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# Potential Role of Promoter in Production Level of *Bacillus sphaericus* Phenylalanine Dehydrogenase in *Escherchia coli* Expression System

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**In order to investigate the Potential role of promoter in production level of phenylalanine dehydrogenase in *E. coli* expression system this study was made. The L-phenylalanine dehydrogenase gene *(pdh*) was isolated from *B. sphaericus*. The *pdh* gene was cloned into two expression vectors; pET-23a and pPR37 differing in their promoter, which were T7 and λPR respectively. Expression of gene was induced by adding 1 mM (final concentration) of IPTG for vector carrying T7 promoter or by temperature shift from 30 to 42 °C for vector carrying λPR. The highest rate of expression was observed in pETpdh/BL21(DE3)plysS construction. The level of expression of other construction was very low. PheDH was purified by Ni-NTA chromatography column. The relative molecular mass of PheDH subunit was about 41KDa by SDS-PAGE 10%. Applying this method for purification the specific activity of enzyme was 705 U/mg protein. The purity and amount of proteins were assessed by SDS-PAGE. Our polyhistidine-tag enzyme can be ideally used for the immobilization on a nickel coated microliter plate surface.**

**Key words:** L-phenylalanine dehydrogenase, Expression, Plasmid, Promoter.

L-phenylalanine dehydrogenase or PheDH [ $NAD^+$ oxidoreductase deaminating; EC 1.4.1.20] was discovered in 1984 [1], and after then, has been found from different microbial sources, including *Bacillus*, *Sporocarcina*[2], *Microbacterium*[3], *Rhodococcus Maris* k-18[4], *Nocardia* [5], and *Thermoactinomyces* [6]. PheDH has both industrial and medical applications. The medicine application of enzyme is identifying and quantifying the exact amount of L-phe in blood serum, in order to PKU (phenylketanuria) screening[7]. The industrial application of PheDH is producing optically pure L-phenylalanine [8], a component of artificial sweetener aspartame [9]. Despite the low concentration of enzyme in wild strains, investigators use recombinant DNA techniques to raise the amount of expressed enzymes[10]. In first attempts to increase the production of PheDH enzyme, the growth condition of *Brevibacterium* sp was optimized [11].

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Using those techniques *pdh* gene from *bacillus badius* IAM was cloned into pBR322 vector and expressed in high level in *E. coli* RR1 [12]. The *pdh* gene of *Bacillus sphaericus* was isolated and inserted into plasmid pUC9 and transformed into *E. coli* JM103 [13]. Isolating *pdh* gene from the genomic library of *Rhodococcus* sp.M4 was done and the gene was cloned in pET-3d vector and expressed in *E. coli* BL21(DE3) [9]. The level of *pdh* expression, which was under control of the wild promoter, was compared in two expression hosts: *B. subtilis* cells and *E. coli* JM109 [14]. In this research, we compare the level of PheDH production in two different expression systems. In the first system, we use PET-23a as a vector and *E. coli* BL21(DE3)plysS as an expression host. In this system PET-23a/BL21(DE3)plysS) *pdh* gene was placed downstream of T7 promoter. In the second system, we use pPR37 vector and *E. coli* TG1 expression host. *Pdh* gene was cloned to downstream of rightward promoter of lambda phage. Promoter of recent vector controlled thermally by the mutant thermo labile *CI857* repressor.

## MATERIALS AND METHODS

**Preparation of *pdh* gene**

As we reported previously, the *pdh* gene was cloned into in pET-16b (construction p ETDH) [14] expression vector and then we used it in this study.

**Sub cloning of *pdh* in pET-23a**

The *pdh* gene from *B. sphaericus* was amplified by Polymerase Chain Reaction (PCR) from plasmid pBDHH1DBL, as a template, a forward primer 22 bp 5´TGGATCCATGGCAAAACAGCTT 3´ and reverse primer 25 bp 5´ GCGAAGCTTCTCTTTTATGTTCCAC 3´. The forward primer carries *Bam* HI site and start codon ATG, while in reverse primer only *Hin*dIII site was inserted. The pET-23a (Novagen) vector carries T7 promoter. The PCR product was cut by *Bam*HI and *Hin*dIII inserted to *Bam*HI and *Hin*dIII sites of pET-23a vector and new construct was named pETpdh.

