Evaluation of heat induction strategy for recombinant human growth hormone expression in fed-batch fermentation

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Abstract
The high cell density cultivation (HCDC) of a recombinant E. coli producing human growth hormone (hGH) under different heat induction strategies has been reported. In this paper the effect of heat shock temperature, its duration and post-induction temperature, at two levels on cell growth and death, hGH production and its degradation, substrate utilization and by-product formation were investigated by full factorial experimental design. The results showed that heat shock temperature was the most effective factor on hGH production having approximately 75.8% of total contribution. There was no significant accumulation of substrate or by-product in culture medium during HCDC in all experiments. Thirty two percent of cells were subjected to lysis during the heat induction at 42°C for 20 min followed by 37°C for 4h. Biologically active recombinant hGH was produced comprising 13% of total cell protein without degradation. An empirical equation was employed to describe the relationship between three factors and hGH production during HCDC.
Keywords: *Heat induction; High cell density culture; Human growth hormone; Recombinant E. coli.*

INTRODUCTION

Escherichia coli (E. coli) is one of the most widely used hosts for the production of heterologous proteins because of its well characterized genetics and physiology as well as its ability to grow rapidly at high cell density cultures on inexpensive substrates (Marr, 1991; Arora and Khanna, 1996; Riesenberg and Guthke, 1999). However, there is no guarantee that a recombinant gene product will accumulate in *E. coli* at high level in full length and biologically active form. A considerable amount of effort has been directed to improve the performance and versatility of this workhorse microorganism (Mattanovich *et al*., 1998; Wang and Lee, 1998; Kim and Cha, 2003). The expression systems of *E. coli* consisted of plasmids and promoters have been reviewed (Makrides, 1996; Hannig and Makrides, 1998; Baneyx, 1999).

The strong, inducible promoter systems such as λP_L, λP_R, trp and T7, commonly used in recombinant *E. coli*, are advantageous for overproduction of recombinant proteins at high cell density fermentation (Arthur *et al.*, 1990; Yoon *et al.*, 1994; Shin *et al.*, 1997; Lim and Jung, 1998). High-level expression systems using the universal stress promoters *uspA* and *uspB* in a fed-batch cultivation based on minimal medium have also been recently designed (Prytz *et al.*, 2003). To achieve optimal productivity, the growth and production phases should be separated, so recombinant organisms with inducible promoters especially heat inducible ones which have no disturbance in downstream processes and no additional cost as chemicals are preferred (Seeger *et al.*, 1995 and Ryan *et al.*, 1996).

We have chosen a model system where the T7 RNA polymerase gene is under the control of λP_L promoter can be induced by heat shock. However, the appropri-

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ate conditions for optimum induction of the λP_L promoter are not clearly addressed in literature. Some authors suggest to increase temperature from 30 to 42°C for 2-15 min, then decrease to 37°C to induce transcription (Hui *et al.*, 1987 and Gupta *et al.*, 1999), while others recommend to increase the temperature to 37°C or 42°C for the remainder of the experiment after a suitable cell density has been achieved (Remaut *et al.*, 1981; Bauer *et al.*, 1990; Hoffmann and Rinas, 2000).

In previous study, we described the high cell density cultivation of a recombinant *E. coli* harboring two plasmids producing hGH under heat induction (Tabandeh *et al.*, 2004a). In this study, for the first time three major factors namely heat shock temperature, its duration and post-induction temperature as well as their interactions were optimized for the production of biologically active form of hGH by full factorial experimental design.

MATERIALS AND METHODS

Bacterial strain and recombinant plasmids: The host-plasmids system used for the production of recombinant hGH in this study named *E. coli* A6-5 consisting of the structural gene for hGH. The plasmids are the T7-based pET21 expression plasmid (pET21-*hgh*) and pGP1-2 (Tabor and Richardson, 1985) carrying the gene 1 of T7 phage under the control of heat inducible λP_L promoter (kan^+, T7 RNA $polymerase^+$), with *Escherichia coli* DH5-α as the host. The *hgh* gene was expressed by heat induction and the rhGH was accumulated as inclusion body in cytoplasm, as described previously (Tabandeh *et al.*, 2004a).

