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Glucose concentration affects recombinant interferon α-2b production in *Escherichia coli* using thermo-induction system

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ABSTRACT

In the present work, a recombinant *Escherichia coli* strain was used for the production of interferon α-2b in both shake flask and in bioreactor. The first part of this research was focused on the investigation of the effect of glucose concentration on the kinetics of cell growth, recombinant protein production and acetate formation. In general, glucose supplementation to culture medium enhanced cell growth when added in concentration between 0-20 g/L. Further increase in glucose level reduces biomass production and enhances acetate accumulation in culture. The results clearly demonstrated that maximal interferon production of 27.7 mg/L was achieved in culture supplemented with 20 g/L glucose. Further improvement in recombinant interferon production process was also achieved by scaling up from shake flask to 16-L stirred tank bioreactor. The maximal volumetric interferon production in bioreactor batch culture was 44.5. mg/L after only 6 hours.

INTRODUCTION

For many years *Escherichia coli* acts as industrially important microorganisms in health industries. This bacteria can produce pharmaceutically important enzymes in both antibiotic industries such as penicillin acylase (El Enshasy *et al.,* 2009, Mohy El deen*et al.,* 2012) and biotherapeutic enzymes for cancer treatment like asparaginase (Swain *et al.,* 1993). Moreover, since the revolution of recombinant DNA technology in the mid-1970s, *E. coli* became one of the main protein biofactories for biopharmaceutical protein production in wellness industries (Sarmidi and Elenshasy, 2012). Since that time *E. coli* showed high potential for different recombinant protein production and enzymes such as β-glucanase (Beshay*et al.,* 2011), human growth hormones (Castan*et al.,* 2002), Insulin (Schmidt *et al.,* 1999); Viral surface antigen (Abbas *et al.,* 2007), and Hirudin (Lu *et al.,* 2013). The superiority of *E. coli* over other Prokaryotic and Eukaroytic hosts was based on its higher growth rate, cheap cultivation medium and better understood of the genetic, molecular biology and cultivation strategies of this microorganism. It showed also high potential for interferon production in laboratory and semi-industrial scale (Luz *et al.,* 2007).

Of different pharmaceutically important recombinant biotherapeutic proteins, interferons (IFs) are glycoprotein cytokines family with particular importance based on their bioactivities as antiviral, anti-proliferative and immunomodulator agent. Based on their wide applications in the treatment of epidemic diseases such as HepB and HepC viruses, the interferons market expect to exceed US$ 10-Billions by 2015 according to the recent interferon market forecast study (Interferon Market Forecast 2015). The level of expression of the desired protein is highly regulated not only by the plasmid expression system and stability but also by the cultivation condition and medium composition. The cultivation medium for recombinant protein production using *E. coli* includes mainly glucose as sole carbon source. During cultivation, the cells utilize glucose for growth and protein production and produce some

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undesired by products such as lactate and acetate. These two compounds can act as inhibitors for cell growth and recombinant protein production. Thus, it is necessary to optimize glucose concentration in batch culture for better cell growth and recombinant protein induction. Thus, the main objective of this work was to study of the effect of initial glucose concentration on the kinetics of cell growth, interferon α-2b production. Based on the obtained results, cultivations were also conducted in bioreactor level to investigate the effect of production scale on the kinetics of cell growth and recombinant protein production.

MATERIALS AND METHODS

Microorganism

The original strain a non-pathogenic *Escherichia coli* K-12 (ATCC 10798) was initially obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). This strain was genetically modified to produce a recombinant strain of r*E. coli* with a plasmid coding Egyferon 4457bp for recombinant human interferon α-2b production. The recombinant strain was preserved in frozen in 50% glycerol solution (v/v) at -80 °C. Before use, the cells were activated twice in LB agar medium of the following composition (g/L): beef extract 5, peptone 10, yeast extract 5, NaCl 5, and agar 20 (pH 7.0). After 24 h day cultivation, the arisen colonies were used to inoculate 250-ml Erlenmeyer flask (of 50 ml working volume) containing LB broth. The inoculated flasks were incubated on rotary shaker at 37°C and 200 rpm for 24 h.

