

Optimization of alkaline phosphatase gene expression in *E. coli*

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Abstract

Alkaline phosphatase is an enzyme with widespread use in research and industry such as protein labeling, dephosphorylation of nucleic acids, and enzyme based biosensors. In the present study, alkaline phosphatase gene was inserted into the pET15b vector. The recombinant DNA was then expressed using IPTG as an inducer. The expression of this enzyme was optimized by changing expression conditions such as IPTG concentration, time, and temperature. The obtained results demonstrate that with the expression system used, large quantities of active alkaline phosphatase can be produced in *E. coli*.

Keywords: Alkaline phosphatase; pET15b; *E. coli* K-12

INTRODUCTION

Alkaline phosphatase is a homodimeric enzyme with a molecular weight of 56 kD. It is a metalloenzyme, binding two zinc atoms and one magnesium ion per monomer. This enzyme catalyzes the hydrolysis of a wide variety of phosphomonoesters and also catalyzes transphosphorylation reaction by transferring phosphoryl group to alcohol in the presence of certain phosphate acceptors (1).

Escherichia coli alkaline phosphatase is a valuable reagent for the removal of terminal monoesterified phosphate from both ribo- and deoxyribo-oligonucleotides. Also highly active bacterial alkaline phosphatase variants can be used as reporter enzymes fused to the foreign proteins via their amino and carbohydrate groups (2). These are convenient tools that can have widespread use such as expression, epitope mapping, histochemistry, immunoblotting, mutant analysis and ELISA (3).

Alkaline phosphatase gene has been cloned and expressed from various species. For example, the alkaline phosphatase gene from *Sphingomonas* sp. (4) and also some thermophilic bacteria (5,6) has been cloned and expressed in *E. coli* and their biochemical

behaviors have been investigated (7). It seems that the source of this enzyme is important for the enzyme activity and characteristics. Therefore, in the present study we decided to express the alkaline phosphatase gene from an available source in Iran, and by optimizing its expression, produce large quantities of this enzyme. This study would not only help to establish a method for the production of large quantities of alkaline phosphatase but also would open the door for its future modifications.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains used in this study were *E. coli* K-12 (PTCC 1268, Iran), *E. coli* Hb101 and BL21 strains (Cinagen, Iran). The pTZ57R cloning vector and the pET15b expression vectors were obtained from Fermentas, Poland.

Media and chemicals

Luria Bertani (LB) media was prepared according to Sambrook and Russel (3). Antibiotic screening was performed on LB agar plates using ampicillin at 100 µg/ml which was obtained from Sigma, USA.

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Different concentrations of isopropyl β -D thiogalactopyranoside (IPTG, 0.1 mM and 0.5 mM) were used for the induction of *phoA* gene. This reagent was purchased from Fermentas, Poland. The restriction enzymes were purchased from Fermentas (Poland) and Roche (Germany) companies.

Cloning of phoA gene

Alkaline Phosphatase gene from *E. coli* K-12 was cloned in pTZ57R vector as described previously (8). This recombinant vector (*phoA/pTZ57R*) and pET15b vector were digested by *NdeI* and *BamHI* enzymes.

The insert and digested pET15b plasmid were isolated from agarose gel and then ligation was performed with T4 DNA Ligase (Fermentas, Poland) at 16 °C overnight in a water bath. Before ligation, the vector was dephosphorylated by treatment with calf intestinal alkaline phosphatase (Roche, Germany) to remove 5'-terminal phosphate and to prevent recircularization of the plasmid. The ligated DNA was then transformed into *E. coli* BL21 competent cells by using CaCl_2 method (3).

The obtained colonies were used for plasmid preparation using High Pure Plasmid Isolation Kit (Roche, Germany) and a portion of the plasmid DNA was digested with the restriction enzymes such as *EcoRI*, *NcoI* and loaded onto a 0.7% agarose gel to verify the presence of the *phoA* insert.

Expression of phoA gene

A culture of *phoA/pET15b* containing bacteria was grown in 5 ml LB/ampicillin (100 $\mu\text{g/ml}$) overnight and 100 μl of the overnight culture was used to inoculate 5 ml of pre-warmed LB/ampicillin. The culture was grown in a shaking incubator at 37 °C until optical density (OD) at 550 nm of approximately 0.5 was obtained.

