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Enhanced Expression of Recombinant Activin A in *Escherichia coli* by Optimization of Induction Parameters

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Abstract

Activin A is a member of the transforming growth factor β super family. Because of its extensive clinical usages, its recombinant production is beneficial. In this study, activin A was expressed in *E. coli* using the pET 21a expression vector. The optimization of the activin A production in *E. coli* was done by using the response surface methodology (RSM). At this stage, the effect of IPTG and lactose concentration as inducers on protein production was investigated. The effect of different post-induction time and temperature on protein production was then studied in two strains of *E. coli* (BL21(DE3) and BL21(DE3) plysS). For enhanced expression, the optimum IPTG and lactose concentrations were 1.5 mM and 0% W/V respectively. In the DE3 strain, the optimum post-induction time and temperature were 10 hours and 30°C respectively while in DE3 (plysS) these were 4 hours and 35°C respectively.

Keywords: Activin A; *E. coli*; RSM; Lactose; IPTG.

Introduction

Activin A, a member of the transforming growth factor β super family is a dimmer of βA subunits (βA βA) that are linked together by disulfide bond [1, 2]. Activin A produced in the body plays critical roles in cellular differentiation, apoptosis, metabolism, wound repair and maintenance of the neurons [3-5]. Because of its extensive biological roles, its recombinant production is beneficial. As its structure is complex with multiple disulfide bonds, its production has been done mostly in eukaryotic cells [6-8]. Despite the production of correctly folded proteins in these expression systems, there are nevertheless some disadvantages such as expensive culture media and low yield. On the other hand, *Escherichia coli* (*E. coli*) is the most widely used expression system for the production of recombinant proteins because it is capable of producing large quantity of proteins with a very low cost compared with other expression systems [9]. However, improved expression of many recombinant proteins in *E. coli* needs optimization of culture conditions and induction parameters [10-12]. It is worth nothing that the optimization of protein production using one factor at a time is time-consuming and a very labor-intensive approach. Response surface methodology (RSM) is the most common statistical method used for the optimization of various biochemical processes considering the effects of more than one factor at a time [13]. Up to now, there are many attempts to find out the

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best inducer concentration, IPTG or lactose, to trigger the protein expression from the T7 promoter in the commonly used pET expression vector [14-18]. Another alternative that is beneficial to study is investigation of effects of both inducers combined. Therefore, in this study we used RSM to determine the optimum values. Moreover, the effects of post-induction time and temperature for improved expression of activin A were evaluated in two strains of *E. coli*.

Materials and Methods

Materials

Anti his-tag monoclonal antibody, H_2O_2, IPTG, ampicillin and BSA were purchased from Sigma-Aldrich (USA) Company. Other reagents were procured from Merck (Germany).

Bacterial strain, expression vector and culture conditions

The BL21(DE3) and BL21(DE3) plysS strains of *E. coli* (Novagene-USA) were used as the host strains. pET21a(+) vector (Novagene-USA) contained histidine-tag sequence was used as an expression vector. Gene synthesis and cloning of the activin A cDNA to the pET21a expression vector was done by ShineGene (China).

pET21a::activin A was transformed in the both strains of *E. coli* using $CaCl_2$ and heat shock method [19]. Transformed cells were cultured in Luria-Bertani (LB) medium supplemented with 100 µg/ml ampicillin at 37°C and 200 rpm.

Protein expression

For protein expression, 1% dilution of an overnight culture of transformants was grown at fresh SOB medium (20 g/l Tryptone, 5 g/l yeast extract, 0.5 g/l NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 10 mM $MgSO_4$) at 37°C and 200 rpm up to OD_{600nm}, 0.6. Then lactose or isopropyl thio-B-D-galactoside (IPTG) at final concentrations obtained by RSM was added for inducing the promoter. Finally, the cells were grown for additional 2, 4, 6, 8, 10 or 12 h at 20, 25, 30, 35 or 37°C individually. The cells were harvested by centrifugation at 5000 *g* for 20 min and re-suspended in urea 8M. Cell disruption was done by sonication in 10 repeating cycles of 30s on/1 min off on ice. Bacterial proteins were obtained after overnight incubation at 37ºC followed by centrifugation at 12000 *g* for 30 min.