Inoculum preparation

Inoculum was prepared in a 250 ml Erlenmeyer flask containing 50 ml YPM medium composed of (g/L): yeast extract, 5, peptone, 3 and mannitol, 25. After sterilization for 15 min at 121°C, 50 ml YPM medium was inoculated with 250 µl of glycerol culture. The inoculated flasks were incubated on the rotary shaker (Innova 4080, New Brunswick Scientific Co., NJ, USA) at 200 rpm and 37°C for 24 h. Cells were used thereafter to inoculate either 250 ml Erlenmeyer flasks or stirred tank bioreactor with inoculum concentration of 10% (v/v).

Fermentation medium

The medium used for interferon production had the following composition (g/L): yeast extract, 10; NaCl, 2.5; Tryptone, 20; $MgSO_4.7H_2O$, 0.12 and pH 7.0. Glucose was sterilized separately and added to the fermentation medium before inoculation. In case of shake flask, cultivations were carried our using 250 ml Erlenmeyer flask with a working volume of 50 ml and inoculated with cells grown for 24 h in vegetative medium as described previously in concentration of 10% (v/v). The inoculated flasks were incubated on rotary shaker at 200 rpm and 37 °C for the first 2 hours and then temperature increased up to 42 °C for the rest of cultivation time.

Bioreactor cultivations

Cultivation in stirred tank bioreactor were conducted using the same medium in shake flask level (supplemented with glucose 20 g/L) and run under the same cultivation conditions in term of inoculums size, temperature and pH. The bioreactor used in this study was 16-L stirred tank bioreactor (Bio-Engineering, Wald, Switzerland) with working volume of 8-L. The stirrer was equipped with two 6-blade Rushton turbine impellers (d_i (impeller diameter) = 85 mm; d_t (tank diameter) = 214 mm, d_i/d_t = 0.397). The agitation speed was adjust to 600 rpm and kept constant throughout the cultivation and aeration was performed using filtered sterile air and supplied continuously to the bioreactor with rate of 0.5 v/v/min. Foam was suppressed by the addition of silicon antifoam grade A (Sigma-Aldrich Inc., MO, USA). During the cultivation process, pH value and dissolved oxygen concentration were determined using pH and DO polarographic electrodes, respectively (Ingold, Mettler-Toledo, Switzerland). The pH was adjusted to 7.0 by cascading the pH controller with acid/base feeding peristaltic pumps connected with 4 M HCl and 4 M NaOH solutions.

Sample preparation and cell dry weight determination

Samples, in form of two flasks containing 50 ml each, or 25 ml of broth in case of bioreactor, were withdrawn at different times during the cultivation in a centrifugation falcon tube (Falcon, USA). Immediately after sampling, the optical density was measured by using spectrophotometer (Novaspec II, Pharmacia Biotech, Sweden) at 600 nm after proper dilution. For all samples, the cultivated broth was diluted to give values less than (1 OD_{600}) for better accuracy. The OD of culture was converted to dry cell mass through a linear correlation standard curve. Based on standard curve of this strain, One OD_{600} was almost equivalent to 0.28 g/L.

Glucose, lactate and acetate determination

Glucose and lactate concentrations were determined using automatic glucose/lactate analyzer (YSI 2700, Yellow Springs Instruments, OH, USA). The measurement is based on a coupled enzymatic-electrochemical process in which the enzyme (glucose oxidase or lactate oxidase) is immobilized between two membrane layers, polycarbonate and cellulose acetate. The substrate is oxidized at the immobilized oxidase layer producing hydrogen peroxide, which then passes through the cellulose acetate membrane to interact with the platinum anode (Pt/AgCl/Ag) producing electrons. The electron flow is proportional to the H_2O_2 concentration and, therefore, to substrate concentration. Acetate was assayed according to the method of Pecina*et al.* (1984) which was modified later by Tomlins*et al.* (1990) using HPLC system (Waters, Milford, MA, USA). This system composed of a pump Waters 600 controller, 2690 Separation Module HPLC (Model 2690, Waters, Milford, MA, USA) and auto sampler fitted with a detection system; Ultraviolet (UV) 2487 Dual Absorbance Detector at 210 nm (Water, Milford, MA USA). Separation was carried out using organic acid column: 300×7.8 mm/ 8 micron (Phenomenex, Torrance, USA) was used to achieve the chromatographic separations. Acetic acid was eluted with 0.005N. sulphuric acid at a flow rate of 0.5 ml/min at 40 °C.

