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Optimized Condition for Enhanced Soluble-Expression of Recombinant Mutant *Anabaena Variabilis* Phenylalanine Ammonia Lyase

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ABSTRACT

Purpose: Recently discovered *Anabaena variabilis* phenylalanine ammonia lyase (*Av*PAL) proved to be a good candidate for enzyme replacement therapy of phenylketonuria. Outstanding stability properties of a mutant version of this enzyme, produced already in our laboratory, have led us to the idea of culture conditions optimization for soluble expression of this therapeutically valuable enzyme in *E. coli*.
Methods: In the present study, the gene encoding mutant version of *Av*PAL was cloned into the pET28a expression vector. Different concentrations of IPTG, induction period, growth temperature, shaking speed, as well as different types of culture media were examined with respect to the amount of recombinant protein produced and specific activity of the enzyme.
Results: Based upon our findings, maximum amount of active mutant enzyme was attained by addition of 0.5 mM IPTG at 150 rpm to the TB culture media. The yield of active enzyme at cluture tempreature of 25 °C and induction period of 18 hour was the highest.
Conclusion: The results of this study indicated that the yield of mutant *Av*PAL production in *E. coli* can be affected mainly by culture temperature and inducer concentration.

Introduction

Phenylketonuria (PKU), the most prevalent in-born disease of amino acid metabolism, is caused by deficiency in phenylalanine hydroxylase (PAH) activity.[1] Two enzymes have been so far proposed for enzyme replacement therapy of PKU, including phenylalanine hydroxylase (PAH), and phenylalanine ammonia lyase (PAL). The first enzyme produces L-Tyr from L-Phe, and the latter converts L-Phe to the much harmless metabolite trans-cinnamic acid. Each of these enzymes has its own advantages and disadvantages. However, phenylalanine ammonia lyase do not need any additional cofactors and is more favorable in PKU enzyme replacement therapy.[2] *Anabaena variabilis* phenylalanine ammonia lyase (*Av*PAL), firstly discovered in 2007,[3] has been the subject of different clinical trials for PKU replacement therapy.[4]

There is need to a suitable host for recombinant production of any therapeutic protein. *E. coli* is the most widely used expression host for production of recombinant proteins, especially for proteins lacking complex post-translational modifications.[5] However a large fraction of recombinant proteins expressed in *E. coli* aggregates inside the bacteria and becomes insoluble. This results in formation of inclusion bodies mainly consisted of inactive proteins. Although it is possible to recover protein from inclusion bodies by in vitro refolding methods, the procedure is laborious and the yield is generally low.[6] Therefore, it is preferred to optimize expression condition to obtain higher levels of soluble and active protein directly, without needing any refolding

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procedures. Various parameters have been tried yet to increase the solubility of recombinant proteins expressed in *E. coli*.[7-10]

Considering the importance of *Av*PAL enzyme in replacement therapy of phenylketonuria, this study devoted to optimize the culture condition to enhance the yield of soluble and active mut*Av*PAL enzyme upon expression in *E. coli*. The mut*Av*PAL is a derivative of *Av*PAL, stabilized by introduction of a disulfide bridge into the structure of wild-type *Av*PAL.[11]

Materials and Methods

Transformation and Expression of mutant AvPAL gene

50 ng of expression cassette pET28a-mut*Av*PAL was added into 70 µl of BL21 (DE3) competent cells, incubated on ice for 30 min, heat shocked for 90 seconds at 42°C and incubated again on ice for 10 min. Then 500 µl of LB media was added to the tube and incubated for 1 hr at 37°C without shaking, transferred to LB agar-plates containing 50 µgml^{-1} of kanamycin and incubated overnight at 37°C. A single colony of recombinant bacteria was transferred to 10 ml of LB-kanamycin (50 µgml^{-1}) and cultivated at 37 °C with shaking (150 rpm) overnight. 100 ml of LB-kanamycin were inoculated with an aliquot of this media.

Optimization parameters

To assess the effect of IPTG concentration on the recombinant protein production and the enzyme specific activity, 20 ml of the recombinant clone culture was grown to OD$^{600\ nm}$ of about 0.7, and induced by adding IPTG in final concentrations of 0.1, 0.25, 0.5 and 1 mM. After 3 hours of induction, relative intensity of recombinant protein bands on the SDS-PAGE and the specific activity of mutant enzyme from three independent and parallel samples were compared.

To examine the effect of media type on the specific activity of recombinant enzyme, 20 ml of different media types (LB and TB media) were inoculated with the same fresh culture of recombinant bacteria. After 3 hours of induction at 37 °C, the specific activity of the recombinant enzyme from three parallel samples was analyzed.