Protein expression was induced by the addition of IPTG with final concentration of 1 mM and the culture was grown for an additional 2 or 3 h. At these time points, 1.5 ml of each culture was transferred to a microfuge tube and cells were harvested by centrifugation at $13000 \times g$ for 1 min at room temperature and after removing supernatant,

the bacterial pellets were re-suspended in 15 μl of $6 \times$ SDS gel-loading buffer. The samples were heated to 100 °C for 3 min. The tubes were then centrifuged at $13000 \times g$ for 1 min at room temperature and were stored on ice before loading into the gel. Protein expression was analyzed using SDS-PAGE. For this purpose, a 12% poly-acrylamide gel was utilized. Proteins were stained with coomassie blue for 20 min and were visualized by destaining with methanol:acetic acid solution (3).

Optimization of the gene expression

Time and concentration of IPTG: Six overnight cultures of *E. coli* BL21 harboring the recombinant plasmids were diluted in a 1:20 (v/v) ratio and grown at 37 °C until the optical density (at 550 nm) of 0.5 was reached. Five cultures were induced by the addition of different concentration of IPTG (0.25, 0.5, 0.75, 1 and 1.5 mM) and one culture was uninduced. These cultures were incubated at 37 °C and after 1, 2, 3, and 4 h 1.5 ml of each culture was transferred into a microfuge tube and cells were harvested by centrifugation at $13000 \times g$ for 1 min at room temperature. After removing supernatants, the bacterial pellets were re-suspended in 15 μl of $6 \times$ SDS gel-loading buffer. The samples were heated at 100 °C for 3 min. The tubes were centrifuged at $13000 \times g$ for 1 min at room temperature and were stored on ice until loading of the samples into a gel.

Temperature: The above experiments were repeated at 35 °C and 39 °C incubation temperature instead of 37 °C. After 1, 2, 3, and 4 h, 1.5 ml of each culture was transferred to a microfuge tube and cells were harvested by centrifugation at $13000 \times g$ for 1 min at room temperature and after removing supernatants, the bacterial pellets were re-suspended in 15 μl of $6 \times$ SDS gel-loading buffer. The samples were heated at 100 °C for 3 min. The tubes were centrifuged at $13000 \times g$ for 1 min at room temperature and were stored on ice before loading into the gel.

Alkaline Phosphatase assay

The standard assay for alkaline phosphatase activity was carried out at room temperature

for 2.5 min, using 2.5 mM *P*-nitrophenyl phosphate (pNPP) as the substrate in 50 mM NaOH-glycine (pH=10.0) buffer, containing 0.5 mM CaCl₂. The release of *P*-nitrophenol in the reaction mixture (1 ml) was measured spectrophotometrically at 410 nm. Enzyme and substrate blanks were also included.

RESULTS

Construction of recombinant plasmids

The cloned alkaline phosphatase gene (8) was digested out of the cloning vector and ligated into the expression vector pET15b. Determination of orientation of the insert was performed using digestion with *Eco*RI and *Nco*I restriction enzymes. The correct orientation should have produced three bands of 6559 bp, 610 bp and 331 bp with *Eco*RI and two bands of 6158 bp and 1023 bp with *Nco*I (Fig. 1 and 2).

These experiments demonstrated that recombinant plasmids containing correctly oriented alkaline phosphatase gene were obtained. One of these plasmids was selected and used for the transformation of *E. coli* BL21 cells.

Expression analysis

Genes having promoters for T7 RNA polymerase could be expressed in *E. coli* BL21 cells using IPTG. Therefore, this substance was employed for the induction of alkaline phosphatase enzyme in *E. coli* BL21 cells. The

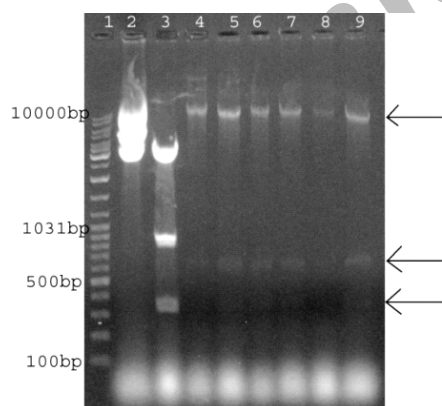


Fig. 1. Agarose gel electrophoresis of recombinant pET15b plasmids (phoA/pET15b) digested with *Eco*RI. Lane 1: ladder, lane 2, 3: products with the wrong orientation, lane 4, 5, 6, 7, 8, 9: products with the correct orientation. Arrows show the position of the obtained bands.

expressed proteins in these cells were then separated using SDS-PAGE. After staining of proteins by Coomassie brilliant blue (R-250) and destaining, in addition to other bands, a band with an estimated size of ~55 kD on lane of the induced samples was obtained (Fig. 3).