Peak heights were measured using a dual channel computer integrator (Water Empower chromatography system, Waters, Milford, MA, USA) and converted to acetic acid concentration based on previously prepared standard.

RESULTS AND DISCUSSION

Effect of glucose concentration on kinetics of cell growth and recombinant interferon production

The aim of this experiment was to study the influence of glucose concentration on cell growth kinetics and interferon production. Thus, the recombinant strain of *E. coli* was used to inoculate different media of different glucose concentrations from 0 to 30 g/L glucose. The inoculated flasks were incubated simultaneously on rotary shaker of 200 rpm. The initial cultivation temperature for all cultures were set at 37 ℃ during the first 2 hours of cultivation and increased thereafter up to 42 ℃ to induce recombinant protein production.

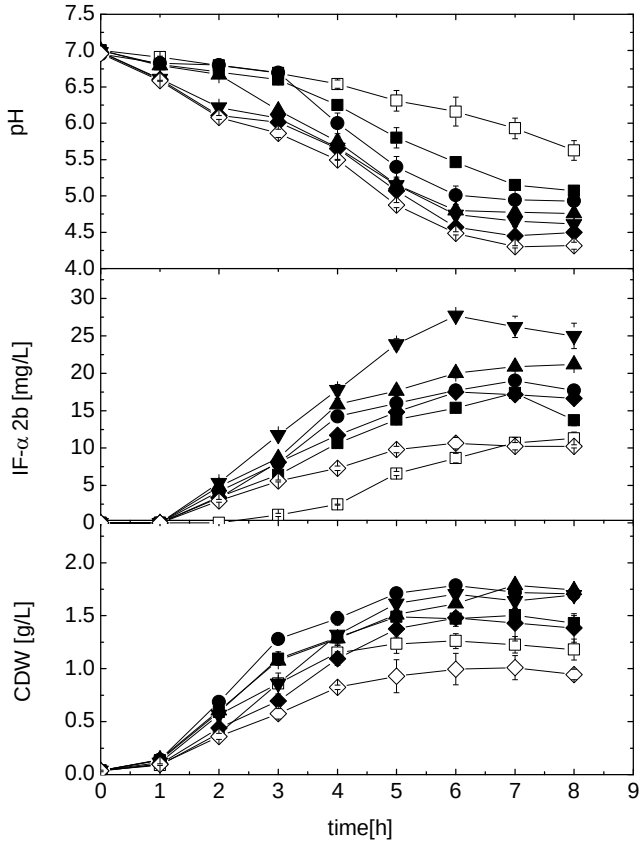

Fig. 1: Changes in cell growth, interferon α-2b production and pH during batch cultivation of r*E. coli* in shake flask level at different glucose concentrations (□, 0 g/L; ■, 5 g/L; ●, 10 g/L; ▲, 15 g/L; ▼, 20 g/L; ♦, 25 g/L; ◊, 30 g/L). Values are mean ± standard deviation, n=2.

Figure 1 demonstrates the dynamic profile of *rE.coli* growth, interferon production and pH during batch cultivation at various levels of glucose concentration (0-30 g/L). As shown, cell growth was enhanced by glucose concentration in range between (0-20 g/L). Further increase in glucose concentration suppresses cell growth. The maximal biomass of about 1.7g/L in 20 g/L glucose supplemented culture after 7 hours. On the other hand, the expression of interferon in culture was enhanced by addition of glucose to culture medium. The rate of interferon production in culture increased from 2.14 mg/L/h in glucose free medium to about 5.54 mg/L/h in 20 g/L glucose supplemented medium. The maximal value of volumetric interferon production of 27.7 mg/L was obtained in 20 g/L glucose supplemented culture. However, in all medium formulations, the pH dropped gradually during cell growth phase and kept more or less constant for the rest of cultivation time. As shown in figure 1, direct proportional relation was observed between the glucose concentration applied and the drop of the pH in culture. The minimal value of pH of about 4.25 was observed in 30 g/L supplemented culture after 8 hours.

