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Over expression of the Interferon β-1b by optimizing induction conditions using response surface methodology

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ABSTRACT

High expression of therapeutic proteins is crucial for industrial scale manufacturing. In the present study, induction conditions for the production of interferon β-1b by recombinant Escherichia coli BL21 (DE3) were optimized using response surface methodology based on the central composite design (CCD). The induction conditions included isopropyl β -D-thiogalactopyranoside (IPTG) concentration, induction start time (OD_{600} nm), post-induction time and temperature. The optimal set of culture conditions for high interferon β-1b was determined: 0.7 mM IPTG, an induction start time (OD_{600} nm) of 0.58, a post-induction time of 5h, and a post-induction temperature of 37°C. A high level of interferon beta-1b protein was expressed in the optimized conditions, which accounted for about 40.2 % of the total cell proteins.

Key words: Over expression, Interferon β-1b, Optimization, Response surface methodology, Escherichia coli BL21 (DE3)

1. INTRODUCTION

Interferon-β is a cytokine shown to have antiviral, anti-proliferative and immune-regulatory properties and is used therapeutically by slowing the progression of disability to multiple sclerosis (MS) (1, 2). Natural hIFN-β is composed of 166 amino acid residues. It is glycosylated and contains three cysteine residues. Recombinant Interferon β-1b (IFNβ-1b) is a non-glycosylated therapeutic protein produced by the cells of *Escherichia coli*. It is biologically active, and has serine instead of cysteine 17 residues to prevent the formation of undesired disulfide bonds (3). *E. coli* BL21 (DE3) is commonly used for the high-yield expression of recombinant proteins. This strain was designed by Studier and Moffatt as a host for the pET expression vectors containing the T7 promoter. The coding sequence for T7 RNA polymerase is present in the chromosome under control of the IPTG- inducible lacUV5 promoter in the chromosome of host cell BL21 (DE3) (4). Over-expression of eukaryotic and disulfide containing proteins in *Escherichia coli* usually results in formation of insoluble aggregates known as inclusion bodies (5). Inclusion bodies have several advantages as follows. Firstly, inclusion bodies can be accumulated in the cytoplasm to much higher levels (usually greater than 25% of total proteins) than soluble proteins. Secondly, inclusion bodies can be initially isolated in a highly purified and concentrated state by simple centrifugation. Thirdly, inclusion bodies have no biological activity, facilitating production of proteins (such as interferon beta) that are toxic to the *E. coli* cell. Fourthly, inclusion bodies are resistant to proteolysis by *E. coli* proteases, allowing high-yield protein production (6). The expression efficiency of foreign proteins in *E. coli* is dependent on a variety of parameters (7). Therefore, it is pivotal to identify the influence of parameters such as temperature, inducer concentration, as well as the point of induction and the duration of the induction phase on the expression of the target protein in *E. coli*. The bioprocess optimization through statistical design, especially for culture conditions, is a common practice in biotechnology. These statistical methods, as compared to the common 'one-factor-at-a-time' method, proved to be useful tools. In this work, attempts

have been made to optimize the induction conditions for the expression of interferon beta-1b using response surface methodology based on central composite design (CCD). The completely optimized parameters, including IPTG concentration, the optical density at 600 nm (OD_{600} nm) at the time of induction, post-induction time, post-induction temperature, were determined by response surface methodology. To our knowledge, previous studies have not described induction conditions in detail for the expression of rhIFN-β-1b (8-12). Hence, the present study becomes important for high yield production of rhIFN-β-1b using *E. coli* as the host system.

2. MATERIALS AND METHODS

2.1. Materials

E. coli strainBL21 (DE3) harboring human IFNβ gene was obtained from Zistdaru Danesh Ltd. (Tehran, Iran). LB (Luria-Bertani) medium, isopropyl-β-D thiogalactopyranoside (IPTG), sodium dodecyl sulfate (SDS) were purchased from Sigma (St. Louis, MO, USA). Glucose was obtained from Scharlau (Scharlab S.L., Barcelona).Water for injection (WFI) was used for solution and buffer preparations. All other chemicals were of reagent grade.

2.2. Cell growth and harvesting

Luria–Bertani (LB) medium (100mL) supplemented with 100 µg/mL kanamycine and 0.1% Glucose was inoculated with 1 ml of glycerol stock of *E. coli* host cells and cultured overnight at 37 °C, 250 rpm. The overnight culture was inoculated into 100 ml of LB supplemented with 100 µg/mL kanamycine and 0.1% Glucose. The cells were cultured at 37 °C at 250 rpm. When the bacterial culture reached an optical density of 0.2, 0.6 or 1at 600 nm (OD_{600} nm), The culture was induced by the addition of IPTG to a final concentration of 0.2, 0.6, or 1.0 mM and incubation at 17 , 27 or 37 °C, 250 rpm for 2 , 4, or 6 h. The culture was centrifuged at 4000 rpm, 5 °C for 10 min, the supernatant carefully removed, and the cell pellet washed by gently suspending in PBS. The washed cell mass was collected by centrifuging at 4000 rpm, 5 °C for 10 min and stored at -20°C.

