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ORIGINAL PAPER

Improved Production of Recombinant Human β-NGF in *Escherichia coli* – a Bioreactor Scale Study

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Abstract

Human nerve growth factor β (β-NGF) is considered a major therapeutic agent for treatment of neurodegenerative diseases. We have previously reported the optimized conditions for β-NGF overproduction in *Escherichia coli* in a shake-flask culture. In this study the optimal %DO (dissolved oxygen) and post induction temperature values for improved production of β-NGF were found in the bioreactor scale using response surface methodology (RSM) as the most common statistical method. Also, for further enhancement of the yield, different post-induction periods of time were selected for testing. In all experiments, the productivity level and bacterial cell growth were evaluated by western blotting technique and monitoring of absorbance at 600 nm, respectively. Our results indicated that %DO, the post-induction time and temperature have significant effects on the production of β-NGF. After 2 hours of induction, the low post induction temperature of 32°C and 20% DO were used to increase the production of β-NGF in a 5-l bioreactor. Another important result obtained in this study was that the improved β-NGF production was not achieved at highest dry cell weigh or highest cell growth. These results are definitely of importance for industrial β-NGF production.

Key words: bioreactor, RSM, β-NGF over production, *E. coli*

Introduction

NGF (Nerve Growth Factor) consists of three subunits, α, β and γ; among them β subunit is responsible for NGF biological activity. The β subunit is a dimer of two similar monomers containing cysteine knot motif, which is formed by three disulfide bonds (Wiesmann and De Vos 2001). β-NGF plays a fundamental role in development and survival of nervous system; so, it can be used as a therapeutic agent for the treatment of neurodegenerative diseases (Heese et al. 2006). NGF can be extracted from its natural source, the male mice submaxillar glands, by different methods of chromatography but it is unsuitable for clinical uses because contains heterogeneous mixtures of partially degraded dimmers (Bocchini and Angeletti 1969). Nowadays many studies have attempted to produce it as recombinant protein using different eukaryotic and prokaryotic hosts (Kurokawa et al. 2001; Choi and Lee 2004; Fan and Lou 2010).

Production of recombinant proteins in prokaryotic hosts is cost effective; among them *E. coli* is widely used expression host both in laboratory and industrial scale (Rosano and Ceccarelli 2014). Today many strategies have been introduced to increase the expression level in *E. coli*. In general, expression can be optimized by varying cultivation media, the proper selection of *E. coli* strain, induction conditions such as time and temperature of induction and the type of promoter and inducer concentration (Schumann and Ferreira 2004; Saez and Vincentelli 2014; Tegel et al. 2011). However, protein expression and production in industrial scale using a bioreactor requires special consideration. Previous studies have reported the significant effects of oxygen level, aeration and agitation rate on the growth rate in liquid medium of bioreactor (Zhong 2010; Wang et al. 2016). Agitation or shaking of culture is necessary to maintain a uniform environment without formation of bacterial aggregates throughout the bioreactor contents. Adequate agitation and aeration are also important

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factors in the success of protein expression (Thiry and Cingolani 2002; Lee et al. 2013).

The aim of this work was to optimize the fermentation parameters to obtain the highest β-NGF production in the bioreactor scale. Therefore, conditions of the fermentation process in the 5-l batch bioreactor were optimized to maximize the yield of the active protein. Response Surface Methodology (RSM) as the most common statistical method for optimization of various biochemical processes (Elibol and Ozer 2002; Wejse et al. 2003; Rui et al. 2009) was used to optimize the induction parameters and dissolved oxygen level (or different agitation rate) for β-NGF over production.

Experimental

Materials and Methods

Reagents. Unless otherwise specified, all reagents were purchased from Merck Company (Germany).

Bacterial strains and vector. The *E. coli* BL21 (DE3) carrying T7 RNA polymerase gene with genotype of *E. coli* B F^- *dcm ompT hsdS*(rB^- mB^-) *gal* λ(DE3) was used as the host strain throughout this study. The β-NGF cDNA was cloned into the expression vector pET39b (+) (Novagen, USA). The resulting vector was transformed to BL21 (DE3) strain using heat shock procedure (Sambrook and Russell 2001).

Culture media. The medium for primary and secondary seed cultures was Luria-Bertani (LB) medium (Sigma, USA) containing tryptone 10 g/l, yeast extract 5 g/l and NaCl 10 g/l. Kanamycin disulphate (Sigma, USA) at a final concentration of 50 μg/ml was added as selective factor to primary and secondary seed cultures. The medium for batch culture was SOB medium: tryptone 20 g/l, yeast extract 5 g/l, NaCl 0.5 g/l, KCl 0.186 g/l, $MgCl_2$ 0.952 g/l and $MgSO_4$ 1.204 g/l.

Culture conditions. The primary seed culture was prepared by transferring a single colony to 5 ml LB medium and incubation for 12 h at 37°C and 200 rpm. The secondary seed culture was prepared by incubating a 100 ml fresh LB medium with 1% (v/v) of the primary seed culture for 12 h at 37°C and 200 rpm.

The batch fermentation was performed in a 5-l bioreactor (Sabaferm110, Zist Farayand Sanat, Iran) containing 2 l medium inoculated with 100 ml secondary seed culture (5% v/v). The pH was kept constant throughout the experiment at 7.0 by the automatic addition of 2*N* NaOH or 2*N* HCl solution. The dissolved oxygen (DO) was maintained at different levels by changing the agitation speed (rotation speed of the mixer). The constant aeration speed of 1vvm measured and controlled by rotameter was used throughout the batch fermentation. The sterile compressed air was delivered to the bioreactor tank after passing through a cellulose filter.