For the induction period, samples were collected in different time intervals (2, 3, 6, and 18 hours) after adding 0.5 mM of inducer at 37°C, and the level of expression was checked by SDS-PAGE.

To explore the effect of shaking speed on the specific activity of the recombinant AvPAL enzyme, induction was performed at 37°C by addition of 0.5 mM IPTG. Three samples of each shaking speed (100, 150 and 200 rpm) were assessed in parallel. After 3 hours of incubation, bacterial cells were harvested and analyzed for the enzyme specific activity.

To examine the effect of temperature, recombinant protein expression was induced by addition of 0.5 mM IPTG at 150 rpm at different temperatures (25, 30 and 37°C). Three parallel samples of each temperature were grown and after 3 hours of induction, the protein expression was checked by SDS-PAGE and the specific activity of the enzymes was analyzed.

Protein Extraction and Purification

The bacteria from induced cultures were collected by centrifugation at 6000 rpm for 15 min at room temperature. For each 100 ml cell culture, pellets were resuspended in 5 ml of buffer A as lysis buffer (50 mM sodium phosphate buffer pH=8.0, 100 mM NaCl, 5 mM 2ME and imidazole 20 mM), and sonicated on ice ten times at the intensity of 60% for 30 seconds. Cell debris was removed by centrifugation at 4°C, 12,000 rpm for 15 min. Supernatants were loaded on a Ni-NTA column, and washed with ten volumes of column of buffer B (the same as buffer A except NaCl 300 mM). Fractions were eluted with buffer C (the same as buffer B except imidazole 300 mM). Protein expression and the purity of the eluted fractions were analyzed by 10% SDS-PAGE and Coomassie Blue staining. Fractions containing the recombinant protein were dialyzed against the Tris buffer 100 mM pH=8.0, NaCl 25 mM, 2ME 5 mM and used for enzyme assay.

Enzyme assay

Enzyme activity of the recombinant phenylalanine ammonia lyase was measured in a 0.5 ml reaction mixture containing 100 mM Tris-HCl buffer pH 8.0, 5 mM L-Phe, and 4 µg of purified mut*Av*PAL enzyme. The reactions were kinetically followed at 280 nm, maximum wavelength of trans-cinnamic acid (molar extinction coefficient of 16,890 litre mol^{-1} cm^{-1}), as mentioned before.[3]

Protein concentration determination

Protein concentration was measured by Bradford assay.[12] Briefly 0, 3, 6, 9, 12 and 15 µg of BSA protein was mixed with Bradford reagent and the absorbance was measured at 595 nm. Standard curve was plotted and the equation produced from linearized fit to the data was utilized for protein concentration determinations.

Data analysis and statistics

Images of the SDS-PAGE were analyzed by ImageJ software.[13] This package assigns a number to each of gel bands based on the intensity. All samples were treated in three independent experiments in parallel. Error bars indicated one standard deviation from the mean value plotted on the graph. Graphs were prepared with SigmaPlot software version 11.

Results

Different concentrations of IPTG were added to the growing culture of BL21 DE3 containing expression

cassette of mut*Av*PAL enzyme. Analysis by SDS-PAGE revealed that maximum amount of recombinant protein was attained by addition of 1 mM of IPTG, as shown in Figure 1, whereas the highest enzyme specific activity was observed in 0.5 mM IPTG (1.6±0.1 µmolmin^{-1}mg^{-1} as compared to the 1.4 ±0.1 for 1 mM of IPTG).