Experimental design

Response surface methodology (RSM) based on the central composite design (CCD) was used to analysis the effects of two independent variables (IPTG and lactose concentration) on the expression yield of recombinant human activin A in BL21(DE3) strain of *E. coli*. Furthermore, the concentration of total proteins extracted from bacteria was determined in each experiment by Bradford method and selected as response 2. Table 1 illustrates the minimum and maximum levels of variables chosen for trials in CCD. Also the values of experimental variables chosen in this study are presented in Table 1; variables were IPTG concentration (0, 0.75, 1.5, 2.25 and 3 mM) and lactose concentration (0, 1, 2, 3 and 4 %W/V). The design

Table 1. Experimental design levels. Values of independent variables (IPTG and lactose concentration) and corresponding levels used in a central composite design (normalized in -2, -1, 0, +1 and +2).

Variable name	Level				
	-2	**-1**	**0**	**1**	**2**
Inducer concentration (IPTG)(mM)	0	0.75	1.5	2.25	3
Inducer concentration (Lactose)(%w/v)	0	1	2	3	4

Table 2. The experimental design of 10 runs with 2 variables (IPTG and lactose concentration) and 2 responses (total protein concentration and recombinant protein expression level).

Run	Inducer concentration (IPTG mM)		Inducer concentration (Lactose %W/V)		Response 1 Recombinant Protein Expression Level (%)	Response 2 Total protein concentration (mg/ml)
1	-1	0.75 mM	-1	1% w/v	7.5	1.14
2	+1	2.25 mM	-1	1% w/v	10.4	3.76
3	-1	0.75 mM	+1	3% w/v	9.9	2.93
4	+1	2.25 mM	+1	3% w/v	5.9	0.32
5	-2	0.00 mM	0	2% w/v	6.9	2.77
6	+2	3.00 mM	0	2% w/v	9.4	3.55
7	0	1.50 mM	-2	0% w/v	22.9	6.59
8	0	1.50 mM	+2	4% w/v	8.0	0.69
9	0	1.50 mM	0	2% w/v	9.0	0.65
10	0	1.50 mM	0	2% w/v	9.1	0.63

consisted of 10 experimental runs with 2 replicated runs (centre points) and corresponding responses are shown in Table 2. The purpose of replication was to estimate the experimental errors. The data obtained from 10 experimental runs was 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 statistical analysis was performed using the soft ware package design expert version 7.1.2 (stat soft, USA). The significance of the model equation and model terms were evaluated by *F*-test. Finally, the optimum values of the variables were obtained by analyzing the response surface contour plots.

Protein concentration determination

The concentration of protein was determined with the Bradford assay using bovine serum albumin (BSA) as standard [20].

Immunoblot analysis

Dot blotting using anti-his tag monoclonal antibody conjugated with horseradish peroxidase was performed to detect activin A in protein samples spotted on nitrocellulose membrane (Millipore-USA). PBS containing 0.05% Tween20 and 3% W/V skimmed milk was used as blocking buffer. A solution of DAB and hydrogen peroxide in PBS was used as enzyme substrate to detect the protein of interest. The area of each spot on the nitrocellulose membrane representing the activin A expression level was calculated using Image J software [21].