Effect of glucose concentration on acetate formation

Figure 2 demonstrates the change in glucose and acetate concentrations during batch cultivation of r*E.coli* in shake flask level. As shown, glucose was fully consumed in culture supplemented with range between 0-20 g/L. In cultures more than 20 g/L, glucose was still available in culture after 8 days cultivation. On the other hand, acetate formation was also followed in this culture to investigate the relation between initial glucose concentration in medium and acetate formation. It was observed that, no acetate was formed in glucose free culture and the amount of acetate produced was directly proportional to the initial glucose concentration.

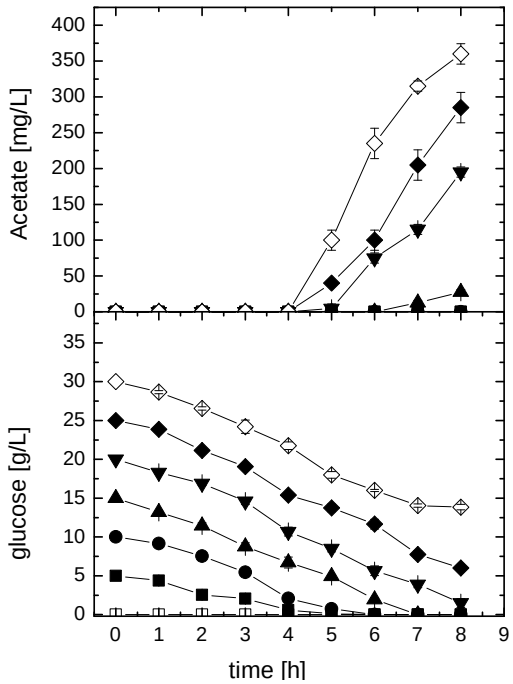

Fig. 2: Glucose consumption and acetate production during batch cultivation of recombinant *rE. coli* in shake flask level at different glucose concentrations (□, 0 g/L; ■, 5 g/L; ●, 10 g/L; ▲, 15 g/L; ▼, 20 g/L; ♦, 25 g/L; ◊, 30 g/L). Values are mean ± standard deviation, n=2.

The maximal acetate formation of 360 mg/L was obtained in 30 g/L glucose supplemented culture. Thus, we can also conclude that the drop in medium pH (figure 1) is directly related to the acetate

formation in culture as the lowest pH was also recorded in culture of high glucose concentration. Based on all these data together (in figures 1 and 2), we can conclude that glucose in concentration of 20 g/L is optimum for interferon production and thus this concentration was used in bioreactor experiment.

Kinetics of cell growth and interferon production in stirred tank bioreactor under controlled pH condition

Based on the results of shake flasks, cultivations were conducted in 16-L stirred tank bioreactor to investigate the scalability of this process. Inoculum size and medium composition were the same as in shake flask culture. As show in figure 3, cells grew exponentially with growth rate of 0.31 g/L/h reaching a maximal cell mass of about 1.9 g/L after 6 h. During that time, dissolved oxygen decreased gradually as a function of cell growth but was not limited during the all stages of cultivations. During the exponential growth phase, glucose consumed in culture with rate of 3.25 g/L/h and was limited in culture after 7 h cultivation. For recombinant protein production, interferon induction was first induced in culture after 2 h by increasing the cultivation temperature from 37 °C to 42 °C. After this temperature shift, interferon produced in culture with rate of about 10.5 mg/L/h and reached a maximal value of 44.5 mg/L after only 6 hours.

