High purity recombinant human growth hormone (rhGH) expression in *Escherichia coli* under *phoA* promoter

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**ABSTRACT**

Recombinant human Growth Hormone (rhGH) is an important protein for human growth and is in high demand in clinics. Hence, it is necessary to develop an efficient fermentation process to produce highly pure rhGH. In this study, rhGH was expressed in *Escherichia coli* under alkaline phosphatase (*phoA*) promoter. The cultivation conditions for high expression level and purity of rhGH were investigated. The best initial phosphate concentration for rhGH expression, out of the 4 levels of initial phosphate concentration tests performed, was 12.6 mmol/L. Subsequently, 2 fed-batch cultivations under low dissolved oxygen (DO) (0%–10%) and high DO (20%–30%) conditions were carried out. High purity rhGH (92%) was obtained from 20%–30% DO-stat cultivation, although the biomass did not show any significant difference. In summary, this research provided an efficient fermentation process for high purity rhGH production from *E. coli* under *phoA* promoter, which can lower the production and purification costs for large-scale production of rhGH.

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**Introduction**

Human Growth Hormone (hGH) is a 22 kDa endogenous and non-glycosylated protein consisting of 191 amino acids and is synthesized by somatotropic cells of anterior pituitary. hGH not only plays an important role in a baby’s growth, but also in the treatment of dwarfism, obesity, wound healing, and burn injury. hGH is in high demand because it is a safe pharmaceutical protein even at high doses. Genetic engineering provides an exciting approach for heterologous protein production from microbes, especially *Escherichia coli*, to overcome the lack of hGH resources. In the past years, significant progress has been made in recombinant hGH (rhGH) production. Zamani et al. optimized the cultivation medium of recombinant *E. coli* through response surface methodology, giving hGH yield of 391 mg/L. However, hGH was expressed as inclusion body in *E. coli*, which was a major hurdle. Nevertheless, optimization of solubilization of rhGH inclusion body from *E. coli* was reported by Patra et al. Heterologous protein in periplasmic space benefits from protein folding, which makes it convenient to purify rhGH. The inclusion body solubilization is unnecessary when heterologous protein is secreted in soluble form. rhGH was secreted into periplasmic space in *E. coli* for easy purification, giving a final yield of 1.4 mg/L. Recently, an efficient *E. coli* system with soluble expression of rhGH fused with thioredoxin and a final yield of 511.2 mg/L was reported by Levarski et al. Another flaw in rhGH production is the presence of impurities, such as the deamidate forms of rhGH, which seriously affect the activity of rhGH. The deamidate forms of rhGH (Asn149 to Asp149 and Asn152 to Asp152), also known as rhGH variants, are major by-products. Furthermore, separation of rhGH deamidation variants from active rhGH is difficult and costly due to their similar structures. Hence, high purity rhGH production by fermentation optimization was necessary and urgent to reduce cost of rhGH production. Fed-batch is widely used in lab-scale protein production. Fed-batch offers the following advantages: 1) feeding rate is a good way to control the microorganism’s growth according to its physiology, such as to reach a high cell density, 2)
fermentation optimization is an important step in preventing by-product formation and inhibition of substrate-associated growth, and 3) productive phase is extendable under controlled conditions, giving high yield. The parameters of fed-batch involved substrate level, temperature, and pH, and these parameters are affected by each other. During fermentation process, dissolved oxygen (DO) was another important parameter in bioreactor operation. The final objective was to keep the DO level above the critical limit, where the product quality was not affected. The DO level significantly affects the formation rate and variability of the product, for example, oxygen-enriched fermentation benefits rhGH expression in E. coli and does not lead to acetate accumulation. The heterologous protein expression system, using alkaline phosphatase (phoA) promoter, was an attractive system owing to its easy control and use of non-toxic chemicals during the induction process. The phoA promoter is inhibited by excess phosphate and is induced on phosphate starvation. Moreover, phosphate promotes assimilation of glucose by microorganisms and stimulates their growth. In this study, the yield and quality of rhGH were enhanced significantly through DO value control. In addition, minimization of rhGH variants was achieved.

**Materials and methods**

**Materials**

*E. coli* W3110 strain was used in this research. The expression plasmid pCS/hGH harbored tetracycline resistance gene and hGH gene, which was under the control of phoA promoter.

Luria-Bertani (LB) medium: 10.0 g/L tryptone, 5.0 g/L yeast extract, and pH adjusted to 7.0 using 1 mol/L NaOH solution.