Media and cultivation: Stocks of the *E. coli* A6-5 was maintained at -20°C and -70°C in 15 and 30 percent glycerol. The inoculum for each bioreactor experiment was prepared by serial subculturing of recombinant cells taken from stock cultures using Luria-Bertani (LB) medium containing yeast extract, 5 g; tryptone, 10 g; NaCl, 10 g; ampicillin, 100 mg; and kanamycin, 30 mgl^{-1}. The complex medium consisted of (gl^{-1}) KH_2PO_4, 3; K_2HPO_4, 6; $(NH_4)_2SO_4$, 2; $MgSO_4.7H_2O$, 1; Thiamin-HCl, 0.02; and glucose, 20; yeast extract, 10 and tryptone 20. Two milliliter of trace metal solution containing the following per liter of 5 M HCl: $Na_2B_4O_7.10H_2O$, 0.02; $FeSO_4.10H_2O$, 10; $CaCl_2.2H_2O$, 2; $ZnSO_4.7H_2O$, 2.2; $MnSO_4.4H_2O$, 0.5; $CuSO_4.4H_2O$, 1; and $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.1 g was added to 1 liter of the medium. The feed solutions used for fed-batch studies had the following compositions (gl^{-1}): glucose, 500 and $MgSO_4.7H_2O$, 15 as feed 1 and yeast extract, 75 and tryptone, 150 as feed 2 which were precisely adjusted to pH 7.0.

Bioreactor operation: All batch and fed-batch bioreactor experiments were conducted in 3 liter Bioflo-3000 laboratory fermenter (New Brunswick Scientific, USA). Unless otherwise mentioned, the bioreactors were operated at pH 7.0 and 30°C. To avoid oxygen limitation, dissolved oxygen was maintained above 40% of air saturation by increasing in the agitation rate from 400 to 900 rpm, followed by the addition of air and pure oxygen mixture to 1 liter per min. The medium feed in the fed-batch cultures was controlled using the pH-stat and constant rate feeding based on the growth kinetics of the recombinant *E. coli* during batch cultivation (Tabandeh *et al.*, 2004b). In the pH-stat method, the feed 1 was automatically controlled by on-off mode depending on the culture pH. The feed 2 was added to the culture medium by a constant-rate feeding mode as 20 mlh^{-1}. In this dual feeding strategy, glucose concentration was maintained under inhibition limit and acetate formed in less than 2 gl^{-1}. On the other hand, complex feed which is necessary for over-expression of hGH (Tabandeh *et al.*, 2004a) was supplied by feed 2.

hGH purification: During heat induction, 40 ml of bacterial culture was taken and centrifuged (Sigma Lab Centrifuge 3K30, Germany) at 11,800 ×g for 10 min at 4°C. The pellet was suspended in lysis buffer consisting Tris-HCl, 12.11 g; EDTA, 37.24 g; Triton X-100, 10 ml per liter, pH 9 and sonicated (Labsonic L, B. Braun, USA) 7 times each for 30 Sec and centrifuged at 11,800 ×g for 30 min at 4°C. The pellet containing inclusion bodies were dissolved at pH 12.2 for 1h and then at pH 11.5 overnight. The solution was centrifuged at 75,000 ×g for 45 min at 4°C. The pH was adjusted to 8.0 at 4°C in order to obtain protein refolding. Human growth hormone purification was carried out by lowering the pH to 4.9, based on hGH isoelectric point, at which all contaminated proteins precipitate and biologically active monomers of hGH remains in the solution (Khodabandeh *et al.*, 2003).

Strategy for heat induction: Variables including heat shock temperature (T_S), its duration (t) and post-induction temperature (T_{in}), each at two levels were of interest in the following study. The full factorial design consisting of 2^3 experiments was considered to investigate all main effects and interactions of the factors. The experiments were duplicated to control the noise

factors. When the optical density at λ=550 nm of the cells reached to 80 during fixed-volume fed-batch fermentation, the temperature was shifted to T_S as 42 or 37°C for 20 or 40 min (t) and then decreased to 30 or 37°C (T_{in}) for 4h.

