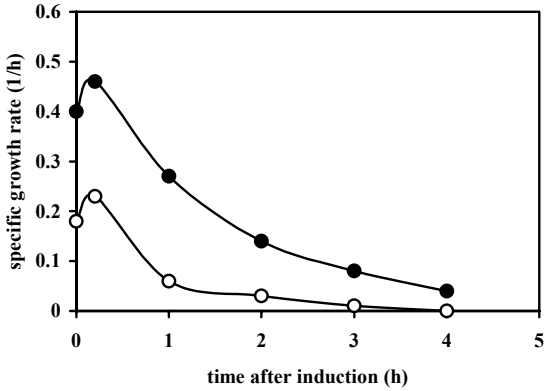

Fig.5. Time profile of specific growth rate of A6-5 batch cultures induced at early- (•) and late- exponential (o) phases

4. DISCUSSION

The recombinant *E. coli* A6-5 was grown on a semi-defined medium in batch fermentation to find the growth kinetics and hGH production pattern for further research. As shown in Fig. 1, the optimal dissolved oxygen level is 25%. This result is similar to findings in [20] which reported that the optimal level of dissolved oxygen is 25% saturation for recombinant hGH production by *E.coli*. The glucose metabolism in *E.coli* has been studied extensively [3]. Aerobic growth conditions with high glucose concentration can result in unbalance between glycolysis and TCA cycle resulting in accumulation of acetate. This has been reported to have detrimental effects on recombinant cells [22]. Our results indicated that the high concentration of glucose decreased $Y_{x/s}$ and substrate was not consumed completely, which may be due to the detrimental effects of the accumulation of acetate. Consequently, the glucose concentration should be less than 5 gl^{-1} during fed-batch fermentation by controlling the feeding rate.

The hGH production pattern was the same in semi-defined and complex media in such a way that hGH was produced at the early-exponential phase when μ is near to μ_{max}. This may be due to the effect of temperature induction on growth and the decrease in specific growth rate at mid- and late-exponential phase. Recombinant *E. coli* with λP_L –inducible expression vector was confronted with a

harsh environment that lead to retardation or cessation of cell growth and protein production. The control of the post-induction specific growth rate (μ_i) on cell growth and productivity of INF-γ has been studied. It was revealed that sustaining μ_i above a certain value after induction was the key factor for high-level production of INF-γ under control of a λP_L promoter [7]. The relation between the pre-induction specific growth rate (μ_g) and cell growth was also revealed in previous works [23]. In the above mentioned cases, expression of foreign proteins was directly under the control of the λP_L promoter. Although hGH was indirectly expressed by λP_L in our study, the same results were obtained. Due to this, we suggest that the control of μ during fed-batch fermentation, both before and after induction, might be feasible for sustaining cell growth and prolonging and increasing the production of hGH in the recombinant *E. coli*.

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