Fermentation medium: 9.0 g/L tryptone, 1.8 g/L yeast extract (Angel Yeast, China), 0.75 g/L glucose, 2.65 g/L KH₂PO₄·3H₂O, 1.0 g/L NaH₂PO₄·2H₂O, 4.375 g/L (NH₄)₂SO₄, 2.7 g/L MgSO₄·7H₂O, 1.3 g/L KCl, 0.25 g/L Na₂C₆H₅O₇·2H₂O, 0.35 g/L L-isoleucine, and 0.5 mL/L trace element solution. Trace element solution contained 54.0 g/L FeCl₃·6H₂O, 7.0 g/L CoCl₂·6H₂O, 2.0 g/L H₃BO₃, 8.0 g/L ZnSO₄·7H₂O, 8.0 g/L CuSO₄·5H₂O, 5.0 g/L MnSO₄·H₂O, and 80.0 mmol/L HCl.

Feeding solution: 600.0 g/L glucose, 58.5 g/L Hycase and 19.5 g/L Amicase (Sigma, USA), and 10.8 g/L yeast extract.

LB medium was supplemented with tetracycline to a final concentration of 100 mg/L. Sterilized phosphate solution was added to LB medium before inoculation. All media were sterilized at 115°C for 20 min.

**Methods**

**Initial phosphate concentration for high rhGH expression**

The recombinant *E. coli* W3110 was preserved in 25% glycerol solution. First, 100 μL of 25% glycerol solution with recombinant *E. coli* W3110 was inoculated in 3.0 mL LB medium and incubated at 37°C and 200 rpm for 8 h in a rotary shaker. Following this, the 3.0 mL LB cultivation broth was transferred to a 250 mL flask with 60 mL LB medium, which was incubated at 37°C and 200 rpm for 8 h in a rotary shaker, and the cell density of the cultivation broth was assessed from absorbance at 600 nm (OD₆₀₀= 2.0). The 60 mL LB cultivation broth was transferred into a 30 L bioreactor (Bioengineering AG, Wald, Switzerland) for rhGH expression using fed-batch cultivation. The bioreactor was equipped with a DO electrode and a pH electrode. Fed-batch culture was carried out on 20 L fermentation medium with various initial phosphate concentrations, which were 10.8, 12.6, 14.4, and 18.0 mmol/L. DO level was kept above 20% by adjusting airflow and agitation. Maximum air flow and agitation rates were 1.2vvm and 1,000 rpm, respectively. A rapid increase in pH indicated that glucose was used up (about 4 h). Feeding solution was added when the pH was more than 6.9. The pH was maintained at 7.0, using 25% (w/v) ammonia solution.

**DO-stat fermentation strategy for enhancement of rhGH yield and purity**

*E. coli* W3110 grew in the fermentation medium at 37°C in all fermentation processes, and the DO value decreased as the fermentation process went on. The feeding solution was fed when DO increased sharply, as the initial glucose was used up. The glucose feeding rate increased with increase in *E. coli* W3110 growth, according to the exponentially-fed-batch culture model. With the growth of *E. coli* and the addition of feeding solution, the DO level decreased gradually. Two DO values were maintained after DO decreased to particular levels. The first DO value was as low as 0% - 10%, and the second DO value was as high as
20% – 30%. The feeding solution was added when the pH was more than 6.9. The pH was adjusted manually at 10 min intervals and was maintained at 7.0, using 25% (w/v) ammonia solution.

**Analytical procedures**

Cell density was monitored by measuring OD\textsubscript{600}. Dry cell weight (DCW) was obtained from the relationship between OD\textsubscript{600} and DCW. rhGH was extracted using the freeze-thaw method twice. The cells were first frozen at \(-20^\circ\text{C}\) overnight and then thawed at 8°C for 8 h. These two steps were repeated twice to break the cell wall. The expression level of rhGH was measured by 12.6% SDS-PAGE quantification, and its purity was assessed by high-performance liquid chromatography (HPLC, HP1100, Agilent, Santa Clara, CA USA), with a reverse C8 column (Zorbax 300 SB-C8 column, 4.6 × 250 mm, 5 μm) and a UV detector at 280 nm. The gradient elution was performed with a mixture of acetonitrile and 0.1% trifluoroacetic acid solution, at 1.0 mL/min. The ratio of acetonitrile to 0.1% trifluoroacetic acid solution was gradually changed from 5:95 to 30:70 in 30 min and then maintained at this ratio to the end. Phosphate level was analyzed using the molybdenum blue colorimetric method.\textsuperscript{22}