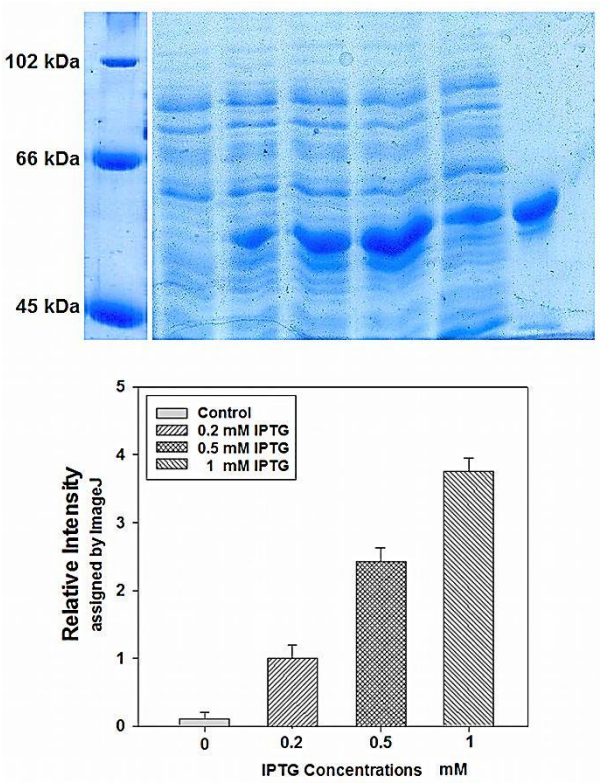

Figure 1. Induction of recombinant mut*Av*PAL by different concentrations of IPTG.
A1 indicates recombinant clone before induction and A2-A5 show induction by addition of 0.2, 0.5, and 1 mM concentrations of IPTG respectively. All samples were cultured at 37°C.

Our findings although showed that using TB media results in higher specific activity of the mut*Av*PAL enzyme (1.35±0.1 for LB media, compared to 1.65±0.1 for the TB media).

As shown in Figure 2, induction period of more than 6 hours resulted in the maximum amount of recombinant protein production. But according to the Figure 3, the specific activity of the enzyme at 37°C and 18 hours of induction dropped significantly probably due to the aggregation of the recombinant enzyme.

For analysis of the effect of shaking speed on the expression level of mut*Av*PAL, the culture of recombinant clone was induced at 37°C for 3 and 6 hours at various speeds (100, 150 and 200 rpm). The specific activity of the mutated enzyme was the same after 3 hours of induction in three speeds (1.3 µmolmin^{-1} mg^{-1}). But after 6 hours of induction, the enzyme specific activity was higher at lower speeds (1.7±0.1, 1.7±0.1 and 1.4±0.1 µmolmin^{-1}mg^{-1} for 100, 150 and 200 rpm, respectively).

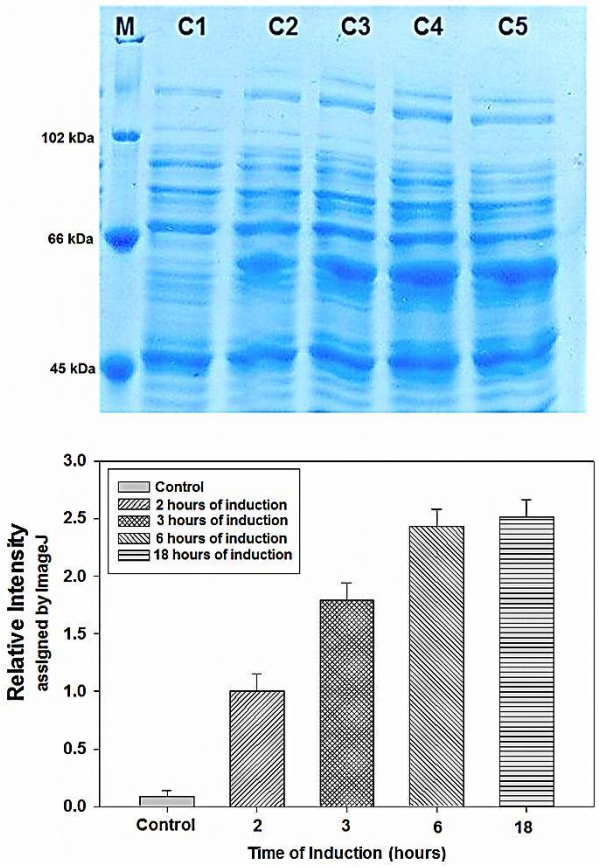

Figure 2. Induction of recombinant mutant *Av*PAL enzyme at different time intervals.
C1 indicates the recombinant clone before induction and the lanes C2-C5 show respectively the 2, 3, 6 hours, and overnight induction of recombinant mutant *Av*PAL, respectively.

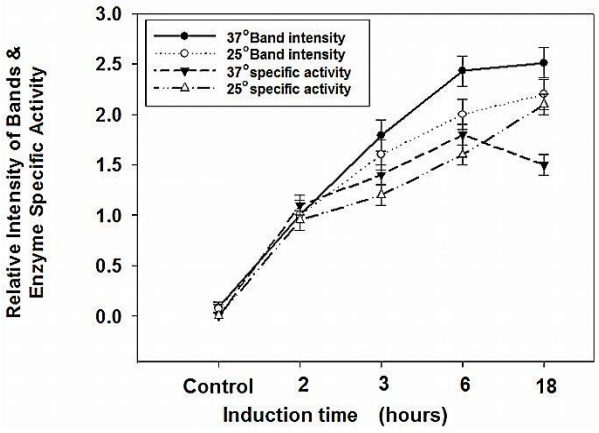

Figure 3. Recombinant protein relative intensity of bands and specific activity at 25 and 37°C.