Optimization of β-NGF production in the bioreactor scale. The process of β-NGF production was conducted in 5-l bioreactor. In each individual experiment, protein expression was initiated by addition of the filter sterilized solution of IPTG (isopropyl β-D-1-thiogalactopyranoside) at a final concentration of 0.3 mM when OD_{600nm} reached the value of 0.6. After desired time of production, cells were harvested by centrifugation at 5 000 g for 15 min. Subsequently, the cells were re-suspended in 8 M urea and broken by an ultrasonic disrupter. After overnight incubation at 37°C, the debris was removed by centrifugation at 12 000 g for 30 min. Finally, the supernatant was collected and used for western blotting analysis.

Determination of the best post induction time. To determine the best post induction time to achieve the highest β-NGF production, the experiments were done at constant and controlled automatically temperature of 37 ± 0.5°C and pH 7.0 ± 0.1. Also, the aeration speed and agitation speed were monitored at 1vvm and 300 rpm, respectively. After induction of the promoter by IPTG as mentioned above, the cells were grown for additional 2, 4, 6, 8, 10 and 12 h. Fermentation samples were collected to extract the proteins in 2 h intervals.

Experimental design and optimization of culture conditions. Response surface methodology (RSM) based on the central composite design (CCD) was used to optimize the culture conditions for β-NGF over production.

The percentage of dissolved oxygen (DO) and the post induction temperature were chosen as the independent variables. The ranges and levels of the two variables are listed in Table I. The cell growth at 600 nm (or the dry cells weigh) and the recombinant β-NGF expression level were chosen as response (output). The experimental data obtained from the design (Table I) were analyzed by the following second order polynomial equation:

$$Y = \beta_\circ + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

Where Y is the measured response, β_o, β_i, β_{ij} and β_{ii} are the regression coefficients and X_i and X_j are the independent variables in coded values.

The software package Design Expert version 11.0.3.0 (StatSoft, USA) was used to find out the interactive effect of the two variables. The significance of the model equation and model terms were evaluated by *F*-test. The fitted polynomial equations were also expressed as three-dimension surface plots to indicate the relation between the response experimental levels

Table I
The values of independent variables (post induction temperature and dissolved oxygen) and the corresponding levels used in a central composite design (normalized in –2, –1, 0, 1 and 2).

Variable	Level				
	–2	–1	0	1	2
A: Post induction temperature (T) (°C)	20	24.25	28.5	32.75	37
B: Dissolved oxygen (DO) (%)	10	20	30	40	50

Table II
The experimental design of 10 runs with two variables (post induction temperature and dissolved oxygen) and two responses (recombinant protein expression level and cell growth measured by absorbance at 600 nm).

Run	Post induction Temperature (T) (°C)		Dissolved Oxygen (DO) (%)		Response 1 Recombinant Protein Expression Level (%)	Response 2 Cell Growth (Abs_{600nm})
1	–1	24.25	–1	20	8.0 ± 0.9	1.83 ± 0.18
2	+1	32.75	–1	20	15.3 ± 1.2	2.60 ± 0.19
3	–1	24.25	+1	40	10.1 ± 1.0	1.02 ± 0.11
4	+1	32.75	+1	40	8.4 ± 0.8	5.60 ± 0.29
5	–2	20.0	0	30	4.6 ± 0.4	1.36 ± 0.11
6	+2	37.0	0	30	11.1 ± 1.1	3.40 ± 0.22
7	0	28.5	–2	10	10.0 ± 0.9	1.87 ± 0.21
8	0	28.5	+2	50	3.8 ± 0.4	2.05 ± 0.20
9	0	28.5	0	30	14.6 ± 1.2	2.45 ± 0.19
10	0	28.5	0	30	14.2 ± 1.2	2.60 ± 0.19

of each factor used. The experimental design matrix is given in Table II. Each experiment was done in different dissolved oxygen level ranging from 10 to 50%. The temperature of culture was kept constant at 37°C before induction of the promoter, but after induction different temperatures ranging from 20 to 37°C were used according to Table II. Automatic controlling of the temperature was via the water jacket. After additional cell growth for 2 h, the culture medium was collected and centrifuged for further protein extraction.

Determination of bacterial biomass. To monitor cell growth, the absorbance of the bacterial cell culture at 600 nm was measured using a spectrophotometer (Thermo, USA). The OD_{600nm} was converted to dry cell weight (g/l) based on a standard curve (Ren et al. 2013).

Protein concentration determination. The concentration of protein in the sample was determined by Bradford method using bovine albumin as standard (Bradford 1976).

Protein identification with western blotting (WB). The proteins were subjected to electrophoresis on a 15% SDS-Polyacrylamide gel according to Laemmli method (Laemmli 1970). Then the separated proteins were electro-transferred onto the nitrocellulose membrane (Millipore, USA) in a transfer buffer (25 mM Tris, 192 mM glycine, 15% methanol) at 200 mA (Burnette 1981) for 3 h. After blocking the membrane with 5% fat-free milk in Tris-buffered saline (TBS, pH 7.4), it was treated with anti-his tag monoclonal antibody (Sigma, USA) conjugated with horseradish peroxidase at a final dilution of 1:1000. Proteins were then detected using a solution of DAB (Biobasic, Canada) and hydrogen peroxide as enzyme substrates. The area of each band on the nitrocellulose membrane which represents the β-NGF expression level was calculated by densitometry analysis (using Image J software) (Schneider et al. 2012).