**Subcloning of pdh in pPR37**

**Preparation of pdh gene**

The *pdh* gene from *B. sphaericus* was amplified by PCR from plasmid pBDHH1DBL, as a template, a forward primer 5´ GCAGATCTATGGCAAAACAGCTT 3´ carries *BgL*II site and start codon ATG, while in reverse primer only *Bam*HI site was designed.The pPR37 vector, which has heat-inducible λPR promoter upstream from *BgL*II(0) and *Bam*HI(190) sites, was cut with *BgL*II and *Bam*HI, inserted to *BgL*II and *Bam*HI sites of pPR37 vector and new construct was named pPRpdh.

**Transformation**

BL21(DE3)plysS (Novagen, Madison, WI, USA) and TG1 (Novagen) cells were transformed respectively with pETpdh and pPRpdh. strain BL21(DE3)plysS is lysogen of λ phage containing T7 gene *1*(coding T7 RNA polymerase) which is controlled by lacUV5 promoter-operator sequence and inducible with isopropyl-λ-D-thioglactoside (IPTG) [15]. *E. coli* DH5β (Novagen) and XL1-Blue (Novagen) was used respectively for pETpdh and pPRpdh amplification and purification.

**Expression of PheDH**

Transforments were grown at 37 °C in Luria-Bertani (LB) medium supplemented with 50 µg/ml ampicillin, until culture reached on $OD_{600}$=0.6, The cell carrying pETpdh was induced by the addition of 1 mM is IPTG and the cell carrying pPRpdh was induced by increasing temperature from 37 °C to 42 °C. In different times, 3h, 5h and 8h after induction, the cells were collected by centrifuge at 3500 rpm for 15 min, washed twice with 0.9% NaCl solution and reserved at -20 °C until use.

**Purification of His-tagged PheDH**

The cell pellets were suspended in a buffer A (50 mM Tris-HCl pH 8.3, 0.1 mM EDTA and 5 mM 2-mercaptoethanol including 1 mg/ml lysozyme. They were left at room temperature for 20 min. The cells were disrupted by ultrasonication for 10 min with a 9-KHZ ultrasonic oscillator. To discard cell debris, the sonication suspension was centrifuged at 15'000 rpm for 30 min at 4 °C. The supernatant was applied to $Ni^{2+}$-chelating-Sepharose column (CL-6B QIAGEN) equilibrated with 20 mM Tris-HCL buffer, pH 8.0, containing 0.5 M NaCl and 2 mM 2-mercaptoethanol. After washing with the same buffer containing 25 mM imidazole, the enzyme was eluted with 20 mM Tris-HCL buffer, pH 8.0, containing 0.5 M NaCl, 2 mM 2-mercaptoethanol, and 250 mM imidazole. The fractions corresponding to PheDH activity pooled

and dialysed against buffer B (25 mM Tris-HCl pH 8.3, 0.05 mM EDTA, 2.5 mM 2-mercaptoethanol). The purified enzyme was identified by means of SDS-PAGE.

**Enzyme assay**

The standard assay of PheDH activity was done at 25 °C measuring the reduction of $NAD^+$ at 25 °C using L-phenylalanine as a substrate in 1 ml reaction mixture containing 100 mM glycine-KCl-KOH buffer (pH 10.5, 2.5 mM $NAD^+$, 10 mM L-phenylalanine and the enzyme sample. To determine The $K_m$ and $V_{max}$ value of PheDH, various concentrations of L-phe and $NAD^+$ were used. Reductive amination was done at 25 °C in reaction mixture containing 100 mM glycine-KCl-KOH buffer pH 9.0, 0.1 mM NADH, 200 mM $NH_4Cl$, 10 mM sodium phenylpyruvate, and enzyme solution (total 1.0 ml). One unite of enzyme was defined as the amount of enzyme that catalyzed the formation 1ìmol of NADH in the oxidative deamination. Protein concentration was determined spectrophotometrically (absorbance at 280 nm using the absorption coefficient $A_{1\%/1cm}$=6.3), or with protein assay kit (Bio-rad laboratories,Inc.).

## RESULTS

**Construction and transformation**

*Pdh* gene has successfully cloned from different sources [3]. In this study, we successfully subcloned *pdh* gene into pET-23a and pPR37. Construct pETpdh and pPRpdh was made as described in experimental procedure. (Fig 1, Fig 2). *E. coli* BL21(DE3)plysS and TG1 transformed with pETpdh and pPRpdh respectively.

**Expression, SDS-PAGE analysis and purification**

The level of PheDH expression in above mentioned constructs were analyzed by means of

**Table 1.** Different level of PheDH expression according to various promoters

| Construct | Promoter | Enzyme activity U/L Culture |
|---|---|---|
| BL21(DE3)plysS/pETpdh | T7 | 17000 |
| TG1/pPRpdh | λ | 4900 |
| E. coli BL21(DE3)/pETDH | T7 lac and wild | 6300 |
| E. coli JM109/pBPDH1-DBL[a] | Lac and wild | 7200 |

[a] Asano *et al.* 1990.