Results and Discussion

Optimization of IPTG and Lactose concentration using response surface methodology

IPTG and Lactose are respectively synthetic and natural inducers of promoter in pET expression vectors. Many studies have been done to find out the best concentration of IPTG for achieving the highest level of specific recombinant protein expression [16-18]. Since

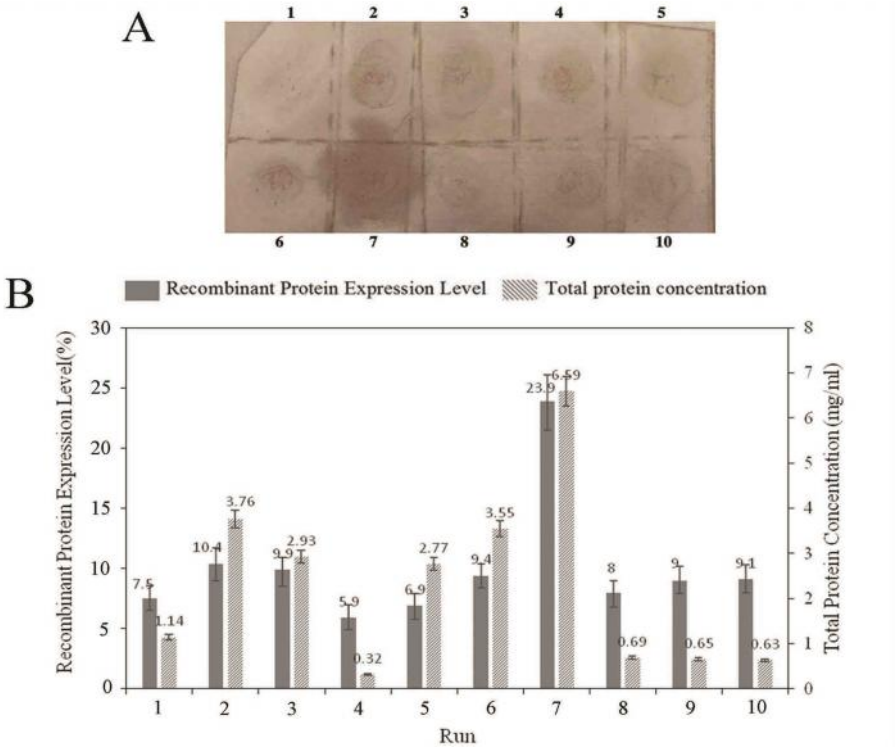

Figure 1. Recombinant protein (activin A) expression level analysis in different IPTG and lactose concentrations as independent variables in 10 experiments designed by CCD. (A) Dot blot analysis of extracted proteins from recombinant bacteria carrying pET21::activin A plasmid using anti-histag.HRP antibody. (B) % Recombinant protein expression level measured by ImageJ software in comparison with the concentration of total proteins extracted from recombinant bacteria measured by Bradford method; the areas of each dot on the nitrocellulose membrane in A section calculated by Image J software were considered as % Recombinant protein expression level. All experiments were repeated three times under the identical experimental conditions.

IPTG is expensive and toxic, some researchers have used lactose as a natural inducer instead of IPTG [14, 15]. As using both inducers in combination can be beneficial, in this study we used response surface methodology (RSM) as a high effective method to identify the interactive effects of IPTG and lactose and also the best concentration of them to obtain high level production of activin A. Therefore, different combinations of IPTG (0-3 mM) and lactose (0-4 %W/V) concentrations were chosen (Tables 1 and 2). As indicated in Table 2, different total protein production ranging from 0.32 to 6.59 mg/ml was obtained as measured by the Bradford method. Since the main purpose of this study was to obtain a high concentration of activin A, we used dot blotting analysis in which an anti-his tag monoclonal antibody recognized recombinant activin A in the extracted protein sample (Fig 1.A). As each colored dot indicated the interaction of antibody with the His-tag tail on the recombinant activin A, the areas of each dot on the nitrocellulose membrane were calculated by Image J software [21]. It is obvious in Figure 1 and Table 2 that the maximum yield of the recombinant activin A (% recombinant protein expression level) was achieved at 1.5 mM of IPTG and 0% W/V lactose. Also at this run (run 7), total protein concentration was at the maximum level. We showed in this study that the very low concentrations of IPTG are not favored for activin A production. Likewise, very high concentrations of lactose but in the absence of IPTG can be suitable both for total protein production and recombinant activin A expression.