**Results and discussion**

**Initial phosphate concentration for rhGH expression in E. coli W3110**

The phosphate level required to trigger phoA promoter was reported to be as low as 0.05 mmol/L, in defined medium. However, this low level of phosphate retards microbial growth.\textsuperscript{21} In complex medium, the residual phosphate level was reported as 0.32 mmol/L, for heterologous protein expression under phoA promoter.\textsuperscript{23} Therefore, residual phosphate levels are different for different cultivation media. Phosphate concentration affects both rhGH expression and E. coli W3110 growth. With the increase in initial phosphate concentration, DCW increased gradually, whereas rhGH expression level showed a bell-shape curve. rhGH expression level reached 365 mg/L for 12.6 mmol/L initial phosphate concentration (Fig. 1). At 18.0 mmol/L initial phosphate concentration, rhGH expression level was 220 mg/L, although DCW was 13.73 g/L because of high residual phosphate concentration (Fig. 2). The residual phosphate levels, during the E. coli W3110 growth phase, decreased significantly to 0.35 mmol/L when initial phosphate level was lower than 14.4 mmol/L. At 18.0 mmol/L of initial phosphate level, the rhGH expression level was lowest and did not increase significantly even after increase in cultivation time to 24 h (data not shown). These data confirmed that E. coli W3110 growth and the rhGH expression contradicted each other because of the phoA promoter. Therefore, it was better to separate these 2 phases of rhGH production. The first phase was E. coli W3110 growth in the presence of sufficient phosphate to obtain high cell density. The second phase was rhGH expression, where phosphate was depleted to induce phoA promoter.

**Enhancement of rhGH expression by DO-stat fermentation process**

Figure 3A shows the DO values maintained in this research. At first, the DO values decreased as the
fermentation process progressed. After 8 h of cultivation, the DO value of high DO-stat fed-batch was maintained between 20% – 30%. Low DO value was maintained between 0% – 10%, after 12 h of cultivation. The glucose feeding profiles are shown in Fig. 3B. In low DO-stat fed-batch, glucose feeding rate increased as fermentation progressed, until the rate reached 126.65 g/h at 12 h. On the other hand, glucose feeding rate increased to 105.65 g/h at 10 h and decreased to 72.0 g/h at 14 h, in high DO-stat fed-batch. Figure 3C shows the profiles of E. coli W3110 growth under the 2 fed-batch modes. Overall, these 2 fed-batch growth curves showed the same profile during the whole cultivation process. The DCW of these 2 cultivation processes reached similar values (10.23 g/L in low

![Figure 3. Fermentation process of E. coli W3110 (pCS/GH). (A) DO curves, (B) glucose feeding rate, (C) DCW profiles, (D) phosphate concentrations.](image)

![Figure 4. Expression level of rhGH during fermentation process. Low DO-stat fed-batch (A), High DO-stat fed-batch (B).](image)
DO-stat fed-batch and 10.35 g/L in high DO-stat fed-batch) at 14 h. However, DCW of low DO-stat fed-batch before 14 h of cultivation was lower than that of high DO-stat fed-batch, whereas after 14 h of cultivation it became higher than that of high DO-stat fed-batch. For phosphate concentration during the process, the 2 modes showed similar curves, although lower phosphate concentration was observed in low DO-stat fed-batch from 6 h to 14 h of cultivation, and the maximum difference reached was 2.64 mmol/L, between the 2 modes. During these 2 fermentation processes, the phosphate concentrations were higher than 0.5 mmol/L and the rhGH was expressed as early as 8 h of cultivation (Fig. 4A). During the low DO-stat fed-batch, the expression level of rhGH increased significantly at 12 h. The highest expression level of rhGH was achieved at 14 h and was kept stable thereafter. Additionally, cells lysed at 20 h, because of low level of DO in low DO-stat fed-batch cultivation. Finally, the expression level of rhGH reached 584 mg/L. During high DO-stat fed-batch, the expression level of rhGH increased gradually from 8 h to 20 h of cultivation (Fig. 4B), giving a yield of 678 mg/L.

