Optimization of culture conditions for a synthetic gene expression in *Escherichia coli* using response surface methodology: the case of human interferon beta

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Abstract

A human interferon beta (hINF-β) synthetic gene was optimized and expressed in *Escherichia coli* BL21-SI using a vector with the T7 promoter. To determine the best culture conditions such as culture medium, temperature, cell density and inducer concentration, we used the response surface methodology and a Box-Behnken design to get the highest hINF-β production. The maximum hINF-β production of 61 mg l^{-1} was attained using minimum medium and the following predicted optimal conditions: temperature of 32.5°C, cell density of 0.64, and inducer concentration of 0.30 M NaCl. We also demonstrated that the response surface methodology is effective for the optimization of recombinant proteins production using synthetic genes.

Keywords: Codon bias, *E. coli* BL21-SI, multiple sclerosis, response surface methodology (RSM), synthetic gene, yeast extract.

1. Introduction

Escherichia coli is the most used host for the over-expression of recombinant proteins. Weak expression of foreign genes has been associated to the difference of codon usage between eukaryotic organisms and those preferred by *E. coli* [1]. There are strategies to overcome the problem of codon bias, such as commercial *E. coli* strains containing copies of tRNA genes, which are rare in *E. coli* but frequently used in other organisms [2]. However, there are not described *E. coli* strains containing all "rare" tRNA genes. Furthermore, the rapid degradation of mRNA proceeding from foreign cDNA causes poor expression in heterologous systems [3]. Thus, the replacement of rare codons in the target gene improves the protein expression [4]. However, culture conditions such as inducer concentration, cell density (induction time), and temperature also affect the recombinant proteins production [5-10].

Human interferon β (hINF-β) is a glycoprotein involved in antiviral, antiproliferative and immunoregulatory processes. The recombinant hINF-β has been approved for the treatment of sclerosis multiple, arthritis, genital condylomata acuminata and malignant melanoma therapy [11-14]. The glycosilated hINF-β produced in animal cells is more stable than the non-glycosilated form produced in bacteria, however, the non-glycosilated hINF-β is used for therapy and *in vitro* studies [15]. In this work, we assessed the production of hINF-β using a synthetic gene optimized for its expression in *E. coli* BL21-SI [16] (a T7 system inducible with NaCl) in minimum medium. The effect of yeast extract and culture conditions such as temperature, cell density and NaCl concentration was evaluated using the response surface methodology (RSM).

2. Materials and methods

2.1. Bacterial strains and plasmids

The pCR4-585 vector containing the optimized synthetic hINF-β gene was purchased from Entelechon GmbH (Regensburg, Germany). The *Nde*I and *Bam*HI sites were added to hINF-β synthetic gene by PCR using pCR4-585 as template and the primers sense 5´-CATATGAGCTATAACCTG-3' and anti-sense 5´-GGATCCTTAATTACGCAG-3´. The *Nde*I-hINF-β–*Bam*HI amplified fragment was cloned in a pET12a (Novagen, Darmstadt, Germany) vector to construct pTPM13 (Fig.1), then *E. coli* BL21-SI (GIBCO, Darmstadt, Germany) was transformed by heat-shock method with the pTPM13 plasmid and transformed clones were selected on LBON medium (Luria-Bertani salt-free) with 100 mg l^{-1} ampicillin.

2.2 Media and culture conditions

The minimum medium contains per liter: 5.0 g glucose, 3.5 g $(NH_4)_2HPO_4$, 3.5 g KH_2PO_4, 1.0 g $MgSO_4$, 40 µg thiamine and 100 mg ampicillin. The pH was adjusted to 7.4 with NaOH prior to sterilization (20 min at 121°C). The supplemented medium consists in minimum medium with 5 g l^{-1} of yeast extract (Difco Laboratories, Franklin Lakes, NJ, USA). For all experiments, pre-inocula were grown in supplemented medium overnight at 37°C and shaken at 250 rpm. Batch cultures were carried out in 500 ml Erlenmeyer flasks with 100 ml of minimum medium or supplemented medium inoculated at a cell density of 0.20 at 620 nm, and shaken at 250 rpm. Each experiment was performed under different conditions of temperature, cell density and

inducer concentration as described in experimental design (Table 1).