2.3. Cell lysis

The cell pellet was suspended in 5 ml of PBS buffer maintained on ice. The cells were disrupted with a sonicator programmed for 5 cycles of 5 min on and 5 min off; The sonicate was then centrifuged at 12000 rpm for15 min. The supernatant was discarded. A compact pellet containing inclusion bodies was obtained. Pellets were washed three times with sterile water. After a final centrifugation at 12000 rpm for 15 min, pellets were stored at -20 °C.

2.4. SDS–PAGE

Pellets were applied to 14% SDS–PAGE gel and the expression yield was analyzed using the Quantity One quantitative software (Bio-Rad) according to the relative band intensities of Comassie blue stain.

2.5. Experimental design and optimization by RSM

Response surface methodology was applied to design experiments. Each variable was considered at two levels (−1, +1) and center point (0) which represents the midpoint of each factor range (Table 1).

Table 1. Experimental range of four variables studied in terms of actual and coded values

Variable	symbol	code level		
		-1 (low)	0 (mid)	1 (high)
Temprature	A	17°C	27 °C	37°C
IPTG Conc	B	0.2 mM	0.6mM	1mM
Induction Time	C	OD600nm=0.2	OD600nm=0.6	OD600nm=1
Induction Period	D	2 hour	4 hours	6 hours

According to the central composite design (CCD) 29 experiments were carried out. All experiments were performed in triplicates. The analysis of variance (ANOVA) was done using the statistical package Design-Expert; version7.0 (Stat-Ease Inc. Minneapolis, MN, USA) (

Table 2).

Table 2. Experimental design and responses for the content of protein by recombinant *E. coli* DE3

Experiment	Factors				Response
	A	B	C	D	interferon beta-1b (%)
1	17	0.6	1	4	3
2	27	1	0.2	4	17
3	27	0.6	0.6	4	24
4	27	0.6	1	6	20
5	27	0.6	0.6	4	25
6	27	0.2	1	4	8
7	17	0.6	0.2	4	12
8	37	0.6	1	4	26
9	37	0.6	0.2	4	28
10	17	0.6	0.6	2	5
11	37	0.2	0.6	4	29
12	17	0.2	0.6	4	6
13	27	0.6	0.6	4	20
14	27	1	0.6	2	11
15	27	1	1	4	18
16	27	1	0.6	6	23
17	27	0.2	0.2	4	6
18	27	0.6	1	2	12
19	27	0.2	0.6	6	17
20	27	0.2	0.6	2	7
21	27	0.6	0.2	2	9
22	17	0.6	0.6	6	14
23	27	0.6	0.6	4	27
24	37	0.6	0.6	2	23
25	27	0.6	0.2	6	23
26	37	0.6	0.6	6	40
27	17	1	0.6	4	9
28	37	1	0.6	4	30
29	27	0.6	0.6	4	24

A: Post induction temperature (°C)
B: IPTG concentration (mmol/l)
C: Induction start time (OD600 nm)
D: Post induction time (h)

3. RESULTS AND DISCUSSION

As shown in Table3, the ANOVA results confirm a satisfactory adjustment of the model and the experimental data as well. The model correlation coefficient (R^2), adjusted R^2, Predicted R^2 and adequate precision were 0.9563, 0.9126, 0.7937 and 17.925, respectively. The lack of fit *F*-value of 1.21 indicated that it is not significant relative to the pure error. Therefore, the model is a good fit for the design space. Analysis of variance data's show *P*-value less than 0.05 indicate model terms are significant. In this case *A*, *B*, *D*, B^2, C^2, D^2 are significant model terms (*P*-value <0.05). From *P*-values of terms in the Table3, it can be seen that the linear term of induction temperature have most significance on the production of interferon β -1b Protein. The linear term of induction start time have least significance on the production of interferon β -1b protein, and the linear and square terms of post-induction time and IPTG were highly significant, and the square term of induction start time is also one of the most influential factors. The model terms of C, AB, AC, AD, BC, BD, CD and A^2 had *P*-values >0.05. Therefore, they were

eliminated from the model (Table 3). Therefore, the simplified quadratic model for overall productivity of interferon β -1b in terms of coded factors was expressed as follows:

Final Equation in Terms of Coded Factors:

$$Y = 24.00 + 10.58\ A + 2.92\ B + 5.83\ D - 5.87\ B^2 - 5.75\ C^2 - 3.00\ D^2$$

Where Y is the response variable (interferon β -1b production), A, B, C and D are the independent variables (Post induction temperature, inducer (IPTG) concentration, Induction start time (OD_{600} nm), Post induction time, respectively).