Protein purification and bioactivity test. The β-NGF was successfully purified using Ni^{+2}-NTA column (ABT, Spain). Purification was done in the following steps: binding, washing and dilution with the appropriate buffers, as follows: the binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH = 8), the washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole, pH = 8) and the elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM Imidazole, pH = 7), respectively. Protein purification was finally evaluated by SDS-PAGE method. To test the biological activity of the purified β-NGF, the PC12 differentiation assay was used (Greene and Tischler 1976). 50 ng/ml of commercial and purified β-NGF was added individually to PC12 cell line for seven days and their differentiation to nerve cells was monitored.

Results

Effect of post induction time on β-NGF production. It has been widely reported that induction parameters can affect protein expression level in shake-flask culture (Azaman et al. 2010; Larentis et al. 2011; Savari et al. 2015; Gholami Tilko et al. 2016). In the present study for the first time, the effect of post-induction time on β-NGF production level was investigated in a 5-l bioreactor. Batch cultivation was performed using SOB medium with constant temperature (37°C), pH (7.0), agitation speed (300 rpm) and aeration speed (1vvm) throughout the experiment. As illustrated in Fig. 1, the maximum yield of β-NGF production (% recombinant protein expression level) was obtained at 2 h post induction time and recombinant protein expression level was reduced over extended periods of time. Therefore, post induction time of 2 h was selected and used for further experiments.

Optimization of post induction temperature and % dissolved oxygen (DO) using RSM. Post induction temperature is one of most important parameters affecting the recombinant protein expression level. Also, DO is a critical factor in the over expression of recombinant proteins, especially in the bioreactor scale. Therefore, to investigate the influence of DO and post induction temperature on β-NGF production, 10 experiments were designed using RSM method. These experiments were conducted by controlling DO (10, 20, 30, 40 and 50%) and post induction temperature (20, 24.25, 28.5, 32.75 and 37°C) (Tables I and II). Experimental error was evaluated at the central point conditions of the experimental plan (28.5°C and 30% DO). The constant cultivation temperature of 37°C was used before induction of promoter in each experiment. In all experiments, the promoter of pET39b::βNGF plasmid was induced by addition of 0.3 mM IPTG, when $OD_{600\ nm}$ reached the value of 0.6. After 2 h, the total proteins produced in recombinant bacteria were extracted. Simultaneously, cell growth (measured by absorbance at 600 nm) was monitored in each case. As the main purpose of this study was production of β-NGF, we used the western blot analysis in which an anti-his tag monoclonal antibody recognized the recombinant β-NGF with his-tag tail in the mixture of total proteins extracted from *E. coli* (Fig. 2A). To evaluate the effects of two variables (temperature and %DO) on the β-NGF expression level, the areas of each band detected on the nitrocellulose membrane were calculated by densitometry analysis (using Image J software) (Table II). Different combinations of post induction temperature (20–37°C) and dissolved oxygen (10–50%) yielded in the β-NGF production level from 3.8% ± 0.4 to 15.3% ± 1.2 as illustrated in Table II. The cell growth, which ranged from 1.02 ± 0.11 to 5.60 ± 0.29 (resulting in dry cell weight of 0.367 and 2.016 g/l) is also presented in Table II.

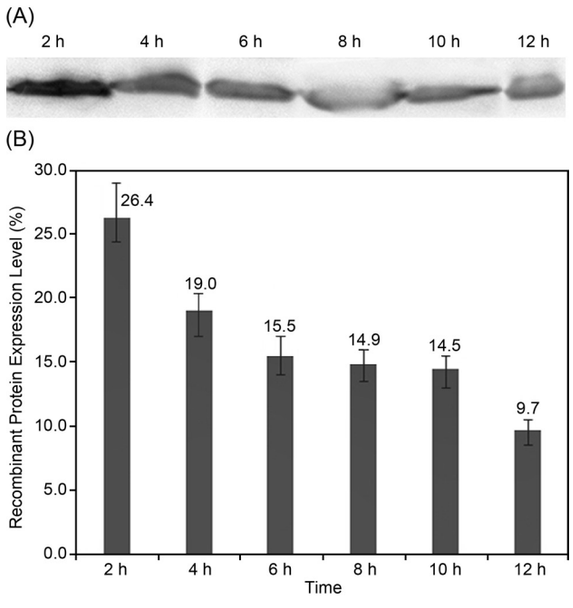

Fig. 1. The recombinant protein expression level analysis in different post induction times. (A) Western blot analysis of the proteins extracted from recombinant bacteria carrying pET39::β-NGF plasmid using anti-histag. HRP antibody. (B) % recombinant protein expression level measured by ImageJ software. All experiments were repeated three times under the identical experimental conditions.