Analytical methods: The optical density of each culture sample was measured at 550 nm using a spectrophotometer (Beckman DU530, USA). The recombinant cell mass concentration was then obtained using a previously developed correlation between optical density and dry cell weight. Cell death was measured by two methods consisting colony counting on LB and DNA extraction of samples taken from the medium (Bylund *et al.*, 2000). Enzymatic test kits were applied to analyze the glucose (Chemenzyme, Iran) and acetate concentrations (Roche, Germany) during cultivation. Expression was determined by subjecting samples taken from the bioreactor to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). The resulting protein bands were scanned with a densitometer (Beckman model R-112) to estimate the rate of recombinant protein to total cell protein. The specificity of the rhGH and its degradation were determined by western blotting (Sambrook *et al.*, 2002). The purified hGH was measured by Protein Assay ESL kit (Roche, Germany) or Lowry method and confirmed by hGH ELISA kit (Roche, Germany). Biological activity of rhGH was compared to hGH standard by cell proliferation assay. The presence of endotoxin in purified samples was detected by LAL kit (Pyramid, USA). Plasmid stability was examined by counting the colonies grown on LB plates containing ampicillin, kanamycin or both by replica plating method.

All chemicals used were of analytical grade, obtained from Merck Co. Standard hGH was purchased from NOVO, Nordisk Co. Denmark.

All statistical calculations consisting of ANOVA, main effects, interactions as well as non-linear regression were performed with SPSS version 9.0, statistical software.

RESULTS

Design of experiments: The experiments were designed based on three major factors of heat induction each at two levels using full factorial design as shown in table 1. They were done randomly and repeated two times to decrease noise errors. Since T7 RNA polymerase expression is directly related to the time for which heat shock is given, it was decided to determine the optimum duration of heat shock. Firstly, cells were grown in shake flask at 30°C, thereby completely repressing the λP_L promoter in LB medium until the optical density at 550 nm reached 1.0. Heat shock was given at this point for different time durations in 1, 5, 10, 20, 30 and 40 min at 42°C (data not shown). The results indicated that *hgh* was expressed after 20 min and the protein degraded after 40 min at 42°C (Fig. 1). Therefore, heat shock duration at two

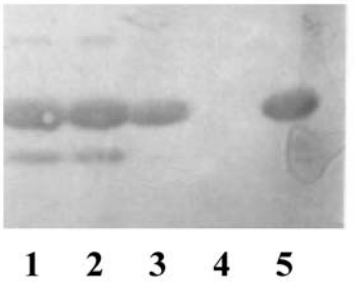

Figure 1. Western blotting of *E. coli* A6-5 expressing hGH under different heat induction duration. Lanes: 1, 40 min; 2, 30 min; 3, 20 min; 4, 5 min; *5*, hGH standard.

levels, 20 and 40 min, were considered.

Effect of heat induction on cell physiology: The cell growth and death under different induction strategies are presented in figure 2. The amount of DNA taken from samples in the medium was considered as an indicator of the cell lysis. The number of CFU's (colony forming units) proportional to the expected cells at that optical density in exponential phase of growth, considering 1 unit OD_{550} is about 8×10^8 cells, indicates the viable cells. The results indicated that the amount of DNA was high when the heat shock temperature was 42°C, especially when the cells remained at 37°C for 4h. The viable cell amounts confirmed the above results. It can be concluded that the higher heat shock temperatures, the more cells are lyzed.

Effect of heat induction on *hGH* expression: Since the heat shock response may have an influence on the aggregation, degradation or proper folding of the product (Bylund *et al.*, 2000), the effect of different heat induction strategies on hGH production (Fig. 3) and its aggregation or degradation (Fig. 4) were studied. The immunoblot analysis showed that there was no hGH degradation in all experiments even at 42°C for 40 min contrary to those results in shake flask. It may be due to detrimental situation for growth and hGH production in shake flask in comparison to fed batch culture in fermenter. As shown in table 2, the soluble form of hGH proportional to inclusion bodies were decreased

Table 1. Full factorial design of experiments for three factors of heat induction at two levels. The hGH production (gl^{-1}) has been shown as a response of the experiments.

Trial No	T_s (°C)	t (min)	T_{in} (°C)	hGH (g l^{-1})
1	42	20	30	0.92
2	42	40	30	1.20
3	42	20	37	1.89
4	42	40	37	2.10
5	37	20	30	0.29
6	37	40	30	0.35
7	37	20	37	0.37
8	37	40	37	0.37

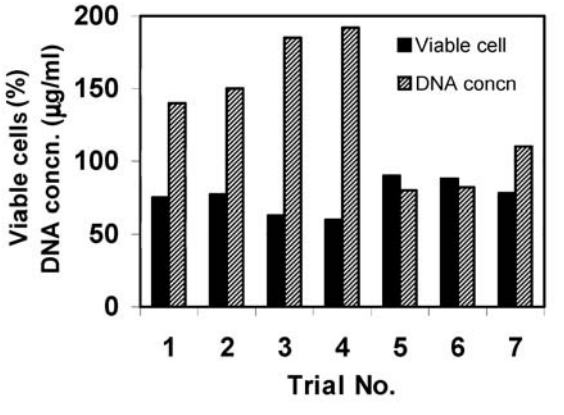

Figure 2. The viability (as cfu number) and lysis (as DNA concentration) of bacterial cells under different induction strategies. Trial Numbers are as described in table 1.