3.1. Response surface of interferon beta-1b protein

To investigate the effects of the four factors on interferon beta-1b protein production, the response surface methodology was used, and the three-dimensional plot was drawn. Figure 1 shows the response surface curves for the four variables in the production of interferon beta-1b protein. The response surface representing the interferon beta-1b protein yield was a function of two induction conditions with the other two conditions being at their optimal levels.

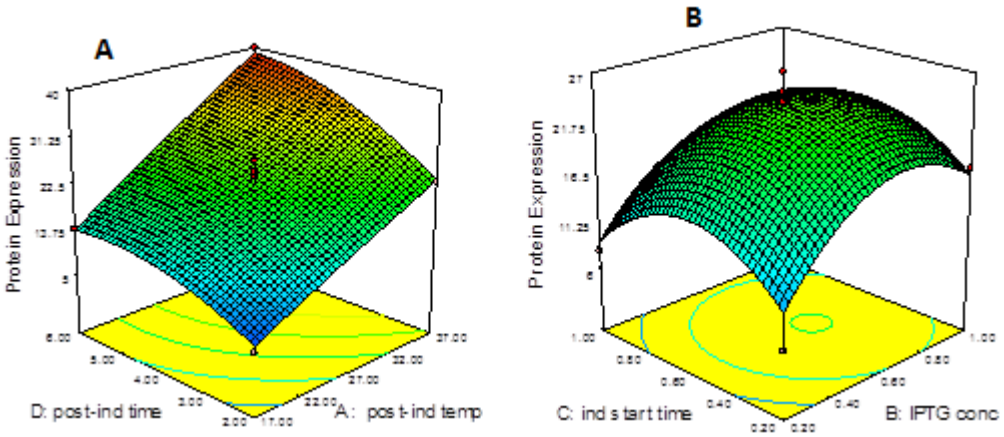

Figure 1. Response surface of interferon beta-1b protein concentration by *E. coli* DE3. A: post-induction temperature vs. post-induction time with constant levels of IPTG concentrations (0.6) and induction start time (0.6). B: Response surface of interferon beta-1b protein concentration by *E. coli* DE3: IPTG concentrations vs. induction start time with constant levels of post-induction temperature (27°c) and post-induction time (4 h).

3.2. Effect of post-induction temperature

Post-induction temperature was an important factor that influenced the expression level of interferon beta-1b (P <0.0001). In general, the cultivation temperature often influences expression levels (13). Figure 1 (A) revealed that 37◦C was the best post-induction temperature for the expression of interferon beta-1b.The explanation for this result could be that in *E. coli*, the maximum cell growth rate is usually prevalent at high temperatures, such as 37°C. However, low temperature will influence the growth of bacteria, resulting in reduction of expression level. At low temperature, the expression rate of target protein is much lower than that of 37 °C. In addition, aggregation of target protein is favored at higher temperatures due to the strong temperature dependence of hydrophobic interactions.

3.3. Effect of inducer concentration

The IPTG concentration used for inducing gene expression ranges from 0.005 to 5 mM (14). Inducer load is a critical factor, both in terms of cost and potential inhibition of cell yield influencing volumetric protein productivity, the product of yield per cell and total biomass productivity. In many cases, the optimal concentration of inducer is chosen to balance the decreasing yields of recombinant cells following induction with the increasing cellular levels of target protein (15). Numerous earlier studies have suggested that the optimal level of inducer for the expression of heterologous genes is product specific (16). In the present study, Figure 1 (B) revealed that expression of the interferon beta-1b gene started increasing as the concentration of IPTG increased from 0.2 to 0.7 mM. Maximum expression was seen at 0.7 mM concentration of IPTG and as the concentration increased from 0.7 to 1 mM the expression reduced slightly. In general, high concentrations of IPTG do not necessarily lead to maximal expression of a target protein (17). Our results revealed that 0.7 mM was the best IPTG concentration for the expression of interferon beta-1b.