By using 0.5 mM concentration of IPTG and induction period of 3 hours, the effect of different temperatures on the expression of recombinant mutated *Av*PAL were examined. As shown in Figure 4, the highest relative intensity of recombinant protein band on the SDS-PAGE gel was observed at 37°C. But the specific activity of mut*Av*PAL enzyme was higher at 25°C than 37°C (Figure 3).

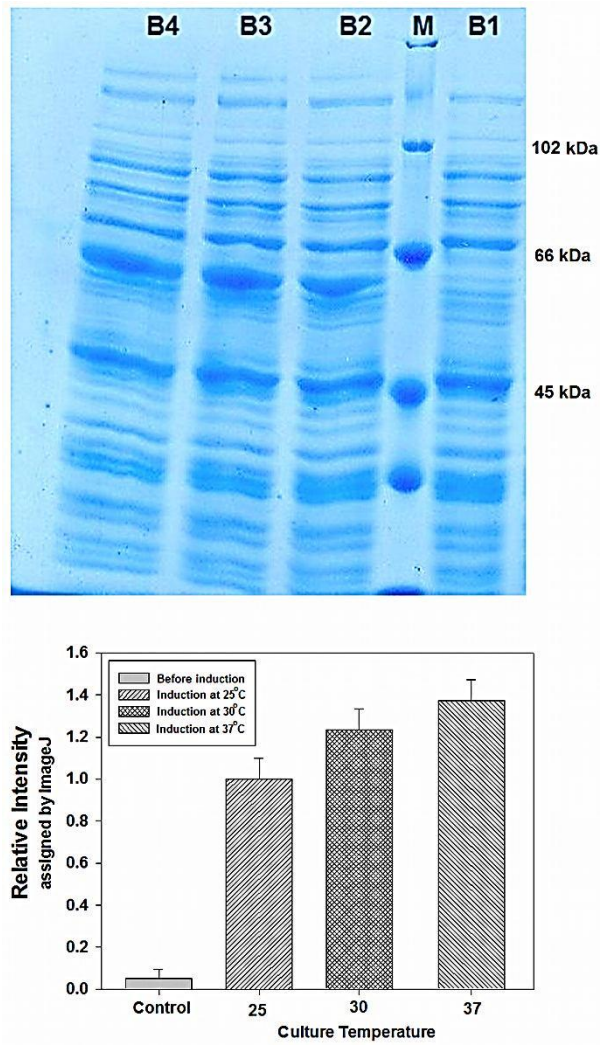

Figure 4. Induction of recombinant mutant *Av*PAL enzyme at different culture temperatures.
B1 indicates the recombinant clone before induction and the lanes B2-B4 show the 25°C, 30°C and 37°C temperatures, respectively. All samples were compared at 3 hours of induction.

Purification of the mut*Av*PAL on the Ni-NTA column resulted in a single band on the SDS-PAGE, which is indicative of the presence of well purified enzyme, as shown in Figure 5.

Discussion

Phenylalanine ammonia lyase enzymes proved to be good alternatives for the natural enzymes PAHs, since they do not require additional cofactor for their catalytic activity. During the early steps of PAL enzymes synthesis and folding, the MIO (methylideneimidazole-5-one) group is simply formed at their active site by autocatalytic cyclization of a conserved Ala-Ser-Gly tripeptide conserved motif.[14]
Various PAL enzymes have been recognized in a wide range of sources from bacteria to yeasts and plants. Prokaryotic PALs are about 20 kDa shorter than their eukaryotic counterparts due to lack of an N-terminal segment.[15,16] This N-terminal extension plays the role of a shield above the active site and alters the kinetic behavior of the enzyme probably by restricting the entrance and exit of substrate and product.[16] The extension also changes the catalytic activity and stability of enzymes; therefor use of bacterial PALs generally is more favorable in replacement therapy of PKU.