Optimization of the gene expression

Three parameters of IPTG concentration, temperature, and time of induction were altered in order to optimize the conditions for the production of alkaline phosphatase enzyme in *E. coli* BL21 cells. As shown in Fig 4, the best condition was incubation of cells for 2 h with 0.5 mM concentration of IPTG at 37 °C.

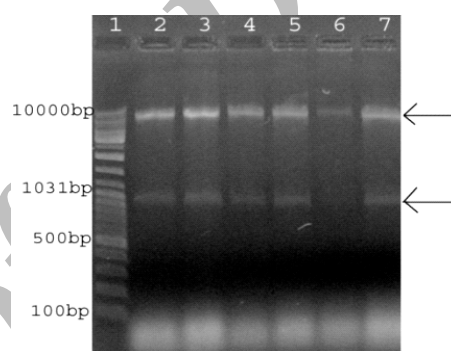


Fig. 2. Agarose gel electrophoresis of recombinant pET15b plasmids (phoA/pET15b) digested with *Nco*I. Lane 1: ladder, lane 2, 3, 4, 5, 6, 7: products with the correct orientation. Arrows show the position of the obtained bands.

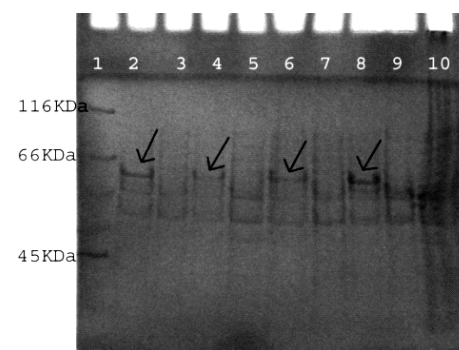


Fig. 3. Effect of IPTG on protein production in *E. coli* BL21 cells transformed with recombinant pET15b plasmid (phoA/pET15b). SDS-PAGE was used for separation of proteins. Lane 1: protein molecular weight marker, Lane 2, 4, 6, 8: samples induced by 1 mM IPTG for 1 h, lane 3, 5, 7, 9: uninduced samples. Arrows show the position of alkaline phosphatase protein.

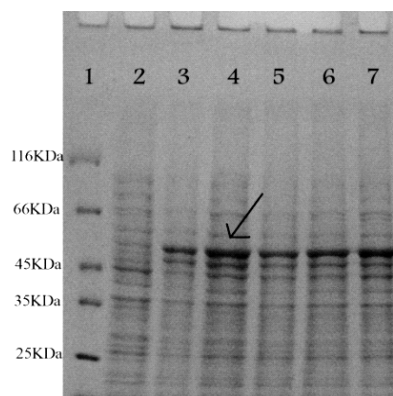


Fig. 4. Effects of different induction conditions on protein expression in *E. coli* BL21 cells transformed with recombinant pET15b plasmid (phoA/pET15b). SDS-PAGE was used for separation of proteins. Lane 1: protein molecular weight marker, Lane 2: uninduced sample, Lanes 3,4,5,6,7: expression of alkaline phosphatase (0.25, 0.5, 0.75, 1, 1.5 mM of IPTG and incubation for 2 h in 37 °C). The arrow shows where the maximum expression of the alkaline phosphatase gene has occurred.

DISCUSSION

In this study, a previously cloned alkaline phosphatase gene from *E. coli* obtained from a source in Iran (8) was inserted into pET15b vector, overexpressed in *E. coli*, and its expression was optimized in order to obtain the maximum level of protein production. Histidine tag was added to the protein for easy purification of the enzyme (7).

In this study different conditions for the induction of expression were utilized including IPTG concentration, temperature, and the time of induction. We also demonstrated that the produced enzyme was active (data not shown). The used vector system and the expression conditions were different from other reports. For example alkaline phosphatase gene from *Pyrococcus abyssi* was cloned in *E. coli* using pARHS vector and the optimized expression conditions were 4 h incubation with 0.5 mM IPTG at 37 °C.

Alkaline phosphatase mutations can be used to obtain better activity from this enzyme. For example a marine bacterium alkaline phosphatase was point mutated and its activity was measured (9). In that study a mutated alkaline phosphatase gene from *Shingomonas* sp. was overexpressed in *E. coli* which could be

potentially used for bioprecipitation of uranium from alkaline solutions. Therefore, based on these studies the next step would be creating point mutations in our cloned gene in order to enhance its activity. Also the effects of cooling and heating on the enzyme activity have been measured (10). These conditions can also be utilized in our experiments to enhance alkaline phosphatase activity.

In conclusion, in the present study alkaline phosphatase was produced in large quantities in *E. coli* and its expression was optimized. Further experiments are needed for its purification and creating mutations for obtaining an enzyme with better characteristics.

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