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Process optimization for human growth hormone biosynthesis by recombinant *Escherichia coli*

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ABSTRACT

Recombinant human growth hormone (r-hGH) can be produced by recombinant *Escherichia coli* a main host for recombinant protein productions. In order to improve the productivity of r-hGH, an orthogonal ($L_{16}(4^3\times2^2)$) experiment was applied to optimize the best culture conditions for r-hGH production by flask cultures of *E. coli* BL (21) harboring a new constructed plasmid (pEHUb-hGH). Based on the results of primary tests, five factors such as culture medium, culture volume, induction starting time, r-hGH expression time, and IPTG concentration were chosen for this present investigation as the main factors to influence the r-hGH expression. The optimum culture conditions were determined as follows: culture medium as the modified TB, culture volume 50 mL, induction starting time 3 h, r-hGH expression time 7 h, and IPTG 0.3 mmol/L. Under this optimized conditions, the r-hGH productivity could reached 18.72 mg/L·h in flask culture. A high r-hGH concentration of 4.3 g/L, resulting in a high productivity of 215 mg/ L·h, could be obtained in the fed-batch culture of recombinant *E. coli*.

KEYWORDS

Human growth hormone;
Recombinant *E. coli*;
Orthogonal experiment;
Process optimization.

INTRODUCTION

Human growth hormone (hGH) is known to be critical for tissue repair, muscle growth, bone strength, brain function, physical and mental health, energy, and metabolism. Recombinant human growth hormone (r-hGH) is identical to hGH, but synthesized from recombinant DNA technology. Although it has been reported that the r-hGH could be expressed correctly in seeds of transgenic tobacco plants[10], animal cells[7], *E. coli*[9,13,14], *lactoccus lactis*[11], and yeasts such as *S. cerevisiae*[6] and *P. pastoris*[3] as a secretary product, recombinant *E. coli* expression systems were the most effective system. The r-hGH can be expressed in a soluble form with activity or inclusion bodies without activity, which is mainly dependent on protein expression systems. Generally, the host, vector, and culture conditions are the three major factors influencing protein expression. Once a recombinant strain was constructed, the subsequent step is usually to optimize culture conditions for getting a high protein concentration or productivity.

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In this paper, a new constructed fusion protein system (plasmid) was used for r-hGH production in recombinant *E. coli*. Flask cultures were carried out to test the expression of r-hGH. Several factors such as induction starting time, r-hGH expression time, IPTG concentration etc. which may probably influence r-hGH expression, were investigated through an orthogonal experimental test. The optimal culture conditions found in flask cultures were further confirmed by carrying out fed-batch culture.

MATERIALS AND METHODS

Microorganism and plasmid

Escherichia coli BL21 (DE3) was used as a host harboring the plasmid *p*EHUb-hGH for the production of r-hGH. The detailed information was showed in a previous work[8].

Culture media and condition

In order to check which culture media is preferred to grow cells, a modified TB media and modified M9 media were used in flask cultures. Their composition is listed in TABLE 1. The trace element solution contains per liter of 2.0 g $FeSO_4$ ($7H_2O$), 0.25 g $CoSO_4$, 0.15 g $CuSO_4$ ($5H_2O$), 1.0 g $MnCl_2$ ($4H_2O$), 0.2 g $CaCl_2$ ($2H_2O$), 0.3 g H_3BO_3, 0.02 g $(NH_4)_6Mo_7O_{24}(7H_2O)$, 0.3 g $ZnCl_2$, 0.45 g Thiamine HCl, and 1.2g citric acid.

TABLE 1 : THE COMPOSITION OF MODIFIED TB MEDIA AND MODIFIED M9 MEDIA

Modified TB media		Modified M9 media	
Glycerol (mL/L)	4.0	$Na_2HPO_4{\cdot}12H_2O$ (g/L)	12.8
KH_2PO_4 (g/L)	2.0	KH_2PO_4 (g/L)	3.0
K_2HPO_4 (g/L)	15.0	NH_4Cl (g/L)	1.0
Yeast extract (g/L)	16	NaCl (g/L)	0.5
Trace element (mL/L)	10	Yeast extract (g/L)	10
		$MgSO_4$ (g/L)	3.5
		Glucose (g/L)	5.0
		Trace element (mL/L)	10

For flask cultures, recombinant *E. coli* cells were inoculated into 10 ml LB media. After overnight growth at 37 °C with shaking, cells were transferred into 250 ml flasks filled with different volume of modified TB or modified M9 media. The volume ratio of cells to culture media was maintained at 1:50. Ampicillin (Ap) with a final concentration of 100 mg/L was added to culture media. The isopropyl-β-D-thiogalactopyranoside (IPTG) with different final concentrations was used for inducing the expression of r-hGH. The initial glucose concentration was 5 g/L.

An orthogonal $L_{16}(4^3\times2^2)$ test design was used to investigate the optimal culture conditions of r-hGH expression in flask cultures of recombinant *E. coli*. Based on the primary study, five factors were chosen. The factors and their levels are showed in TABLE 2. The r-hGH productivity was the dependent variable.