As illustrated in Table II and Fig. 2, high β-NGF expression levels were observed in Runs 2 and 9 (15.3% ± 1.2 and 14.6% ± 1.2, respectively). Run 10 was a repetition of Run 9 and resulted in the same value. In these runs, the cell growth (or dry cell weight) was not at maximum level. In other words, the increased cell growth was not necessarily accompanied by an increase in recombinant production level. It is necessary to mention that the highest value of β-NGF production was obtained at post induction temperature of 32.75°C and % DO of 20.

In contrast, the lowest value of β-NGF production level (3.8% ± 0.4) was obtained at post induction temperature of 28.5°C and 50% DO (Run 8). When comparing the runs 8 and 9 with constant post induction temperature (28.5°C) and different %DO (also runs 2 and 4 with constant post induction temperature of 32.75°C and different %DO) it could be observed that the recombinant β-NGF production was significantly reduced at high degree of DO. It can be a result of high agitation speed because higher speed of stirring could cause a breakdown of the cells (Banerjee et al. 2009).

Analysis of variance. The recombinant β-NGF production was the most important response measured in this study. Interaction of two parameters examined in this study (post induction temperature and %DO) had pronounced effect on the β-NGF production indi-

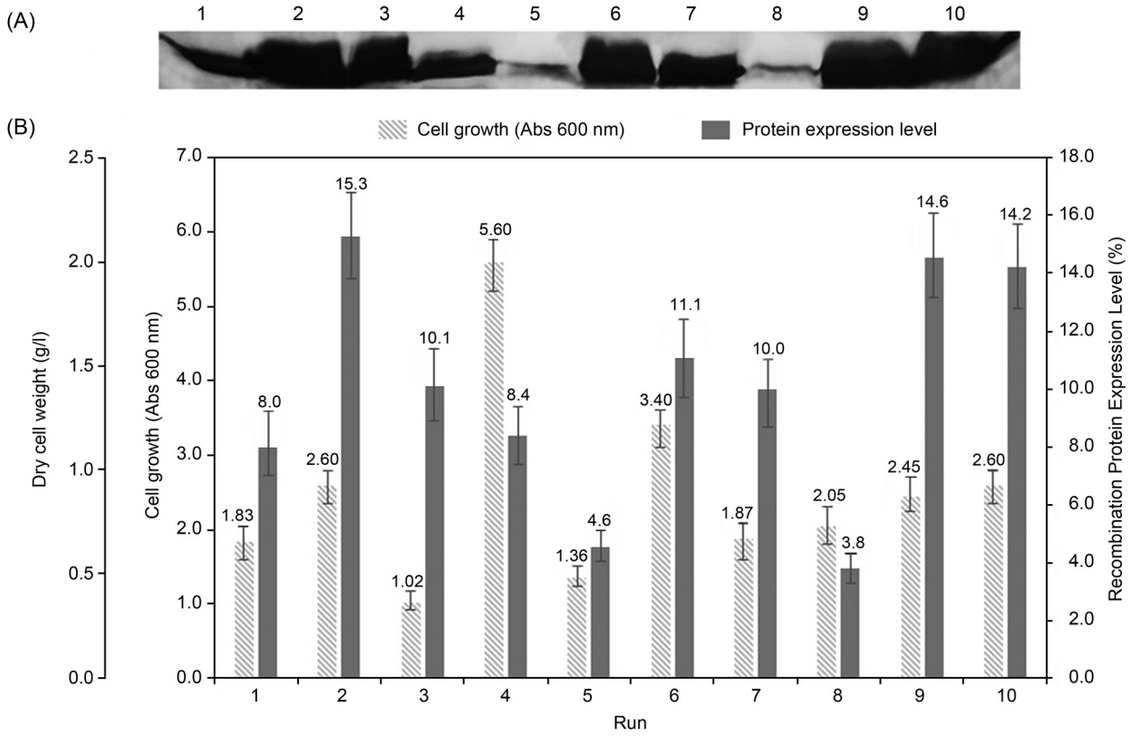

Fig. 2. The recombinant protein expression level analysis in different % DO and post induction temperature. (A) Western blot analysis of the proteins extracted from recombinant bacteria carrying pET39::β-NGF plasmid using anti-histag.HRP antibody. (B) % recombinant protein expression level measured by ImageJ software and comparison with the cell growth measured by absorbance at 600 nm. All experiments were repeated three times under the identical experimental conditions.

cating the importance of these factors for enhancement of the recombinant protein production. The response under different combinations was analyzed by ANOVA technique (Table III). The model *F*-value of 120.36 from ANOVA of the recombinant protein expression level indicated that this model was significant. The *p* values

Table III
Parameter estimates and analysis of variance (ANOVA) of the model for recombinant production of β-NGF in *E. coli* and for cell growth.

	Source of variation	Degree of freedom	Sum of squares	Mean squares	*F*-value	*p* value (Probe > *F*)
Recombinant Protein Expression Level (%)	Model	5	141.50	28.30	120.36	0.0002
	A-Temperature (°C)	1	29.14	29.14	123.94	0.0004
	B-Dissolved Oxygen (%)	1	24.65	24.65	104.85	0.0005
	AB	1	20.25	20.25	86.13	0.0007
	A^2	1	42.94	42.94	182.62	0.0002
	B^2	1	56.17	56.17	238.90	0.0001
	Error	1	0.058	0.058	–	–
	Total	9	142.44	–	–	–
Cell Growth (Abs 600 nm)	Model	3	11.58	3.86	6.82	0.0232
	A-Temperature (°C)	1	7.41	7.41	13.10	0.0111
	B-Dissolved Oxygen (%)	1	0.54	0.54	0.96	0.3655
	AB	1	3.63	3.63	6.41	0.0445
	Error	1	0.011	0.011	–	–
	Total	9	14.98	–	–	–