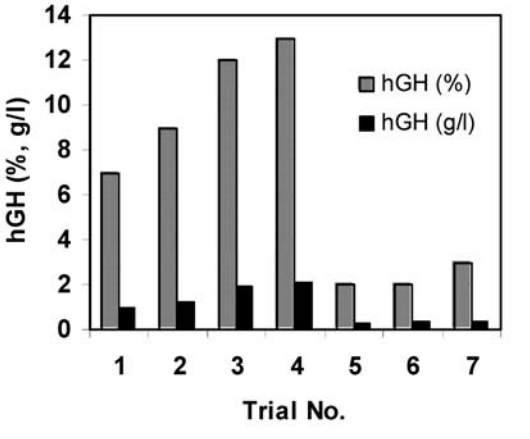

Figure 3. The production of recombinant hGH at different temperature up-shift according to two level full factorial design of experiments . The hGH production was measured as the hGH percentage of total cell protein as well as the amount of the purified recombinant protein as gl^{-1}.

by temperature up-shift. It seems that the small amount of hGH, which was produced at low induction temperature, has a chance for proper folding using protein machinery of the cell.

ANOVA and optimum conditions: The magnitude of interactions was measured by the extent of no parallelism of the effects. The severity of the interactions (SI %) was 26.28, 4.73, 4.40 for the two-factor interactions *viz.* $T_s \times T_{in}$, $T_{in} \times t$ and $T_s \times t$, respectively. Therefore, it was found that the interaction between T_s and T_{in} was of interest. The ANOVA for hGH production is shown in table 3. The sum of squares column indicates that factor T_s makes the largest contribution. It should be noted that percentage probability for every observation was 90. The variance ratio, denoted by F, is the ratio of the mean square due to a factor and the error mean square. A value of F larger than two means the factor effect is not quite small. Regarding to the percent of contribution of each factor shown in the final column, T_S is the most important factor in influencing the hGH production. Heat shock duration was not significant and should be ignored. The optimum condition was suggested as T_s, 42°C and T_{in}, 37°C.

Regression: According to the ANOVA table, three independent variables consisting of T_s, T_{in} and $T_s \times T_{in}$ were considered. The following non-linear model was suggested to correlate the variables:

$$P = B_0 + B_1 T_s + B_2 T_{in} + B_3 T_s \times T_{in}$$

where P is the hGH amount (gl^{-1}) as a dependent variable and B_0-B_3 are the constant parameters. The non-linear statistics and parameter estimations are summarized in table 4 where, R squared was 0.99988.

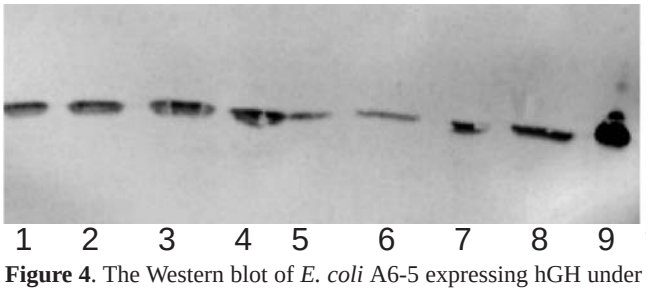

Figure 4. The Western blot of *E. coli* A6-5 expressing hGH under different heat induction strategies. The numbers (1-8) indicate the trial numbers and the last lane(9) shows the hGH standard.

Table 2. Substrate utilization, by-product formation, hGH folding, cell and product productivities at different temperature up-shift.

	1	2	3	4	5	6	7
Acetate (g l^{-1})	0.2	0.2	0.8	0.9	0.8	0.8	0.2
Glucose (g l^{-1})	0	0	0	0	0	0	0
hGH (sol/IB)	0.17	0.18	0.19	0.2	0.47	0.42	0.35
P_{cell} (g l^{-1} h^{-1}) [a]	1.55	1.52	1.61	1.64	1.52	1.53	1.69
P_{hGH} (g l^{-1} h^{-1}) [b]	0.033	0.043	0.065	0.075	0.01	0.012	0.013

[a] cell productivity is defined as the units of cell density (gl^{-1}) per unit time.
[b] product productivity is defined as units of product formed (gl^{-1}) per unit time.