### Analysis of rhGH expression during the 2 fed-batch processes

The total expression levels of rhGH were 584 mg/L and 678 mg/L in low DO-stat and high DO-stat fed-batch fermentations, respectively. However, the significant difference between these 2 cultivations was the purity of rhGH, as shown in Table 1. The purities of rhGH were 27% and 92% in low DO-stat and high DO-stat fed-batch fermentations, respectively (Fig. 5). Therefore, the active rhGH obtained were 157.7 mg/L and 623.8 mg/L. The 92% active rhGH was the highest value obtained through fermentation optimization. Only 8% rhGH deamidation variants occurred. Total ammonia required for low DO-stat fed-batch was 625 mL, and it was 1.36 folds more than that used for high DO-stat fed-batch. The high ammonia demand in the low DO-stat fed-batch process was due to acetate production by *E. coli* W3110. High DO value fed-batch helped produce high purity rhGH, which provides an important advantage to purification process.

| fermentation     | Yield of rhGH (mg/L) | Purity of active rhGH (%) | Yield of active rhGH (mg/L) | Ammonia demanded (mL) |
|------------------|-----------------------|---------------------------|------------------------------|-----------------------|
| Low DO-stat      | 584                   | 27                        | 157.7                        | 625                   |
| High DO-stat     | 678                   | 92                        | 623.8                        | 458                   |

**Table 1.** Yield and purity of rhGH from 2 fed-batches.

![Figure 5. RP-HPLC analysis of rhGH.](image-url)
High cell density cultivation is important to improve the yield of recombinant protein. Additionally, high DO value of fermentation ensured proper nutrient feeding of cells in the fed-batch process. It not only affects the maximum cell concentration, but also the protein productivity. For *E. coli* with *phoA* promoter, cell density and protein expression level contradicted each other. Hence, the maximum rhGH productivity was often achieved when the growth and production phases were separated.

**Conclusion**

An efficient fermentation process for production of high purity rhGH from *E. coli*, under *phoA* promoter, was reported. This fermentation process was divided into 2 phases: the first stage was cell growth where phosphate was sufficient to ensure high cell density and the second stage was protein expression where phosphate was depleted to induce protein expression with 20% – 30% DO value. Using this 2-stage fermentation approach, both high cell density (10.78 g/L DCW) and high expression level of rhGH (678 mg/L) were obtained. Additionally, up to 92% pure rhGH was obtained, which greatly benefitted the purification process. The quality of rhGH was significantly affected by the DO level, based on this research. High DO level supported low deamidation of rhGH, which led to high quality rhGH from the *E. coli* W3110. High quality of rhGH production provides a possibility for high rhGH productivity, which can provide a bright future for rhGH production.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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**References**

[1] Lewis U, Sinha Y, Haro L. Variant forms and fragments of human growth hormone in serum. Acta Paediatrica 1994; 83:29-31; http://dx.doi.org/10.1111/j.1651-2227.1994.tb13282.x

[2] Isaksson OG, Edén S, Jansson JO. Mode of action of pituitary growth hormone on target cells. Ann Rev Physiol 1985; 47:483-99; http://dx.doi.org/10.1146/annurev.ph.47.030185.002411

[3] Van LK. Safety of high doses of recombinant human growth hormone. Hormone Res 1998; 49:78-81; http://dx.doi.org/10.1159/000053092

[4] Weng S, Zhou L, Han L, Yuan Y. Expression and purification of non-tagged recombinant mouse SPP1 in *E. coli* and its biological significance. Bioengineered 2015; 5:405-8; http://dx.doi.org/10.4161/bioe.34424

[5] Chen R. Bacterial expression systems for recombinant protein production: *E. coli* and beyond. Biotechnol Adv 2012; 30:1102-7; PMID:21968145; http://dx.doi.org/10.1016/j.biotechadv.2011.09.013

[6] Olson KC, Fenno J, Lin N, Harkins RN, Snider C, Kohr WH, Ross MJ, Fodge D, Prender G, Stebbing N. Purified human growth hormone from *E. coli* is biologically active. Nature 1981; 293:408-11; PMID:7024824; http://dx.doi.org/10.1038/293408a0

[7] Zamani M, Berenjian A, Hemmati S, Nezafat N, Ghoshoo M, Dabbagh F, Mohkam M, Ghasemi Y. Cloning, expression, and purification of a synthetic human growth hormone in *Escherichia coli* using response surface methodology. Mol Biotechnol 2014; 57:241-50; http://dx.doi.org/10.1007/s12033-014-9818-1

[8] Patra AK, Mukhopadhyay R, Mukhija R, Krishnan A, Garg LC, Panda AK. Optimization of inclusion body solubilization and renaturation of recombinant human growth hormone from *Escherichia coli*. Protein Exp Purification 2000; 18:182-92; PMID:10686149; http://dx.doi.org/10.1006/prep.1999.1179

[9] Missiakas D, Raina S. Protein folding in the bacterial periplasm. J Bacteriol 1997; 179:2465-71; PMID:9098040