These results are in agreement with the previous results obtained by others in which moderately high inducer concentrations were introduced [16, 17].

The three-dimensional response surface plots were generated to investigate the interaction between IPTG and lactose concentrations and their effects on the response of total protein concentration and percentage of recombinant protein (activin A) expression level. As shown in Figure 2, activin A production increased with increasing the IPTG concentration. In addition, when lactose concentration was increased, in the absence of IPTG, activin A expression level increased (Fig 2A and B). The best IPTG and lactose concentration for maximum activin A production were 1.5 mM and 0%

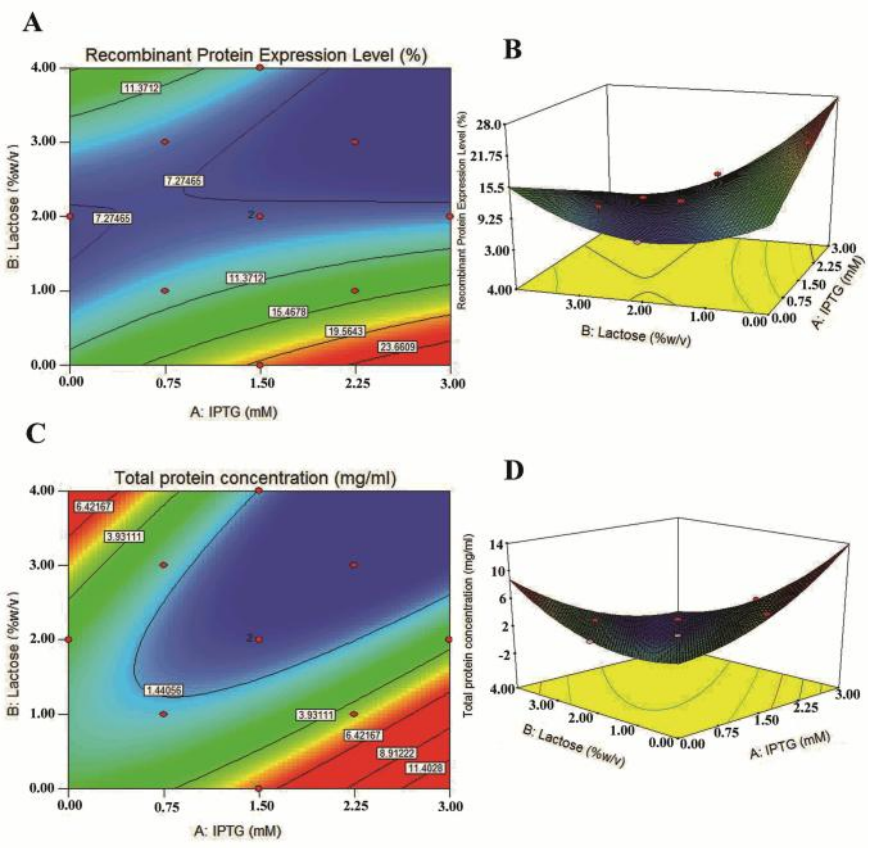

Figure 2. Response surface plot and its contour plot show the effect of IPTG and lactose concentration on recombinant protein (activin A) production (A and B) and on total protein concentration (C and D).

Table 3. Estimated parameters and analysis of variance (ANOVA) of the design for recombinant production of activin A in *E. coli*. A and B factors are IPTG and lactose variables respectively.