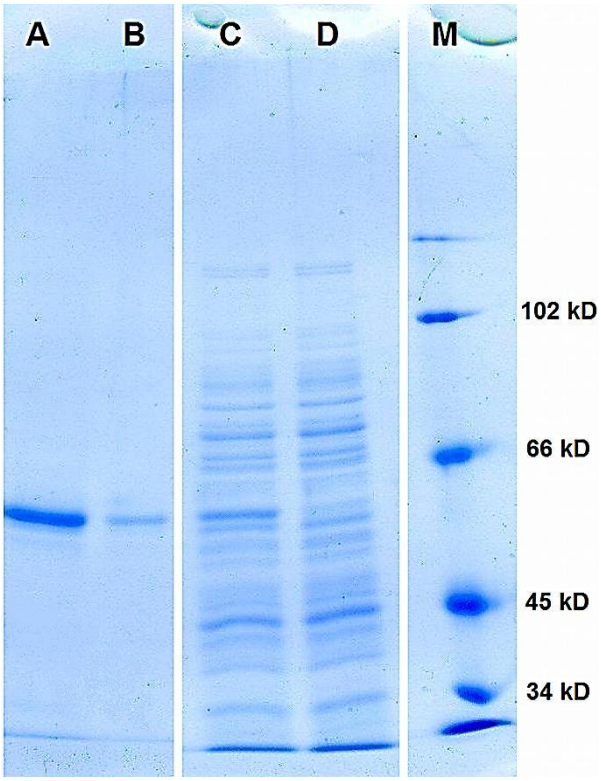

Figure 5. Purification of mut*Av*PAL by Ni-NTA affinity column. Lanes A-M indicate first eluted fraction, second eluted fraction, soluble soup before running on the column, flowthrough, protein molecular weight marker, respectively.

Anabaena variabilis phenylalanine ammonia lyase (*Av*PAL) has been recently discovered by Moffitt *et al.*[3] This enzyme proved to be a good candidate in clinical trials of phenylketonuria enzyme replacement therapy.[4] The prokaryotic source of this enzyme makes bacterial expression system suitable for production of recombinant enzyme. The higher catalytic activity of *Av*PAL is the other advantage of this enzyme in compare to other bacterial PALs.[3] We have already mutated this enzyme in our laboratory to increase stability properties.[11] This mutated enzyme has shown promising catalytic and stability properties for use in treatment of PKU. This study was devoted to optimize the condition for high level expression of recombinant mut*Av*PAL in *E. coli*. Various parameters have been optimized including culture media, culture temperature, inducer concentration, aeration (shaking speed) and induction period.
It has been shown that the level of recombinant protein expression is affected by inducer concentration. Generally most of recombinant proteins tend to be aggregated at high concentrations of IPTG, while some

other proteins are less sensitive to aggregation due to their inherent higher solubility. Based upon our findings, 1 mM IPTG resulted in the highest amount of recombinant protein, but the specific activity of the mutant enzyme was higher at 0.5 mM concentration of IPTG, maybe indicative of aggregation of proteins due to more rapid biosynthesis in bacteria.

Generally the optimum temperature for the recombinant protein production in *E. coli* is 37°C and several studies reported 37°C as the best temperature for maximum protein production.[9] In the other hand, studies showed that rate of expression and culture temperature can affect the proper folding of recombinant proteins and inclusion body formation.[17] Reducing culture temperature usually leads to slower growth of bacteria, slower rate of protein production and lower aggregation of target protein.[18] For recombinant mut*Av*PAL enzyme, as shown in Figure 3, culture at 25°C resulted in more active protein than 37°C. This finding is consistent with reports of Li *et al* that has shown less aggregation of recombinant protein in lower temperatures.[17] Likewise, Cirkovas and colleagues showed that culture of recombinant *E. coli* at lower temperature leads to more solubility and less aggregation of the mink growth hormone.[19]

Our findings show that induction period of more than 6 hours results in the highest amount of recombinant protein production, but the specific activity of the enzyme was decreased at 37°C probably due to aggregation of recombinant proteins.

Shaking of bacterial culture at lower speeds has been shown useful for better protein expression.[9] Upon our findings, shaking speeds higher than 150 rpm for 6 hours of induction decreased the yield of active enzyme whereas prolonged cultivation at lower speeds provided better results.

It has been reported that types of culture media can affect the yield of recombinant protein production.[20] Our results showed that using TB media resulted in higher protein production and enzymatic activity. Hartinger *et al* evaluated the effect of different parameters on the activity of a recombinant aminotransferase enzyme among them, culture under lower temperature was the only parameter affected the yield of active enzyme.[21]

Conclusion

In conclusion, various parameters affect the expression of recombinant proteins but there is not any consensus condition for optimized expression of all recombinant proteins. Hence, the best condition for production of any given protein must be optimized experimentally.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Abbreviation

PAL, phenylalanine ammonia lyase; PAH, phenylalanine hydroxylase; IPTG, isopropylthio-β-galactoside; LB medium, Luria-Bertani medium; 2ME, 2-mercaptoethanol.

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