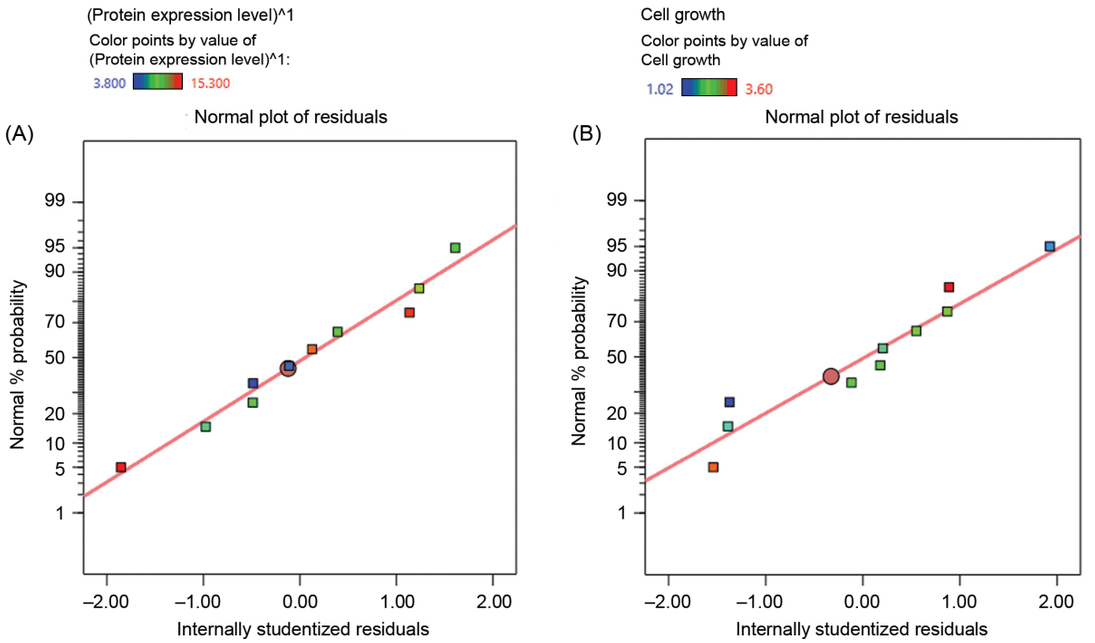

Fig. 3. Normal (%) probability plot of the studentized residuals for the model of recombinant β-NGF production (A) and cell growth (B).

were used as a tool to test the significance of each coefficient. The smaller the magnitude of *p* value, the corresponding coefficient was more significant. Also, a *p* value less than 0.05 indicated that the model terms were significant. The *p* values of terms presented in Table III indicated that both terms (A and B) had a significant influence on β-NGF production. In addition, the model is significant because *p* value is less than 0.05 (probe > F is 0.0002).

The other response was cell growth measured by absorbance at 600 nm and the effects of two parameters (post induction temperature and %DO) on this parameter were also examined. The model *F*-value of 6.82 from ANOVA of cell growth indicated that this model was also significant. The ANOVA indicated that this model and term of A (post induction temperature) were significant (*p* value < 0.05) but %DO did not significantly influence cell growth.

Model diagnostics. The normal (%) probability plot of the studentized residuals is a major tool to detect that errors are normally distributed and independent of each other (Luzier 1992). It can be seen from Fig. 3 that the errors of the models for the recombinant β-NGF production were normally distributed and insignificant.

Optimization of the process. The response surface plots and their corresponding contour plots described by the second-order polynomial equation were illustrated in Fig. 4. Each contour curve represents an infinite number of combinations of two tested variables. Fig. 4A and 4B indicate that the interaction of post induction temperature and %DO was significant. The β-NGF production increased with both post induction temperature and %DO till certain point and then decreased. The best post induction temperature and %DO for the maximum β-NGF production were 32°C and 20%, respectively. Fig. 4C and 4D show that post induction temperature had positive effect on cell growth but the effects of % DO on cell growth was not significant.

Large scale purification of β-NGF and bioactivity test. It must be noted that the β-NGF produced by pET39b::βNGF vector has his-tag tail; therefore it was purified using an affinity chromatography column (Ni^{+2}-NTA agarose). To evaluate whether the purified β-NGF is functional or not, differentiation of PC12 cell line in the presence of the purified β-NGF was studied. Our data showed that the purified β-NGF was capable of differentiate PC12 cell line to the nerve cells similarly to the standard β-NGF (data not shown) (Hajihassan et al. 2017).