**Table 2.** PheDH enzyme enzyme purification from E. coli BL21(DE3)/pETDH

| Step | Activity (U) | Protein (mg) | S. activity (U/mg) | Yield (%) |
|---|---|---|---|---|
| Crude extract | 10850 | 1409 | 7.7 | 100 |
| Ni-NTA column | 6350 | 9 | 705 | 60 |

**Table 3.** Substrate activity of recombinant *B. spharicus* PheDH.

| Amino acid | Relative activity(%) |
|---|---|
| L-phenylalanine | 100.0 |
| L-tyrosine | 74.0* |
| L-norleucine | 4.5 |
| L-Valine | 3.0 |
| L-methionine | 2.0 |
| L-tryptophan | 1.8 |
| L-leucine | 1.0 |

* Measured at 0.3 mM

**Table 4.** Compare the characteristics of lambda and T7 promoter

| Studied characteristics | T7 promoter | λ promoter |
|---|---|---|
| Level of expression | 60% | 40% |
| Cost benefit | No | Yes |
| Use of IPTG | Yes | No |
| Different host of *E. coli* strains | Limited | Optional |
| Cell lysation methods | Very usual | Usual |
| Time of cultivation | 6 hours | 6 hours |

SDS-PAGE 10% (Fig. 3 and Fig. 4). To quantitative analysis of PheDH, the gel was stained with coomassie Brilliant Blue R250 and scanned. The expression level in yield of PheDH was quantified by densitometry scan using Quanti-Scan software. The results showed that the production of PheDH for BL21(DE3)plysS/pETpdh was 65/4%(Fig. 3, lane 4) and for TG1/pPRpdh was 15.6% of total bacterium protein (Fig4, lane4).

**Protein determination and Enzyme assay**

Also the amount of enzyme expression in BL21(DE3)plysS/pETpdh and TG1/pPRpdh were determined by measurement of the oxidative activity of NADH as indicator (Table 1).

Data indicates that the highest level of PheDH observed in pETpdh construct. For this

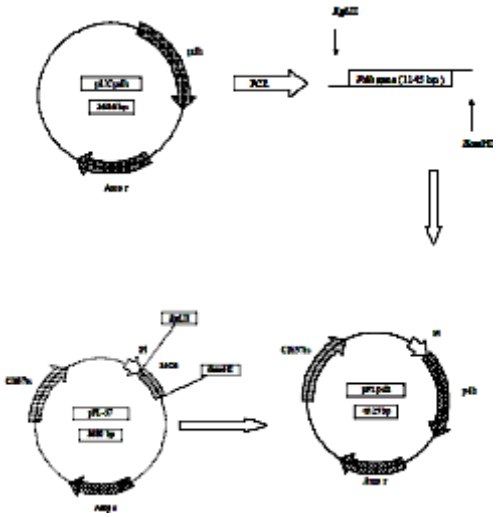

**Fig 1**. Schematic of subclonig of *pdh* gene in pPR37 expression vector (pPRpdh). The PCR product was digested with *BgL*II/*Bam*HI enzyme and inserted into the *BgL*II/*Bam*HI sites of pPR37 vector ( DNA SIS MAX v.3 software)

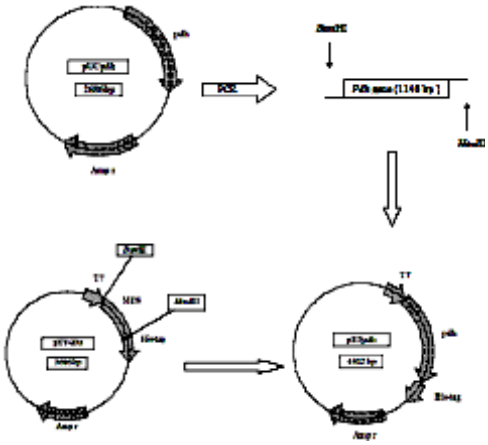

**Fig 2**. Schematic of subclonig of *pdh* in pET-23a expression vector (pETpdh). The PCR product was digested with *Bam*HI/*Hin*dIII enzyme and inserted to *Bam*HI/*Hin*dIII sites of pET-23a vector (DNA SIS MAX v.3 software)