	Source of variation	Sum of squares	Mean squares	*F*-value	*P* value (Probe > *F*)
Recombinant Protein Expression Level (%)	Model	187.90	46.98	5.30	0.0481
	A-IPTG	1.27	1.27	0.14	0.7208
	B-Lactose	95.77	95.77	10.81	0.0218
	AB	11.90	11.90	1.34	0.2989
	A^2	0.089	0.089	8.04	0.9328
	B^2	78.97	78.97	8.91	0.0306
Total Protein Concentration (mg/ml)	Model	32.98	6.60	8.48	0.0297
	A-IPTG	0.21	0.21	0.26	0.6344
	B-Lactose	15.08	15.08	19.39	0.0117
	AB	6.84	6.84	8.79	0.0413
	A^2	6.56	6.56	8.44	0.0439
	B^2	9.30	9.30	11.96	0.0258

W/V respectively (Fig 2A and B). Also, Figure 2C and D show the interactive effects of IPTG and lactose on total protein production. This is obvious that increasing the lactose or IPTG concentration alone has positive effect on the total protein production. The statistical significance of the model was evaluated by the *F*-test (ANOVA). The models were significant with both *P* values less than 0.05 (Table 3).

Effect of post induction time and temperature on activin A production in two strains of E. coli

It has been widely reported that post-induction time and temperature have effects on recombinant protein expression level [16, 17, 22]. Also, some strains may be preferred in the production of recombinant proteins [17]. In this study we used different post-induction times ranging from 2 to 12 hours in two strains of *E. coli*, namely BL21 (DE3) and BL21 (DE3) plysS. Induction of promoter in each experiment was done with 1.5 mM IPTG. As indicated in Figure 3, the highest activin A production was obtained with post-induction time of 4h in the plysS strain and 10h in DE3 strain.

In addition, the effect of post-induction temperature

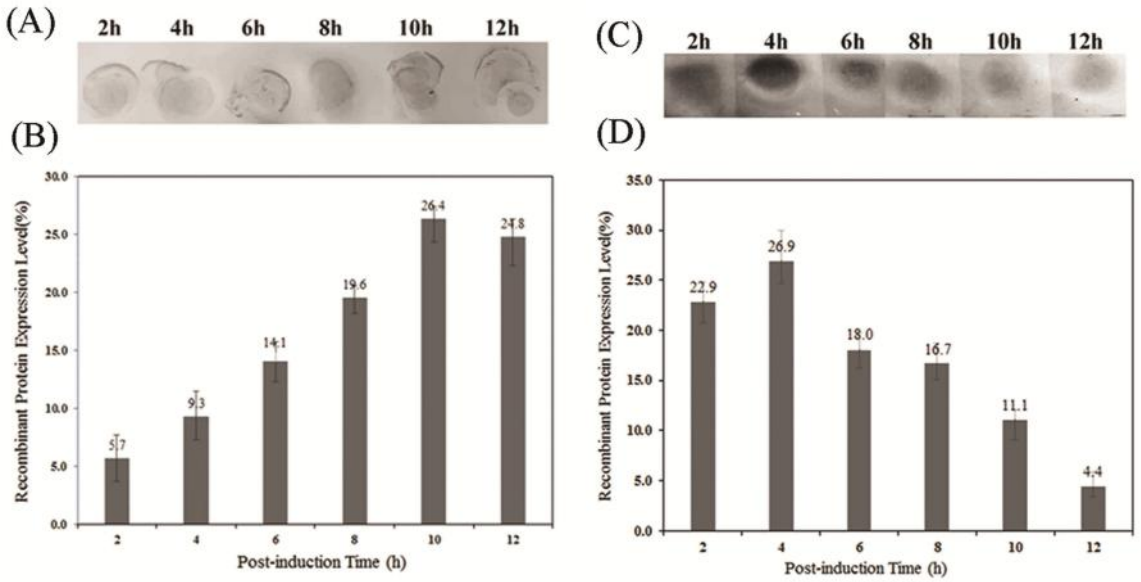

Figure 3. Recombinant protein expression level analysis in different post induction times (2, 4, 6, 8, 10 and 12 hours after induction with IPTG). A and C are respectively dot blot analysis of extracted proteins from DE3 and DE3 (plys S) strains carrying pET21::activin A plasmid using anti-histag.HRP antibody. B and D are % Recombinant protein expression level measured by ImageJ software in DE3 and DE3 (plys S) strains respectively; the areas of each dot on the nitrocellulose membrane in A and C sections calculated by Image J software were considered as % Recombinant protein expression level. All experiments were repeated three times under the identical experimental conditions.