Discussion

Nowadays, recombinant production of functional proteins is in high demand in modern biotechnology. *E. coli* is the most widely used expression host employed to produce many recombinant proteins. Optimization of culture conditions and induction parameters allows us to achieve the high quantity of the interest protein in this valuable host. Most of the studies in this area are in a shake-flask culture (Azaman et al. 2010; Lar-

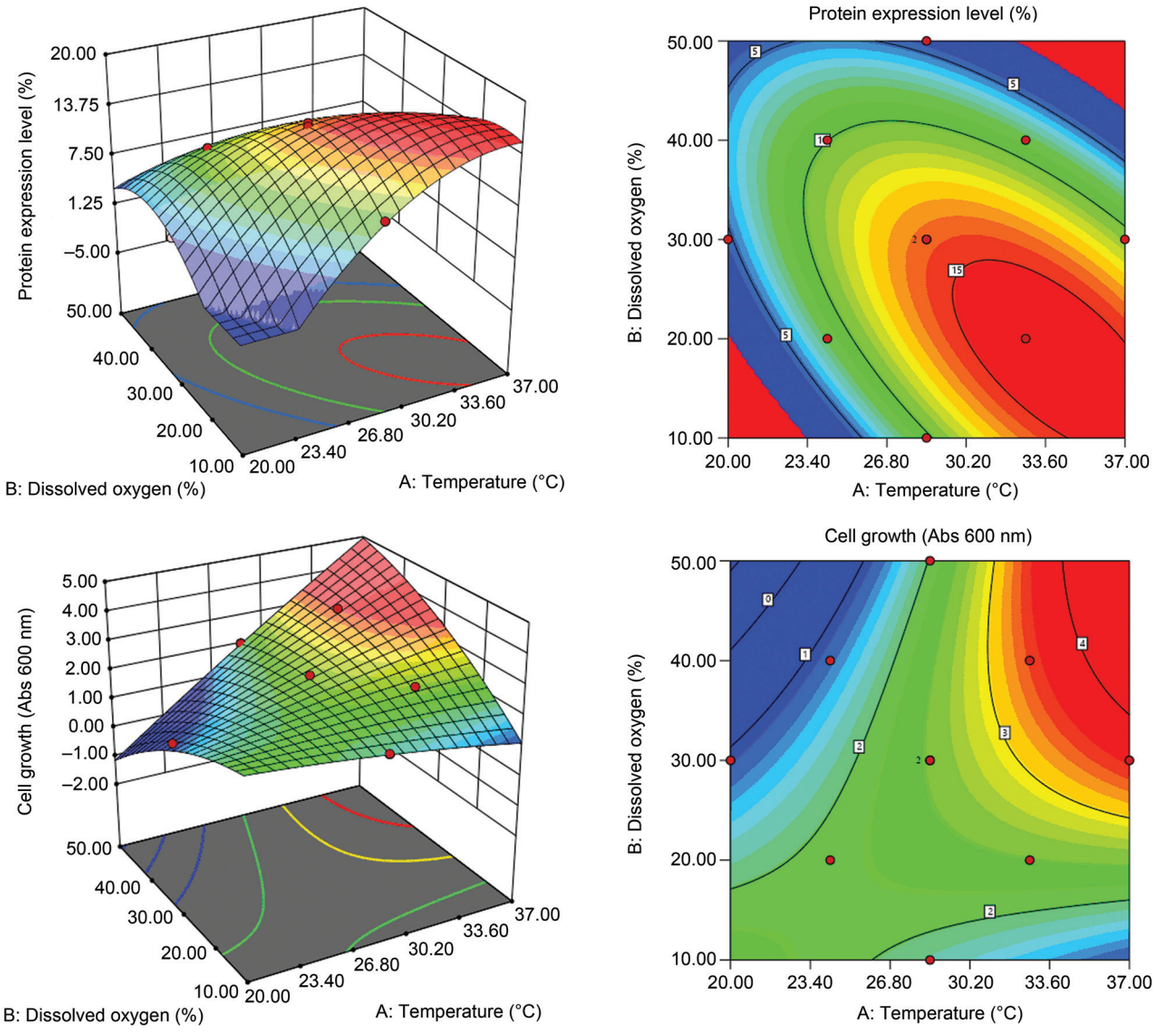

Fig. 4. Response surface plots and their counter plots show the effect of post induction temperature and % dissolved oxygen on the protein (β-NGF) expression level (A and B) and cell growth (C and D).

entis et al. 2011; Papaneophytou and Kontopidis 2012; Papaneophytou et al. 2013; Savari et al. 2015; Gholami Tilko et al. 2016). For example, in a survey recently conducted by our laboratory optimization of recombinant β-NGF production in shake-flask culture was done (Gholami Tilko et al. 2016). In the above study, the optimum post induction time and temperature were 2 h and 25°C, respectively. However, cell growth and protein production in the bioreactor scale are influenced by different parameters affecting each other (Zhong 2010). Since very few studies have been done to optimize protein production, especially recombinant proteins in industrial scale, our aim in this study was to find out the optimal conditions for high level production of recombinant β-NGF in *E. coli* grown in a 5-l bioreactor. Among the most important factors influencing the recombinant protein production level, the post induction time, post induction temperature and %DO play critical roles. Optimization of these parameters results in higher productivity and reduces cost of fermentation. It is of interest to note that the best induction time can be established for each recombinant protein by a trial and error. However, in many reports the expression is reduced over extended induction times, and this phenomenon is likely due to the recombinant protein degradation by bacterial proteinases (Joshi and Puri 2005; Choi et al. 2006; Papaneophytou et al. 2013; Gholami Tilko et al. 2016). In this study to produce high level of recombinant β-NGF in the bioreactor scale, the post induction times between 2 to 12 hours was examined. According to our results, the post induction time of 2 h is optimal for improved recombinant β-NGF production level.