TABLE 2 : FACTORS AND LEVELS FOR ORTHOGONAL TEST

Variables	levels			
	1	2	3	4
(A) Induction starting time (h)	3	4	5	6
(B) r-hGH expression time (h)	4	5	6	7
(C) IPTG concentration (mmol/L)	0.2	0.3	0.4	0.5
(D) culture medium	TB	M9		
(E) Culture volume (ml)	50	100		

In order to further confirm the results found in flask culture, a fed-batch culture was done in a 5-L fermentor. The gas flow-rate was maintained at 1 vvm based on the initial culture medium volume, and the pH and temperature was maintained at 6.7 and 37ºC, respectively. The dissolved oxygen concentration was maintained at higher than 30 % of air saturation by automatically increasing the agitation speed up to 950 *rpm* and supplying high purity oxygen from oxygen cylinder. The feed solution contained (per liter) 30 g yeast extract and 520 g glycerol was added with the exponential feeding strategy[4].

Analysis methods

The cell optical density (OD) and the dry cell weight (DCW) were measured using the same methods described elsewhere[12]. Protein samples were analyzed by electrophoresis on a 12% (w/v) SDS-PAGE. And protein bands on the SDS-PAGE gels were quantified by densitometry (ImagerMaster™; Pharmacia Biotech, Uppsala, Sweden). The amount of total proteins was determined according to the Bradford method using bovine gamma globulin as a standard[2]. The glucose concentration in M9 media was measured by the 3, 5-dinitrosalicylic acid reducing sugar assay[5].

RESULTS AND DISCUSSION

Cell growth in different media

The culture conditions such as culture medium composition, pH, temperature, etc., can influence cell growth and the expression of recombinant protein. In order to investigate the effects of culture media on the cell growth, recombinant *E. coli* was cultivated in a modified TB and modified M9 media, respectively. Figure 1 shows the time course of OD in different media with/without IPTG (0.4 mmol/L) induction. It can be seen that OD could reached about 5.7 in the modified TB media without IPTG induction, whereas it just reached about 4.7 in the modified M9 media, and the cells grew slowly after 3 h. This may be resulted from the fast growth of cells in initial 3 h and quickly decreases of glucose concentration in culture media. Also it can be found that the cell growth in TB media became a little slow after the IPTG induction, however the amount of expressed r-hGH increased along with the induction time (as shown in Figure 2), where the highest amount of r-hGH was expressed at 7 h after the IPTG induction.

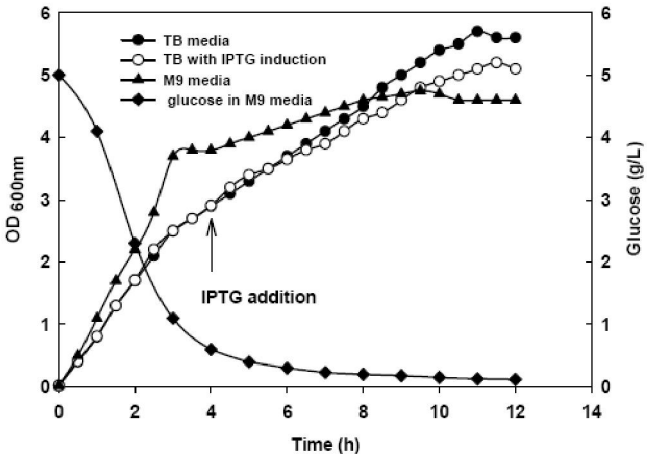
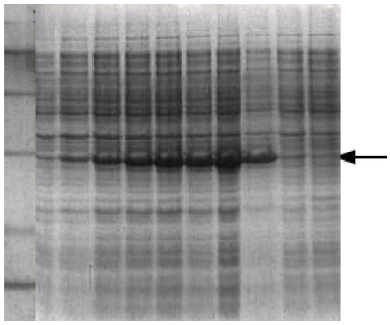


Figure 1 : Cell growth in modified M9 and modified TB media with/without IPTG induction



Lane 1, MW markers; Lane 2-9, induced cells after 1, 2, 3, 4, 5, 6, 7, and 8 h of IPTG induction; Lane 10, same to lane 2; and lane 11, without IPTG induction.

Figure 2 : r-hGH expression in the culture of recombinant E. coli in a modified TB media at different time.

Optimization of r-hGH expression

Several parameters influence the r-hGH expression in cultures of recombinant *E. coli*. In order to check the effects of each parameter on r-hGH expression, five factors (i.e. culture media (modified TB or M9), induction starting time, r-hGH expression time, IPTG concentration, and culture volume in 250 ml flask) were considered here. An orthogonal $L_{16}(4^3\times2^2)$ experiment was designed and well carried out. Each experiment was repeated three times, and the r-hGH was quantitatively analyzed according to the methods mentioned above. The experimental results and several parameters calculated based on the data are shown in TABLE 3.