Table 3. ANOVA of the experiments.

Factor	DOF	SS	Variance	F-ratio	Pure sum	Percent
T_s	1	8.544	8.544	2114.176	8.540	75.792
T_{in}	1	1.460	1.460	361.325	1.456	12.923
$T_s \times T_{in}$	1	1.179	1.179	291.795	1.175	10.429
t	1	0.007	0.007	1.818	0.003	0.029
Standard						0.827

DOF: degree of freedom, SS: sum of square.

DISCUSSION

In this study, the high cell density cultivation of *E. coli* A6-5 producing human growth hormone using the T7 - λP_L system under different heat induction strategies was studied. The plasmids were stable during HCDC of *E. coli* up to 90% and hGH was biologically active in all experiments (data not shown).

It is well established that the expression of the recombinant protein under the control of the T7 promoter is highest when the expression of the T7 RNA polymerase is suboptimal. This is primarily because the T7 RNA polymerase has a high transcriptional efficiency and only a small amount is required for high expression (Chamberlin *et al.*, 1970). Usually the rate-limiting step for protein synthesis does not lie at the transcriptional level of T7 based expression system. Higher levels of T7 RNA polymerase seem to be toxic to the cell (Tabor and Richardson, 1985) and do not serve useful purpose while diverting the essential metabolites away from recombinant protein production.

Acetate, a major by-product, which is formed during HCDC, has been reported to have a detrimental effect on recombinant cells and recombinant protein production (Lee, 1996). It is possible that acetate represses the synthesis of DNA, RNA, proteins and lipids (Hoffmann *et al.*, 2001). Acetate formation was kept under 1 gl^{-1} (Table 2) by pH-stat feeding strategy. Substrate feed, which maintained nutrient quality and also prevented acetate build up, helped increase the exponential phase of growth, thereby allowing heat induction at high cell density (40 gl^{-1}). This, in turn helped in achieving a final product concentration of 2 gl^{-1} of biologically active hGH.

Fed batch cultures are widely employed in bioprocesses to achieve high cell density concentrations, and improve volumetric productivity. Whereas, much efforts have been made to control and find feeding strategies for HCDC (Riesenberg and Schulz, 1991; Kleman and Strohl, 1994; Lee, 1996) There have been few articles covering microbial death in energy-limited cultures (Mason *et al.*, 1986 and Anderson *et al.*, 1996) especially during induction phase (Bylund *et al.*, 2000). The advantages of the temperature inducible expression systems are the strong promoter as well as the ease of induction by a temperature up-shift. However, the high induction temperature favors the formation of inclusion bodies (Hoffmann and Rinas, 2000). This aggregated form can stabilize the sensitive proteins against proteases (Strandberg and Enfors, 1991). Formation of inclusion bodies is also accounted as an advantage for purification purposes because the recombinant proteins are concentrated in this form. The mechanism behind inclusion body formation is not well understood. Early hypothesis includ solubility limitations, protein size, type of promoters and improper disulfide bond formation (Strandberg and Enfors, 1991). The temperature up-shift has also been reported as a parameter to promote the aggregation of recombinant protein as inclusion bodies (Strandberg and Enfors, 1991).

Table 4. Estimation of the non-linear equation parameters that regressed using SPSS 9.0.

Parameter	Estimate	Asymptotic std. error	Asymptotic 95% confidence interval Lower	Upper
B_0	21.512857143	0.571334229	19.926579021	23.099135265
B_1	-0.577857143	0.014435274	-0.617935889	-0.537778397
B_2	-0.897428571	0.016962424	-0.944523811	-0.850333332
B_3	0.024428571	0.000428571	0.023238668	0.025618475

Sum of square = 10.90505 , Mean square = 2.72626, R squared = 0.99988

To find the optimum strategy of heat induction for the production of hGH and prevention of cell death, three major factors each at two levels were considered and the experiments were designed by full factorial approach. The factorial design of experiments has been described as the most powerful technique in technological research due to its simplicity, independently estimation of the effect of each factor (main effect) and all interactions without confounding (Haaland, 1989).