[10] Sockolosky J, Szoka F. Periplasmic production via the pET expression system of soluble, bioactive human growth hormone. Protein Exp Purification 2013; 87:129-35; http://dx.doi.org/10.1016/j.jep.2012.11.002

[11] Levarski Z, Soltýsová A, Krahulec J, Stuchlík S, Turňa J. High-level expression and purification of recombinant human growth hormone produced in soluble form in *Escherichia coli*. Protein Exp Purification 2014; 100:40-7; http://dx.doi.org/10.1016/j.jep.2014.05.003

[12] Bischoff R, Kolbe HV. Deamidation of asparagine and glutamine residues in proteins and peptides: structural determinants and analytical methodology. J Chromatography B: Biomedical Sci App 1994; 662:261-78; http://dx.doi.org/10.1016/0378-4347(94)00203-7

[13] Gellerfors P, Pavlu B, Axelsson K, NyhlÉN C, Johansson S. Separation and identification of growth hormone variants with high performance liquid chromatography techniques. Acta Paediatrica 1990; 79:93-100; http://dx.doi.org/10.1111/j.1651-2227.1990.tb11682.x

[14] Lin P, Xiao Y, Li C, Cai Y, Yun C, Yang H, Yang J, Jian J, Li H. Enhanced recombinant factor VII expression in Chinese hamster ovary cells by optimizing signal peptides and fed-batch medium. Bioengineered 2016; 7(3):189-97:00-00 (in press).
[15] Akesson M, Hagander P, Axelsson J. Avoiding acetate accumulation in *Escherichia coli* cultures using feedback control of glucose feeding. Biotechnol Bioengineering 2001; 73:223-30; PMID:11257604

[16] Lee J, Lee S, Park S, Middelberg A. Control of fed-batch fermentations. Biotechnol Adv 1999; 17:29-48; PMID:14538142; http://dx.doi.org/10.1016/S0734-9750 (98)00015-9

[17] Bhunia B, Basak B, Bhattacharya P, Dey A. Process engineering studies to investigate the effect of temperature and pH on kinetic parameters of alkaline protease production. J Biosci Bioengineering 2013; 115:86-9; http://dx.doi.org/10.1016/j.jbiosc.2012.08.003

[18] Farrell P, Sun J, Champagne PP, Lau H, Gao M, Sun H, Zeiser A, D’Amore T. The use of dissolved oxygen-controlled, fed-batch aerobic cultivation for recombinant protein subunit vaccine manufacturing. Vaccine 2015; 33:6752-6; PMID:26518402; http://dx.doi.org/10.1016/j.vaccine.2015.10.071

[19] Castan A, Näsman A, Enfors SO. Oxygen enriched air supply in *Escherichia coli* processes: production of biomass and recombinant human growth hormone. Enzyme Microbial Technol 2002; 30:847-54; http://dx.doi.org/10.1016/S0141-0229(01)00490-2

[20] Phue JN, Shiloach J. Impact of dissolved oxygen concentration on acetate accumulation and physiology of *E. coli* BL21, evaluating transcription levels of key genes at different dissolved oxygen conditions. Metab Engineering 2005; 7:353-63; PMID:16099189; http://dx.doi.org/10.1016/j.ymene.2005.06.003

[21] Lübke C, Boidol W, Petri T. Analysis and optimization of recombinant protein production in *Escherichia coli* using the inducible phoA promoter of the *E. coli* alkaline phosphatase. Enzyme Microbial Technol 1995; 17:923-8; http://dx.doi.org/10.1016/0141-0229(94)00130-J

[22] Heimann AC, Jakobsen R. Filtration through nylon membranes negatively affects analysis of arsenic and phosphate by the molybdenum blue method. Talanta 2007; 72:839-41; PMID:19071695; http://dx.doi.org/10.1016/j.talanta.2006.11.012

[23] Wang Y, Ding H, Du P, Gan R, Ye Q. Production of phoA promoter-controlled human epidermal growth factor in fed-batch cultures of *Escherichia coli* YK537 (pAET-8). Process Biochem 2005; 40:3068-74; http://dx.doi.org/10.1016/j.procbio.2005.03.010

[24] Lee S. High cell-density culture of *Escherichia coli*. Trends Biotechnol 1996; 14:98-105; PMID:8867291; http://dx.doi.org/10.1016/0167-7799(96)80930-9

[25] Choi J, Keum K, Lee S. Production of recombinant proteins by high cell density culture of *Escherichia coli*. Chem Engineering Sci 2006; 61:876-85; http://dx.doi.org/10.1016/j.ces.2005.03.031