Another factor that influences the expression level is post induction temperature. As the best cultivation temperature for *E. coli* is between 35–37°C, lowering the

temperature causes a lower growth rate. On the other hand, in the case of expression of complex heterologous proteins, usually a decrease in the temperature during protein synthesis especially after IPTG induction and, consequently, slower growth rate of bacteria favor the production of the active protein form. It is also important to note that in the literature different post induction temperatures have been reported for overproduction of different heterologous proteins in the bioreactor scale or shake-flask cultures (Azaman et al. 2010; Papaneophytou and Kontopidis 2012; Morowvat et al. 2015; Savari et al. 2015; Su et al. 2015; Gholami Tilko et al. 2016). Therefore, the evaluation of the best post induction temperature for β-NGF production on bioreactor scale was one of the goals of this study. Also %DO is a critical factor in the over expression of recombinant proteins, especially in the bioreactor scale, because it can affect directly cell growth (Ram et al. 2015; Wang et al. 2016). Previous studies showed that bacterial growth and protein production were dependent on the degree of oxygenation; distribution of oxygen to the bacteria in the bioreactor was related to the intensity of aeration and stirring or agitation (Çalik et al. 2004; Kaya-Çeliker et al. 2009). On the other hand, at high degree of DO as a result of high agitation speed break down of the cells takes place (Banerjee et al. 2009). Recently, Zaslona et al. (2015) showed that production of recombinant 1,3-β-glucanase in *E. coli* increased when moderate stirring and oxygenation was applied. Therefore, to evaluate the effects of %DO and post induction temperature on β-NGF expression level and cell growth rate, we used response surface methodology (RSM) as a high effective method (Elibol and Ozer 2002; Wejse et al. 2003; Rui et al. 2009). Thus, various combinations of %DO (10–50) and post induction temperature (20–37°C) as two independent variables were examined (Tables I and II). Our results indicated that the highest β-NGF production in the bioreactor scale was obtained at post induction temperature and %DO of 32.75°C and 20%, respectively (Fig. 1). On the other hand, in this run the maximum cell growth or dry cell weigh were not obtained. It can be concluded that increasing cell growth does not necessarily increase recombinant protein expression level. The most important result obtained here was that the highest β-NGF production was achieved at moderate %DO (20%) and post induction temperature of 32°C. These results are in agreement with the previous data presented by others on the production of various proteins in *E. coli* in the bioreactor scale (Zaslona et al. 2015).

In summary, the response surface methodology was successfully used for the optimization of recombinant β-NGF production by varying the induction parameters and %DO. Our results could be beneficial for industrial production of β-NGF.

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Conflict of interest

The authors declare that they have no conflict of interest.

Literature

Azaman SNA, Ramakrishnan NR, Tan JS, Rahim RA, Abdullah MP, Ariff AB. 2010. Optimization of an induction strategy for improving interferon-α2b production in the periplasm of *Escherichia coli* using response surface methodology. Biotechnol Appl Biochem. 56(4):141–150.

Banerjee A, Dubey S, Kaul P, Barse B, Piotrowski M, Banerjee UC. 2009. Enantioselective nitrilase from Pseudomonas putida: cloning, heterologous expression, and bioreactor studies. Mol Biotechnol. 41(1):35–41.

Bocchini V, Angeletti PU. 1969. The nerve growth factor: purification as a 30 000-molecular-weight protein. Proc Natl Acad Sci USA. 64(2):787–794.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72(1–2):248–254.

Burnette WN. 1981. "Western Blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem. 112(2):195–203.

Çalik P, Yilgör P, Ayhan P, Demir AS. 2004. Oxygen transfer effects on recombinant benzaldehyde lyase production. Chem Eng Sci. 59(22–23):5075–5083.

Choi JH, Keum KC, Lee SY. 2006. Production of recombinant proteins by high cell density culture of *Escherichia coli*. Chem Eng Sci. 61(3):876–885.

Choi JH, Lee SY. 2004. Secretory and extracellular production of recombinant proteins using *Escherichia coli*. Appl Microbiol Biotechnol. 64(5):625–635.

Elibol M, Ozer D. 2002. Response surface analysis of lipase production by freely suspended *Rhizopus arrhizus*. Process Biochem. 38(3):367–372.

Fan BS, Lou JY. 2010. Recombinant expression of human nerve growth factor beta in rabbit bone marrow mesenchymal stem cells. Mol Biol Rep. 37(8):4083–4090.

Gholami Tilko P, Hajihassan Z, Moghimi H. 2017. Optimization of recombinant β-NGF expression in *Escherichia coli* using response surface methodology. Prep Biochem Biotechnol. 47(4):406–413.

Greene LA, Tischler AS. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci USA. 73(7):2424–2428.

Hajihassan Z, Abdi M, Roshani Yasaghi E, Rabbani-Chadegani A. 2017. Optimization of recombinant beta-NGF purification using immobilized metal affinity chromatography. Minerva Biotecnol. 29:126–132.

Heese K, Low JW, Inoue N. 2006. Nerve growth factor, neural stem cells and Alzheimer's disease. Neurosignals. 15(1):1–12.

Joshi BH, Puri RK. 2005. Optimization of expression and purification of two biologically active chimeric fusion proteins that consist of human interleukin-13 and Pseudomonas exotoxin in *Escherichia coli*. Protein Expr Purif. 39(2):189–198.