It can be seen that the experiment No. 13, 8, 11, and 2 showed r-hGH productivity in the decreasing order of 18.72, 15.66, 15.36, and 14.94 mg/L·h, which are higher than that obtained in the other experiments. The averages of each level of factors (indicated as t_1, t_2, t_3, t_4) and ranges were calculated and showed in TABLE 3 too. According to the orthogonal method, the factor becomes more important as the range increases[1]. Therefore, the ranges for the averages in decreasing order were E, B, C, A, and D (as indicated in the last row in TABLE 3). The factor E, culture volume in a 250 mL flask, indicates the influence of dissolved oxygen levels in culture media on the r-hGH productivity. Generally, the dissolved oxygen level in culture media decreased with the increase of culture volume in a flask. The results obtained in this work show that enough oxygen supply is a key role for getting a high r-hGH productivity. Similar result was reported in a previous research[13]. According to the orthogonal method, the level of the averages with the highest r-hGH productivity corresponded to the optimum conditions. The highest r-hGH productivities of levels are highlighted in bold in TABLE 3 (including level one of A, D and E, and level four of B, and level two of C). Therefore, the optimal conditions determined in this study were A1, B4, C2, D1, and E1, under which the r-hGH productivity reached 18.72±0.19 mg/L·h. The optimal levels

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TABLE 3 : Analysis of L16($4^3\times2^2$) test results

No.	(A) Induction starting time(h)	(B) r-hGH expression time (h)	(C) IPTG concentration (mmol/L)	(D) Culture medium	(E) Culture volume (ml)	r-hGH productivity (mg/L·h)
1	1	2	3	1	2	9.24±0.11
2	3	4	1	1	1	14.94±0.21
3	2	4	3	2	2	10.02±0.17
4	4	2	1	2	1	12.9±0.12
5	1	3	1	2	2	8.94±0.16
6	3	1	3	2	1	13.62±0.18
7	2	1	1	1	2	6.84±0.14
8	4	3	3	1	1	15.66±0.20
9	1	1	4	2	1	12.48±0.11
10	3	3	2	2	2	10.26±0.12
11	2	3	4	1	1	15.36±0.18
12	4	1	2	1	2	7.5±0.11
13	1	4	2	1	1	18.72±0.19
14	3	2	4	1	2	9.06±0.11
15	2	2	2	2	1	13.2±0.12
16	4	4	4	2	2	9.9±0.11
t_1	12.35*	10.11	10.91	12.17	14.61	
t_2	11.36	11.1	12.42	11.42	8.97	
t_3	11.97	12.56	12.14			
t_4	11.49	13.4	11.7			
Range	0.99	3.29	1.51	0.75	5.64	
Order**	4	2	3	5	1	

*** The highest r-hGH productivity of the levels is highlighted in bold; ** It is the ordinal numeral for the range sequence of the five factors in decreasing order**

of A and B show that the earlier induction and the increase of r-hGH expression time were import for enhancing the r-hGH biosynthesis.

r-hGH expression in fed-batch culture

The use of fed-batch cultures has been shown to significantly increase the cell-density and specific protein production. Here, a fed-batch culture of recombinant *E. coli* in the modified TB medium was carried out for further checking the optimal conditions found in the orthogonal experiments, where IPTG was added in 0.3 mmol/L at the 6th hour, and high purity oxygen was supplied when the dissolved oxygen concentration lower than 30%. As shown in Figure 3, the dry cell density could reach 77.1 g/L, while the r-hGH reached 4.3 g/L, which is very close to result obtained in a fed-batch culture with complex medium[13]. This shows that the productivity could reach 215 mg/L.

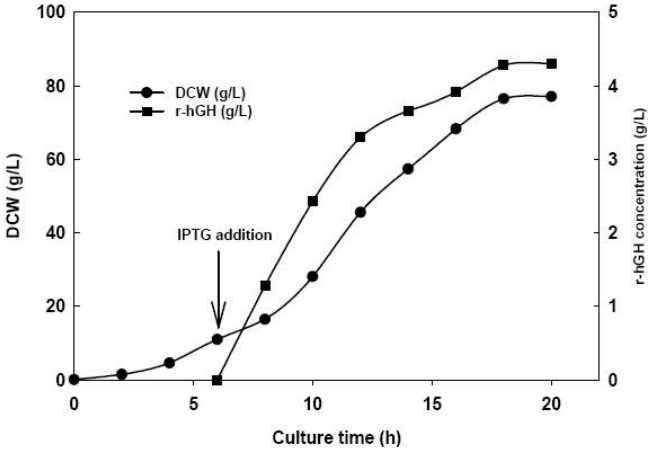

Figure 3 : 5-L scale fed-batch culture of recombinant *E. coli* for r-hGH biosynthesis

SUMMARY

This study investigated the effects of several parameters on the r-hGH expression in flask cultures of recombinant *E. coli* by an orthogonal test. It was found that the oxygen supply would play a key role on the r-hGH expression. The optimal culture conditions for achieving a highest r-hGH productivity were found by an orthogonal $L_{16}(4^3\times2^2)$ test. A high r-hGH concen-

tration (4.3 g/L) was obtained in the fed-batch culture carried out under the optimal conditions. These results will be useful for the further study on the biosynthesis of r-hGH.

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