2.3. Experimental design and optimization by RSM

To study the effect of three independent variables on the production of hINF-β in *E. coli* BL21-SI/pTPM13, we constructed a random experimental design via Box-Behnken strategy [17]. The independent variables were temperature (factor A), cell density (factor B) and inducer concentration (factor C). Each variable was divided into three levels and twelve treatments were made in accordance to Box-Behnken factorial design (Table 1). Three additional experiments were included in order to analyze the effect of non-adjustable data. The production of hINF-β was tested using both minimum medium and supplemented medium via the Box-Behnken design. The analysis of RSM and Analysis of Variance (ANOVA) was done using Minitab^{TM} v 14.0 software (Minitab, Inc., Pennsylvania, USA). The significance of each coefficient (linear or quadratic) was determined with the Student's *t*-test, at 0.05 probability level. The optimal values were obtained solving the regression equation by the Newton-Raphson method and analyzing the response surface contour.

2.4. Analytical methods

Cell density was measured at 620 nm (OD_{620}) in a Varian Cary Bio-50 spectrophotometer (Varian, Inc., Palo Alto, CA, USA). Glucose concentration was determined by the dinitro-salicilic acid (DNS) method for reducing sugars, using glucose as standard [18]. Protein concentration was determined by the method of Lowry et al. [19], using bovine serum albumin

(BioRad, Hercules, CA, USA) as standard. Cell samples of 2 ml were collected, centrifuged at 5000 x *g* for 10 min, resuspended in PBS (0.1M pH 7.8) and lysed by sonication. Then, proteins were separated in a 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (15% SDS-PAGE) in a Miniprotean III System (BioRad). Proteins were stained with Coomassie Blue R-250 (BioRad). For Western blot, proteins were transferred from gel onto a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA) using a Semi-Dry Transblot (BioRad). The membrane was blocked with Svelty milk (3% w/v in PBS). The membrane was incubated with the rabbit anti-hINF-β polyclonal antibody (PBL Biomedical Lab., Piscataway, NJ, USA), followed by goat anti-rabbit IgG antibody conjugated to alkaline phosphatase (BioRad), and visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP, Amersham Biosciences). hINF-β concentration was measured by densitometry using the Quantity One™ v 4.5 software (BioRad). Recombinant non-glycosilated hINF-β (PBL Biomedical Lab.) was used as standard.

3. Results and discussion

3.1. Design of hINF-β synthetic gene

The wild-type hINF-β gene has 27 “rare codons” compared to those preferred by *E. coli*, from which eight are classified as low- usage codons according to Makrides classification [4]. Since, the codon context of specific genes could have adverse effects on both the quantity and quality of proteins levels [4]. Therefore, using codon bias we designed a synthetic hINF-β gene to be expressed in *E. coli* (Fig. 2). The resultant optimized gene had 77.25% of identity with respect to

the wild-type gene. A summary of codon preference in *E. coli* based on the Ikemura classification [20] and codon usage in the wild-type and the optimized synthetic hINF-β gene is shown in Table 2. The most significant changes were for Arginine, Leucine, Glycine and Proline codons. Leucine is the most abundant aminoacid in hINF-β sequence, similar to "typical" *E. coli* proteins [21]. It is likely that similarity on amino acid usage would improve the performance of synthetic hINF-β in *E. coli*. Previous reports showed that expression of mammalian genes in *E. coli* increased after codon-optimization [22-24].

3.2. Kinetic of hINF-β production

A typical batch culture of *E. coli* BL21-SI/pTPM13 in supplemented medium is shown in Fig. 3 (experiment 8 from the Box-Behnken design). Cell density increased to a maximum of 3.9, and thereafter it remained constant until the end of the culture (Fig. 3A). During this period, glucose was consumed and cell growth ceased upon glucose depletion (Fig. 3B). The hINF-β concentration increased from 0 to 11 mg l^{-1} after the induction with 0.15 M NaCl (Fig. 3C). Cultures maintained at other culture conditions showed a similar trend to those in Fig. 3, although the parameters measured, their maximum concentrations, and times to reach them were different in each case. Fig. 4A shows the protein patterns obtained from experiment 10. The identity of the hINF-β was confirmed by Western blot using recombinant hINF-β as standard (Fig. 4B). There are several reports on the production of recombinant proteins using salt induction systems in LBON [16, 25, 26]. However, in our knowledge, there are not previous reports using minimum medium for expression proteins using *E. coli* BL21-SI.

3.3. Optimization of hINF-β production

The Box-Behnken design, the experimental and predicted results for hINF-β production in minimum medium and supplemented medium are summarized in Table 1. The cultures in minimum medium showed a higher hINF-β concentration than those cultures in supplemented medium carried out under the same operational conditions. The highest hINF-β production was attained in minimum medium for the experiment 10. For the cultures using supplemented medium, the significant factors were B, A^2 and B^2 (Table 3), whereas for cultures in minimum medium the significant factors were B, A^2, B^2, C^2, AB and AC interaction (Table 4). To estimate the optimal region of hINF-β production in minimum medium or supplemented medium, second-order models were fitted to the hINF-β observed results (data from Table 1) and the response surface graphs are shown in Fig. 5.