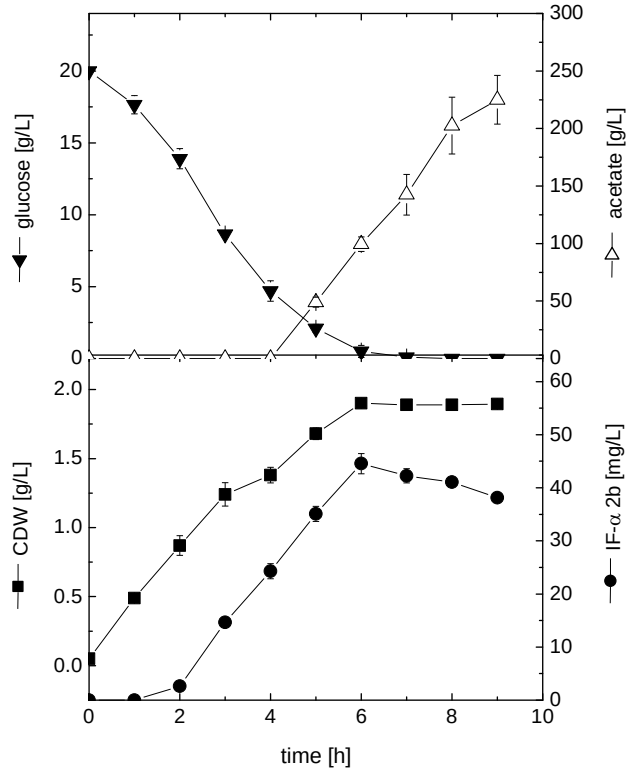

Fig. 3: Changes in cell growth, interferon α2b production, glucose and acetate concentration during cultivation of r*E.coli* in 16-L stirred tank bioreactor (values are mean ± standard deviation, n=2).

The significant increase in the interferon production rate in case of bioreactor culture was due to the better mixing (aeration/agitation) compared to shake flask. In addition, the control of induction temperature is more easy and accurate in bioreactor compared to shake flask. These together results in the increase in metabolic activities and recombinant protein expression in case *E. coli*. However, table 1 shows clear comparison between the kinetics of cell growth, substrate consumption, acetate production, and interferon production for better understanding of the improvement done in this study by process transfer from shake flask to bioreactor.

Table. 1: Different growth and production parameters during interferon production by r*E.coli* in shake flask and bioreactor cultures in production medium supplemented with 20 g/L glucose.

	X_{max} [g/L]	dx/dt [g/L/h]	μ [1/h]	ds/dt [g/L/h]	P_{max} [mg/L]	dp/dt [mg/L/h]	$Y_{p/x}$ [mg/g]	Act_{max} [mg/L]	dAc/dt [mg/L.h]
SF	1.7	0.31	0.39	2.31	27.7	5.54	16.3	195	63.5
BR	1.9	0.31	0.31	3.33	44.5	10.48	23.4	225	45.0

X_{max}, maximal cell growth; dx/dt, growth rate; μ, specific growth rate; ds/dt, glucose consumption rate; P_{max}, maximal volumetric interferon production; $Y_{p/x}$, specific interferon production; Act_{max}, maximal acetate concentration; dAc/dt, acetate production rate.

Thus, it is clearly observed that, bioprocess transfer from shake flask to bioreactor increased the volumetric interferon production by about 61%. However, not only the volumetric production increased but also the specific interferon production $Y_{p/x}$ was increased by about 44%. Thus, we can conclude that the observed increase in interferon production in bioreactor is not only due to the increase in biomass but mainly due to the increase in cell performance. This was also indicated by higher glucose consumption rate in bioreactor which was higher by about 44% compared to shake flask cultures. On the other hand, the rate of acetate production was less in bioreactor compared to shake flask by about almost about 41%.

CONCLUSION

Considering the results achieved, we can conclude that the initial glucose concentration in culture medium have significant influence on the cell growth and expression of recombinant protein in *E. coli* understudy. The maximal interferon production was achieved in 20 g/L glucose supplemented culture. In addition, cultivation in semi-industrial scale 16-L bioreactor showed not only significant improvement in growth and production kinetics but also decrease the production of the undesired metabolites such as acetate. Further studies on bioprocess improvement using different fed-batch cultivation strategies to increase interferon production in bioreactor level are under study in our laboratories.

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