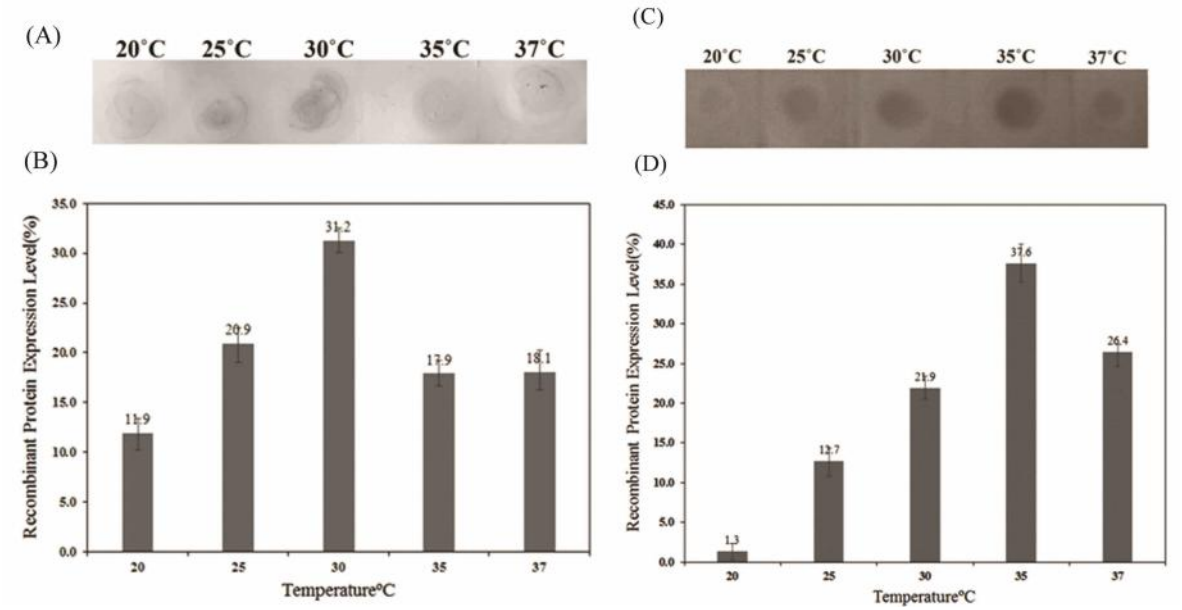

Figure 4. Recombinant protein expression level analysis in different post induction temperatures (20, 25, 30, 35 and 37 °C). A and C are respectively dot blot analysis of extracted proteins from DE3 and DE3 (plys S) strains carrying pET21::activin A plasmid using anti-histag.HRP antibody. B and D are % Recombinant protein expression level measured by ImageJ software in DE3 and DE3 (plys S) strains respectively; the areas of each dot on the nitrocellulose membrane in A and C sections calculated by Image J software were considered as % Recombinant protein expression level. All experiments were repeated three times under the identical experimental conditions.

was studied when post induction time was kept constant (4h for plysS and 10h for DE3). Results presented in Figure 4 indicated that the highest recombinant activin A expression level was obtained at 35 ºC and 30 ºC in plysS and DE3 strains respectively. These results indicate that the effects of post-induction time and temperature on recombinant activin A expression level are totally different in these two strains.

Conclusion

In conclusion, IPTG alone (without lactose) is sufficient for enhancing the expression of recombinant activin A expression in *E. coli*. We found that 1.5 mM of IPTG gives the highest level of recombinant activin A production. Our results also confirmed that expression conditions are strain-specific where those found to be optimum in one strain may not be effective for another strain. We suggest that the best post induction time and temperature for activin A production in *E. coli* DE3 strain was 10 hours and 30 ºC respectively but in DE3 (plysS) strain post induction time and temperature of 4 hours and 35 ºC was preferred.

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