For cultures in supplemented medium the model is described by Eq. (1), where the variables are specified in their original units as follows:

$$Y \text{ (supplemented medium)} = -264.22 + 17.45\,A + 38.33\,B - 70.74\,C - 0.288\,A^2 - 34.90\,B^2 - 37.04\,C^2 + 0.42\,AB + 2.96\,AC - 20.83\,BC \quad (1)$$

Where Y is the response variable (hINF-β production), and A, B and C are the independent variables (temperature, cell density and inducer concentration, respectively). The standard error of the model was 3.884 and according to R^2 value, the predictors included in the model explain 79.0% of the variance in hINF-β production. For the set of experiments in supplemented medium,

the maximum hINF-β concentration was attained when temperature, cell density and inducer concentration were 31.6°C, 0.69 and 0.15 M, respectively, giving a predicted hINF-β concentration of 19 mg l^{-1}.

The mathematical model representing the hINF-β production in minimum medium in the experimental region studied is explained by Eq. (2).

$$Y\ (\text{minimum medium}) = -1354.58 + 78.51\ A + 243.33\ B + 420.74\ C - 1.20\ A^2 - 173.18\ B^2 - 787.04\ C^2 - 0.83\ AB + 1.48\ AC + 12.5\ BC \quad (2)$$

The standard error was 0.695 and the correlation coefficient (R^2) was 99.9%. These values indicated a good fitting between the model and the experimental data, which explains almost the total variance in the hINF-β production in minimum medium. In this case, the maximum concentration of hINF-β was attained when temperature, cell density and inducer concentration were 32.5 °C, 0.64 and 0.30 M, respectively, giving a predicted hINF-β production of 61 mg l^{-1}. These operational conditions are the optimal values predicted to improve the hINF-β production. The culture conditions suggested by the provider for *E. coli* BL21-SI are: LBON medium, 37°C, 0.6 and 0.3M NaCl [27], which those the hINF-β production was 12 mg l^{-1} (data non-shown), whereas using a minimum medium and the optimal conditions proposed by the RSM, the amount of hINF-β increased 5-fold. Goeddel et al. [28] reported a maximum hINF-β production of 0.2 mg l^{-1} using the wild-type gene cloned in *E. coli* and using minimum medium with casaminoacids, while we produced 95 and 305-fold more hINF-β in supplemented medium and minimum medium, respectively. Skoko et al. [29] reported a maximum hINF-β production of 12 mg l^{-1} in *Pichia pastoris* cultures. Aforementioned, we attained the highest hINF-β production using

minimum medium. Similar to the results obtained here, Ling [30] reported that recombinant protein ZZT2 production by *E. coli* was higher using an aminoacids-free medium than cultures supplemented with aminoacids. While Shin et al. [31] reported that the addition of yeast extract onto the culture medium enhanced the production of human proinsulin in *E. coli* cultures. Hence, we strongly suggest assaying the use of aminoacids or yeast extract to design the culture medium for the production of recombinant proteins in each expression system. RSM has been used previously for the optimization of secondary metabolite production such as xylitol by *Candida guilliermondii* [32], native proteins such as xylanase by *Bacillus circulans* [33] and recombinant proteins such as endochitinase by *E. coli* [9]. Our work is the first report showing that the response surface methodology is effective for the optimization of recombinant proteins production using synthetic genes.

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Figure captions

Fig. 1. Schematic representation of pTPM13. The T7 promoter drives the hINF-β synthetic gene.

Fig. 2. Alignment of nucleotide sequence of wild-type and the optimized synthetic hINF-β gene used in this work.

Fig. 3. Growth kinetics of *E. coli* BL21-SI/pTPM13 in supplemented medium for the experiment 8: A) Cell density [●](OD_{620nm}), B) Glucose concentration [■] (g l^{-1}), C) hINF-β concentration [▲](mg l^{-1}). Arrow shows induction time with NaCl.

Fig. 4. Protein patterns and Western-Blot analysis for hINF-β obtained in *E. coli* BL21-SI/pTPM13 cultures from the experiment 10. A) Typical protein patterns in minimum medium. Lane 1, protein ladder (Invitrogen); lane 2, culture before induction; lanes 3 to 7, total cell protein of six samples after induction. B) Western-Blot for samples described above. Lane 1, standard hINF-β (PBL Biomedical Lab); lane 2-7, six samples after induction.

Fig. 5. Response surface plot of hINF-β concentration as a function of temperature and cell density: A) Cultures in supplemented medium induced with 0.15M NaCl. B) Cultures in minimum medium induced with 0.30 M NaCl.