The results showed that the heat shock duration (t) should be at least 20 min to start the hGH production. Although some authors have suggested sufficient heat shock duration, the suboptimal (but not maximal) expression of T7 RNA polymerase (about 2 min) is required for high recombinant protein expression (Hui *et al.*, 1987 and Gupta *et al.*, 1999), we found that maximal amount of T7 RNA polymerase should be expressed in this machinery. This may be due to the presence of the *lac* operator in pET21 which repress T7 promoter at high temperatures. As described, the cell death was slightly increased at the post-induction temperature of 37°C in comparison to 30°C. The recombinant hGH was intracellularly accumulated as inclusion bodies at higher temperatures. The higher amount of hGH was finally purified in the samples treated at higher T_s and T_{in}. The ANOVA table showed that the effect of heat shock duration greater than 20 min on hGH production is not significant. But the heat shock temperature is a key factor on the recombinant protein expression. The interaction of heat shock and post-induction temperatures affect on the hGH production was found to have the coefficient of about 0.02.

The proposed equation predicts the hGH is produced at the higher amount of T_S and T_{in}, but it should be noted that cell lysis is increased at higher temperatures and this in turn decreased the cell density and product productivity. In summary, it is suggested that the heat induction strategy of 42°C for 20 min followed by 37°C for 4h is sutiable for production of high levels of hGH in the recombinant *E. coli*.

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References

Anderson L, Strandberg L, Enfors S-O (1996). Cell segregation and lysis have profound effects on the growth of *Escherichia coli* in high cell density fed batch cultures. *Biotechnol Prog*. 12: 190-195.

Arora D, Khanna N (1996). Method for increasing the yield of properly folded recombinant human gamma interferon from inclusion bodies. *J Biotechnol*. 52: 127-133.

Arthur PM, Duckworth B, Seidman M (1990). High level expression of interleukin-1 beta in a recombinant *Escherichia coli* strain for use in a controlled bioreactor. *J Biotechnol*. 13: 29-46.

Baneyx F (1999). Recombinant protein expression in *Escherichia coli. Curr Opin Biotechnol.* 10: 411-421.

Bauer KA, Ben-Bassat A, Dawson M, De LaPuente VT, Neway JO (1990). Improved expression of human interleukin-2 in high cell density fermentor cultures of *E. coli* K12 by a phosphotransacetylase mutant. *Appl Environ Microbiol*. 56: 1296-1302.

Bylund F, Castan A, Mikkola R, Veide A, Larsson G (2000). Influence of scale-up on the quality of recombinant human growth hormone. *Biotechnol Bioeng*. 69: 119-128.

Chamberlin M, McGrath J, Waskell L (1970). New RNA polymerase from *Escherichia coli* infected with bacteriophage T7. *Nature*, 228: 277-237.

Gupta JC, Jaisani M, Pandey G, Mukherjee KJ (1999). Enhancing recombinant protein yields in *Escherichia coli* using T7 system under the control of heat-inducible λP_L promoter. *J Biotechnol*. 68: 125-134.

Haaland PD (1989). *Experimental design in biotechnology*. Marcel Deker Inc. USA.

Hannig G, Makrides SC (1998). Strategies for heterologous protein expression in *E. coli*. *Trends Biotechnol*. 16: 54-60.

Hoffmann F, Rinas U (2000). Kinetics of heat shock response and inclusion body formation during temperature-induced production of basic fibroblast growth factor in high cell density cultures of recombinant *E. coli*. *Biotechnol Prog*. 16: 1000-1007.

Hoffmann F, Posten C, Rinas U (2001). Kinetic model of *in vivo* folding and inclusion body formation in recombinant *Escherichia coli*. *Biotechnol Bioeng*. 72: 315-321.

Hui A, Jhurani P, de Boer HA (1987). Directing ribosomes to a single mRNA species: a method to study ribosomal RNA mutations and their effects on translation of a single messenger in *Escherichia coli*. *Methods Enzymol*. 153: 432-452.

Khodabandeh M, Yakhchali B, Rahimi M, Vaseli N, Moghaddasi Jahromi Z, Zomorrodipour A, Deezagi A, Ansari Majd S, Sanati MH (2003). Purification of large quantities of biologically active recombinant human growth hormone. *Iranian J Biotechnol.* 1: 207-212.