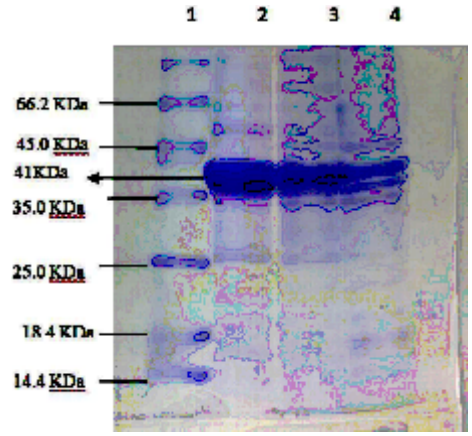


**Fig 3**.SDS-PAGE of level of expression of PheDH in PETpdh construt in different times after induction. Lane 1: protein size marker. Lane 2:3 hours after induction. lane 3: 5 hours after induction. Lane 4: 8 hours after induction

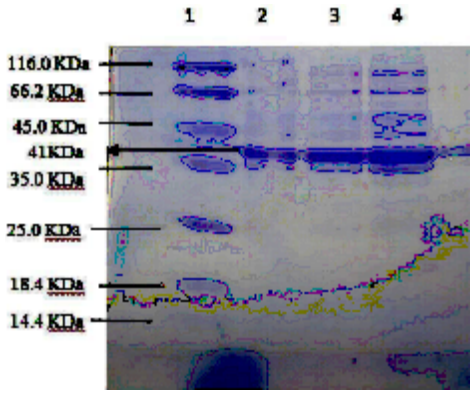


**Fig 4**. SDS-PAGE of level of expression of PheDH in PRpdh costruct in different times after induction. Lane 1 : protein size marker. Lane 2 : 3 hours after induction. Lane 3 :5 hours after induction. Lane 4 : 8 hours after induction

reason, pETpdh construct was chosen for purification and enzyme activity staining (Table 2).

The specific activity of enzyme for the oxidative deamination of L-phenylalanine was 705 U/mg protein. Interestingly, BL21(DE3) plysS/ pETpdh showed PheDH activity (17000U/L) that was over 386 times greater than wild type of *B. sphaericus* SCRCR79a, (44U/L).

**The substrate specificity of recombinant PheDH enzyme is indicating in Table 3.**

The $K_m$ value determined from secondary plot of intercepts against reciprocal concentration of substrate. $K_m$ value for L-phenylalanine, L-tyrosine and $NAD^+$ were 0.24, 0.48 and .019, respectively. It seems that decreasing in $K_m$ value of PheDH enzyme for L-phenylalanine depends on locating His-tag in C-terminal of expressed PheDH.

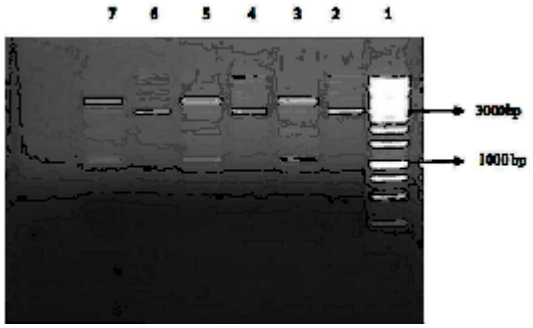


**Fig 5**. Agarose gel analysis of PETpdh construct and vector from different clones. Lane 1:1Kbp marker. Lane 2: PET-23a uncut. Lane 3: PETpdh cut with *Bam*HI , *Hin*dIII . Lane 4, 5 and lane 6 ,7 are respectively PET-23a uncut and PETpdh cut with *Bam*HI and *Hin*dIII from different clones

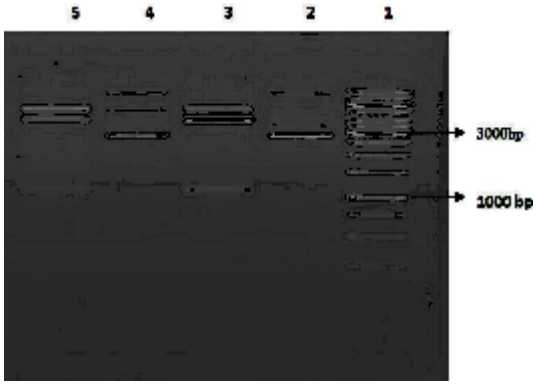


**Fig 6.** Agarose gel analysis of pPRpdh construct and vector from different clones. Lane 1: 1Kbp marker. Lane2: pPR37 uncut. Lane 3 pPRpdh cut with *Bam*HI , *BgL*II. Lane 4 and 5 are respectively pPR37 uncut and pPRpdh cut with *Bam*HI and *BgL*II from different clones.