3.4. Effect of induction-starting time

For determination of the optimal time of interferon beta-1b induction, interferon beta-1b production was induced at $OD_{600\ nm}$ 0.2 (end of lag phase), 0.6 (early exponential phase) and 1(mid exponential phase) of cell cultivation. Fig.1 (B) revealed that interferon beta-1b production was maximal when induced at the initial exponential phase of growth and after that it decreased. Chen *et al.* (18) investigated a study of the timing of IPTG addition in the production of chitinase by recombinant *E. coli*. Their findings also showed that chitinase production was maximal when induced at the initial exponential phase of growth (OD600 nm 0.58) and decreased rapidly after that. A similar result has been reported by Durany *et al.* (19) and Lim *et al.* (20). In this study, the analysis of variance

indicated that the induction-starting time gave insignificant effect toward the expression level of interferon beta-1b (**Table 3**), which was similar to the report given by Yan *et al.* (21) and Pana *et al* (22).

Table 3. ANOVA for Response Surface Quadratic Model

Source of variation	Sum of squares	Degree of freedom	Mean square	F-value	p-value
Model	2295.84	14	163.99	21.88	<0.0001
A-Post induction temperature	1344.08	1	1344.08	179.35	<0.0001
B-IPTG concentration	102.08	1	102.08	13.62	0.0024
C-Induction start time	5.33	1	5.33	0.71	0.4131
D-Post induction time	408.33	1	408.33	54.49	<0.0001
AB	1.00	1	1.00	0.13	0.7204
AC	12.25	1	12.25	1.63	0.2219
AD	16.00	1	16.00	2.14	0.1660
BC	0.25	1	0.25	0.033	0.8577
BD	1.00	1	1.00	0.13	0.7204
CD	9.00	1	9.00	1.20	0.2916
A2	0.91	1	0.91	0.12	0.7324
B2	223.89	1	223.89	29.88	<0.0001
C2	214.46	1	214.46	28.62	0.0001
D2	58.38	1	58.38	7.79	0.0144
Residual	104.92	14	7.49		
Lack of Fit	78.92	10	7.89	1.21	0.4609
Pure Error	26.00	4	6.50		
Cor Total	2400.76	28			

P-value less than 0.05 indicates that the term was significant.

3.5. Effect of post-induction time

The post-induction time was a significant factor (P <0.0001) that influenced the expression of interferon beta-1b (Table3). Based on Fig.1 (B), the expression of interferon beta-1b was lower with shorter post-induction time; in contrast, the longer duration resulted in a higher production of interferon beta-1b, which is similar to the report given by Loa *et al*. (23).In the present study the optimum 5h post-induction time was selected as one of the optimized culture conditions.

3.6. Effect of interactions among the induction conditions

Generally, the interactions between the induction conditions would not give a significant effect towards the production of protein. In this study, none of the interactions was significant (Table3), this finding is similar to the results obtained by Loa *et al*. (23). They reported that interactions among the induction conditions were insignificant.

3.7. The optimum range of desired factors and high-level expression of interferon beta-1b

The optimum induction conditions for maximum production of interferon beta-1b were found to be, 0.7 mM IPTG, an induction start time (OD_{600} nm) of 0.58 , a post-induction time of 5h, and a post-induction temperature of 37°C, the predicted yield of the protein under the optimum conditions was 39.7 % of the total cell protein. Verification experiment was carried out using the predicted optimum induction conditions. The recombinant *E. coli* produced about 40.2 % interferon beta-1b in the optimized conditions (Table 4). It should be noted that this amount of IFN-β production was also higher than the central point as well as any of those in the 29 experiments indicating that the optimization was quite successful.

Table 4. The optimum range of desired factors

Factors				Content of interferon beta-1b protein (%)	
A	B	C	D	Predicted value	Actual value
37	0.7	0.58	5	39.7	40.2

A-Post induction temperature (C)
B-IPTG concentration (mM)

C-Induction start time (OD600nm)
D-Post induction time (h)

As reported in previous works, the amount of rIFN-β production was always low. Goeddel *et al* (1980) reported a maximum hINF-β production of 0.2 mg/L using the wild type gene cloned in *E. coli*. The amount of IFN-β production under the control of *trp* promoter was about 2-4% of total cell protein (24).Warne *et al.* (1986) reported 7-9.1% and with modified *trp* promoter the yield of IFN-β production was about11-14 % (25). Skoko *et al* (2003) reported a maximum rhINF-β production of 12 mg/L in *Pichia pastoris* culture (26). While in the present study a high level of interferon beta-1b protein was expressed in the optimized conditions, which accounted for about 40.2 % of the total cell proteins.

4. CONCLUSION

In conclusion, judicious optimization of induction conditions led to the attainment of rhIFN-β-1b yield (40.2 %) that is, to the best of our knowledge, highest recorded to date in the literature for this important therapeutic protein.

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AUTHORS CONTRIBUTION

This work was carried out in collaboration among all authors.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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