Kim JYH, Cha HJ (2003). Down-regulation of acetate pathway through antisense strategy in *Escherichia coli*: improved foreign protein production. *Biotechnol Bioeng*. 83: 841-853.

Kleman GL, Strohl WR (1994). Developments in high cell density and high productivity microbial fermentation. *Curr Opin Biotechnol*. 5: 180-186.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680-685.

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

Lim HK, Jung KH (1998). Improvement of heterologous protein productivity by controlling postinduction specific growth rate in recombinant *Escherichia coli* under control of the P_L promoter. *Biotechnol Prog.*14: 548-553.

Makrides SC (1996). Strategies for achieving high level expression of genes in *E. coli*. *Microbiol Rev*. 60: 512-538.

Marr AG (1991). Growth rate of *Escherichia coli*. *Microbiol Rev*. 55: 316-333.

Mason CA, Hamer G, Bryers JD (1986). The death and lysis of microorganisms in environmental processes. *FEMS Microbiol Rev*. 39: 373-401.

Mattanovich D, Kramer W, Luttich C, Weik R, Bayer K, Katinger H (1998). Rational design of an improved induction scheme for recombinant *Escherichia coli*. *Biotechnol Bioeng.* 58: 296-298.

Prytz I, Sanden AM, Nystrom T, Farewell A, Wahlstrom A, Forberg C, Pragai Z, Barer M, Harwood C, Larsson G (2003). Fed batch production of recombinant β-galactosidase using the universal stress promoters *uspA* and *uspB* in high cell density cultivation. *Biotechnol Bioeng.* 83: 596-603.

Remaut E, Stanssens P, Fritsch W (1981). Plasmid vectors for high-efficiency expression controlled by the P_L promoter of coliphage lambda. *Gene*, 15: 59-76.

Riesenberg D, Schulz V, Knorre WA, Pohl HD, Korz D, Sanders EA, Ross A, Deckwer WD (1991). High cell density cultivation of *Escherichia coli* at controlled specific growth rate. *J Biotechnol.* 20: 17-28.

Riesenberg D, Guthke R (1999). High cell density cultivation of microorganisms. *Appl Microbiol Biotechnol*. 51: 422-430.

Ryan W, Collier P, Loredo L, Pope J, Sachdev R (1996). Growth kinetics of *Escherichia coli* and expression of a recombinant protein and its isoforms under heat shock conditions. *Biotechnol Prog.* 12: 596-601.

Sambrook J, Fritsch EJ, Maniatis T (2002). *Molecular cloning, a laboratory manual*. Cold Spring, Harbor Laboratory Press.

Seeger A, Schneppe B, McCarthy JEG, Deckwer WD, Rino U (1995). Comparison of temperature and IPTG-induced synthesis of basic fibroblast growth factor in high-cell-density cultures of recombinant *Escherichia coli*. *Enzyme Microbiol Technol.* 17: 947-953.

Shin CS, Hong MS, Bae CS, Lee J (1997). Enhanced production of human mini-proinsulin in fed-batch culture at high cell density of *Escherichia coli* BL21 (DE3) [pET-3aT2M2]. *Biotechnol Prog.* 13: 249-257.

Strandberg L, Enfors S-O (1991). Factors influencing inclusion body formation in the production of a fused protein in *Escherichia coli*. *Appl Environ Microbiol*. 57: 1669-1674.

Tabandeh F, Shojaosadati SA, Zomorodipour A, Khodabandeh M, Sanati MH Yakhchali B (2004a). Heat induced production of human growth hormone by high cell density cultivation of recombinant *Escherichia coli*. *Biotechnol Lett*. 26: 245-250.

Tabandeh F, Yakhchali B, Shojaosadati SA, Khodabandeh M, Sanati MH (2004b). Growth kinetics and human growth hormone production of a heat inducible recombinant *Escherichia coli* during batch fermentation. *Iranian J Sci Technol*. 28(A1): 11-17.

Tabor S, Richardson CC (1985). A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc Natl Acad Sci.* USA 82: 1074-1078.

Wang F, Lee AY (1998). High cell density culture of metabolically engineered *Escherichia coli* for the production of poly (3-hydroxybutyrate) in a defined medium. *Biotechnol Bioeng*. 58: 325-328.

Yoon SK, Kang WK, Park TH (1994). Fed-batch operation of recombinant *Escherichia coli* containing trp promoter with controlled specific growth rate. *Biotechnol Bioeng.* 43: 995-999.