Kaya-Çeliker H, Angardi V, Çalık P. 2009. Regulatory effects of oxygen transfer on overexpression of recombinant benzaldehyde

lyase production by *Escherichia coli* BL21 (DE3). Biotechnol J. 4(7):1066–1076.

Kurokawa Y, Yanagi H, Yura T. 2001. Overproduction of bacterial protein disulfide isomerase (DsbC) and its modulator (DsbD) markedly enhances periplasmic production of human nerve growth factor in *Escherichia coli*. J Biol Chem. 276(17):14393–14399.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227(5259):680–685.

Larentis AL, Argondizzo APC, Esteves GS, Jessouron E, Galler R, Medeiros MA. 2011. Cloning and optimization of induction conditions for mature PsaA (pneumococcal surface adhesin A) expression in *Escherichia coli* and recombinant protein stability during long-term storage. Protein Expr Purif. 78(1):38–47.

Lee EJ, Lee BH, Kim BK, Lee JW. 2013. Enhanced production of carboxymethylcellulase of a marine microorganism, *Bacillus subtilis* subsp. subtilis A-53 in a pilot-scaled bioreactor by a recombinant *Escherichia coli* JM109/A-53 from rice bran. Mol Biol Rep. 40(5):3609–3621.

Luzier WD. 1992. Materials derived from biomass/biodegradable materials. Proc Natl Acad Sci USA. 89(3):839–842.

Morowvat MH, Babaeipour V, Rajabi Memari H, Vahidi H. 2015. Optimization of fermentation conditions for Recombinant Human Interferon Beta production by *Escherichia coli* using the response surface methodology. Jundishapur J Microbiol. 8(4):e16236.

Papaneophytou CP, Kontopidis GA. 2012. Optimization of TNF-α overexpression in *Escherichia coli* using response surface methodology: purification of the protein and oligomerization studies. Protein Expr Purif. 86(1):35–44.

Papaneophytou CP, Rinotas V, Douni E, Kontopidis G. 2013. A statistical approach for optimization of RANKL overexpression in *Escherichia coli*: purification and characterization of the protein. Protein Expr Purif. 90(1):9–19.

Ram KS, Babu KS, Tabitha G, Rajeshwari K, Lakshmi GJ, Gowd BB, Peravali JB, Rao MS, Rao PV. 2015. High cell density cultivation for the production of industrially important engineered Bi-functional recombinant staphylokinase variant from salt inducible *Escherichia coli* GJ1158. I.J.B.S.B.T. 7:327–338.

Ren Q, Henes B, Fairhead M, Thöny-Meyer L. 2013. High level production of tyrosinase in recombinant *Escherichia coli*. BMC Biotechnol. 13(1):18–18.

Rosano GL, Ceccarelli EA. 2014. Recombinant protein expression in *Escherichia coli*: advances and challenges. Front Microbiol. 5:172.

Rui L, Wei-chang C, Wei-peng W, Wen-yan T, Xue-guang Z. 2009. Optimization of extraction technology of *Astragalus polysaccharides* by response surface methodology and its effect on CD40. Carbohydr Polym. 78(4):784–788.

Saez NJ, Vincentelli R. 2014. High-throughput expression screening and purification of recombinant proteins in *E. coli*. Methods Mol Biol. 1091:33–53.

Sambrook J, Russell DW. 2001. Molecular cloning: a laboratory manual 3rd edition. Cold Spring Harbor, New York (USA): Cold Spring-Harbour Laboratory Press.

Savari M, Zarkesh Esfahani SH, Edalati M, Biria D. 2015. Optimizing conditions for production of high levels of soluble recombinant human growth hormone using Taguchi method. Protein Expr Purif. 114:128–135.

Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. Nat Methods. 9(7):671–675.

Schumann W, Ferreira LCS. 2004. Production of recombinant proteins in *Escherichia coli*. Genet Mol Biol. 27(3):442–453.

Su L, Huang Y, Wu J. 2015. Enhanced production of recombinant *Escherichia coli* glutamate decarboxylase through optimization of induction strategy and addition of pyridoxine. Bioresour Technol. 198:63–69.

Tegel H, Ottosson J, Hober S. 2011. Enhancing the protein production levels in *Escherichia coli* with a strong promoter. FEBS J. 278(5):729–739.

Thiry M, Cingolani D. 2002. Optimizing scale-up fermentation processes. Trends Biotechnol. 20(3):103–105.

Wang D, Wang C, Wu H, Li Z, Ye Q. 2016. Glutathione production by recombinant Escherichia coli expressing bifunctional glutathione synthetase. J Ind Microbiol Biotechnol. 43(1):45–53.

Wejse PL, Ingvorsen K, Mortensen KK. 2003. Xylanase production by a novel halophilic bacterium increased 20-fold by response surface methodology. Enzyme Microb Technol. 32(6): 721–727.

Wiesmann C, de Vos AM. 2001. Nerve growth factor: structure and function. Cell Mol Life Sci. 58(5):748–759.

Zaslona H, Trusek-Holownia A, Radosinski L, Hennig J. 2015. Optimization and kinetic characterization of recombinant 1,3-β-glucanase production in *Escherichia coli* K-12 strain BL21/pETSD10 – a bioreactor scale study. Lett Appl Microbiol. 61(1): 36–43.

Zhong JJ. 2010. Recent advances in bioreactor engineering. Korean J Chem Eng. 27(4):1035–1041.