## DISCUSSION

The demand for various uses of proteins in medical and industrial purposes cannot be gotten from natural reservoirs. The ability of *E. coli* to grow rapidly, ease of cultivation on inexpensive media, its well-characterized genetic and the availability of the large number of various expression vectors made it the most attractive host for producing recombinant proteins. When expressing a protein in vivo, we usually wish to get as high yield as possible. Some important parameters affecting the level of expression of heterologous proteins in *E. coli* such as plasmid copy number, mRNA stability and its folding, promoter, elements affecting translation, toxicity of protein, codon usage [16]. In IPTG-inducible expression systems, like pET system, the chemical inducer IPTG needs to producing recombinant protein. In this system T7 RNA polymerase, encoded by T7 gene *1* which is located on *E. coli* strain BL21(DE3)plysS. The synthesis of T7 RNA polymerase is controlled by *Lac*UV5 promoter: to induce the synthesis of T7 RNA polymerase for activating T7 promoter; chemical inducer IPTG is used to disable LacI repressor. The pET system which was use, have three advantages: 1) strong T7 promoter of PET-23a vector which is mentioned above. 2) This vector has His.tag coding sequence adding six consecutive histidine residues to carboxyl-terminal end of recombinant protein. Therefore, we can easily purify the heterologous proteins with $Ni^+$-NTA chromatography column. 3) The *E. coli* strain BL(DE3)plysS has plysS plasmid that encode T7 lysozyme, binding to any residual T7 RNA polymerase made in the absence of induction and preventing leaky expression of recombinant protein. This system has some deficiencies such as high cost and potential toxicity of IPTG which precludes the use of it in industrial-scale, or therapeutic applications. In thermo-inducible systems, like pPR, the production of recombinant protein is done by a temperature up

shift, without using toxic and high cost inducer. At 30 °C or lower, the CI repressor produces by vector is active and repressing the promoter. Raising the temperature inactive the repressor and leading to promote of gene transcription and PheDH synthesis [17; 18]. This system has two benefits: 1) we can induce the promoter in a simple and cost-effective manner, because we don't need high cost chemical inducer like IPTG [10]. 2) Using this recombinant protein in therapeutic application, due to using nontoxic inducer. Analysis of total expressed proteins from the cell lysate in pETpdh and pPRpdh construct showed that the amount of protein production in pETpdh system significantly higher than pPRpdh system. However, the benefit of the temperature-inducible λPR is facile of induction and lack of cost inducer [19]. In previous studies, the different amount of enzyme obtained from:1) *Brevibacterium* sp (3342 U/l) [11]. 2) recombinant PheDH of *B. badius* IAM (2390 U/l) [12]. 3) recombinant PheDH of *B. sphaericus* SCRC-R97a (7200 U/l) [8]. 4) recombinant PheDH was expressed in *B. subtilis* ISW1214 (4700 U/l) and *E. coli* BL21(DE3) (6300 U/l) [14]. Nevertheless, Hanson *et al* [20], reported a huge amount of production over 19000 U/L culture for the recombinant *T. intermedius* PheDH in *E. coli.* Apparently, the different results were gotten to the type of promoters, mRNA construction and its topology and condition of expression, e.g. optimization of IPTG induction, type of cell line, media, and incubation circumstances. In this study, the *pdh* gene was sub cloned in two different expression vectors, which have different promoters. The level of PheDH expression in pETpdh was 6900 U/l and in pPRpdh was 4900 U/l. The highest level of expression found in pETpdh/ *E. coli* BL21(DE3)plysS. Therefore, the pET/pdh product was purified with Ni-NTA chromatography column to near homogeneity with a final yield 60%.

**Characterizations of PheDH**

The molecular mass of PheDH subunits is estimated between 36 and 42 KDa by SDS-PAGE depend on different strains of bacteria [9]. This 41KDa recombinant estimated from its SDS-PAGE mobility agreed with previously reported [2]. There is a significant variation in the quaternary structure of this enzyme. The enzymes from *Rhodococcus maris* K-18 and *Rhodococcus* sp.M4 have been reported dimmer [21]. On the other hand, the *B. badius*, *B. sphaericus*, *Microbacterium* sp. DM 86-1, *S. ureae* have been shown to be octamers [3]. The *Nocardia* sp. 239 has been found as a monomer [5].

In brief, the *pdh* gene of *B. sphaericus* has been successfully cloned and expressed in pET and pPR system in *E. coli*. The amount of expressed protein was compared in two systems. The results showed that the amount of protein, expressed in pET system was more than pPR. Therefore, we used pET system for downstream processing and our polyhistidine-tag enzyme can be ideally used for the immobilization on a nickel coated microliter plate surface.

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