MOLECULAR CLONING AND EVALUATION OF WILD PROMOTER IN EXPRESSION OF *BACILLUS SPHAERICUS* PHENYLALANINE DEHYDROGENASE GENE IN *BACILLUS SUBTILIS* CELLS

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Abstract

To evaluate the role of wild promoter of L-phenylalanine dehydrogenase (PheDH) gene, referred to as *pdh*, from *Bacillus sphaericus* in expression, cloning of *pdh* gene in *Bacillus subtilis* was performed. The whole *pdh* gene was cloned in pHY300PLK shuttle vector and amplified, construct (pHYDH) then transformed in *B. subtilis* ISW1214 and *E. coli* JM109. The *pdh* endogenous promoter presented no effect on transcription of the target gene in *E. coli* JM 109. But *B. subtilis* ISW1214/pHYDH produced PheDH enzyme (4700 U/L). The level of PheDH protein expression with native promoter was about 12%. It was purified to near homogeneity as judged by SDS-polyacrylamide gel electrophoresis (M_r 41 kDa) and the result was 18 fold with a yield of about 31%. The M_r of PheDH was estimated to be approximately 340 kDa (octamer) by a gel filtration on a G-200 sephadex column. Apparent K_m values for L-phenylalanine, L-tyrosine and NAD^+ were 0.24 mM, 0.48 mM and 0.19 mM respectively. The optimum pH of the recombinant enzyme was 11 for the oxidative deamination, 10.2 for the reductive amination. The features of recombinant PheDH enzyme were compatible with the wild type PheDH protein.

Introduction

L-Phenylalanine dehydrogenase (NAD^+ Oxidoreductase, deaminating; EC 1.4.1.20) was found in relatively few Gram-positive and aerobic bacteria such as *Bacillus*, *Sporosarcina* [1-3], *Microbacterium* [4] and *Rhodococcus* [5]. PheDH has been used as a reagent in the colorimetric microdetermination of L-phenylanine in neonates' blood to detect phenylketonuria [7] in some commercial kits in recent years.

The low yield of PheDH enzyme in wild strains prompted researchers to use recombinant DNA techniques to increase the yield of enzyme expression. Synthesis of functional recombinant enzymes depends upon transcription of the appropriate gene, efficient translation of the mRNA, post-translational processing

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and compartmentalization of the nascent polypeptide. Transcription of a cloned inserted gene requires the presence of a promoter recognized by the host RNA polymerase. Most promoter sequences are ideally located at the transcription termination site at the 3′ end of the gene [8]. It is clear that the first requirement for *E. coli* or *B. subtilis* expression is to place the structural genes under the control of a strong promoter.

In the present study, the role of wild type and plasmid-based promoters were examined for the expression of *pdh* gene in two different host strain, *E. coli* and *B. subtilis* cells.

Materials and Methods

Strains, Media and Plasmids

Escherichia coli JM109, BL21(DE3), C600 and *B. subtilis* ISW1214 were used as host strains for subcloning, plasmid construction and protein expression. Transformants were grown at 37°C in Luria and Bertani (LB) medium containing appropriate antibiotics (ampicillin 0.1 mg/ml and tetracycline 0.25 μg/ml). Vectors pUC19, pET16b (Novagen Co., Ltd. USA) and pHY300PLK (Yakult Co., Ltd. Japan) were applied for subcloning, sequencing and expression. Restriction enzymes and shrimp alkaline phosphatase were from Boehringer Mannheim (FRG). Reagents for ligation were supplied in a kit by Takara Corp. (Shuzo, Japan). DNA sequencing was performed using the 7-deza-dGTP kit and Sequenase product number US78500 (United States Biochemicals, USA). Long ranger agarose solution was from FMC Corp. (Rockland, USA). Other chemicals for sequencing were obtained from Wako Corp. (Osaka, Japan). Plasmid purification was done by QIAGEN kit (GmbH, FRG). All other chemicals were from commercial sources and analytical grade.

Recombinant DNA Techniques and Subcloning

All basic recombinant DNA techniques were carried out according to standard methods [10]. Restriction endonucleases and other enzymes were used as recommended by the suppliers. The plasmid pBDH1DBL (*pdh* gene of *B. sphaericus* inserted in the *Bam*H I site of pUC8) was provided by Biotechnology Research Center, (Toyama Prefectural University, Toyama, Japan). It was amplified in *E. coli* JM109, isolated and restricted with *Bam*H I to yield a 1.5 kb fragment which was subcloned into pHY300PLK and pET16b. Both plasmids were digested in a same fashion. The resulting constructs were designated as pHYDH and pETDH. The pHYDH construct has been transformed in *E. coli* C600 cells. The recombinant clones containing *pdh* gene were screened on LB plate containing ampicillin (0.1 mg/ml), and some colonies were selected for digestion with *Bam*H I. After confirming the length integrity of insert, it was subcloned into pUC19. Recent construct (pUCDH) was chosen for sequencing.

Transformation and Expression

The pHY300PLK as a promoterless shuttle vector used to express the *pdh* gene under wild promoter of *pdh*. The competent cells of *B. subtilis* ISW1214 were prepared. The purified pHYDH construct from *E. coli* C600, and then transformed with chemical standard technique [11] and electroporation (BTX Inc. USA) as well. On the other hand, the pETDH and pHYDH constructs have been transformed in *E. coli* BL21(DE3) and *E. coli* JM109 cells, respectively.

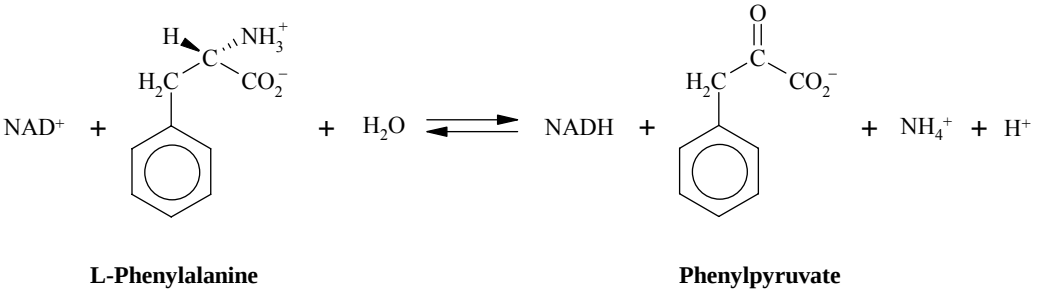
Screening of Recombinants

The visualization of the PheDH activity expressed in the transformants [on the plate culture or cell free extract (CFE) loaded in the well of non-denaturing-PAGE] was carried out as described by Asano [12]. The production of insoluble formazan was initiated by the catalysis of PheDH to yield NADH. The hydride of NADH was transferred non-enzymatically to 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride to form water-insoluble formazan in the presence of phenazine methosulfate as an electron carrier.

A sheet of nitrocellulose filter (Advantech, 82 mm in diameter) with about 200-300 colonies of recombinants was floated on the surface of 1 ml of 10 mg/ml lysozyme solution containing 10 mM EDTA at pH 6.0 until it wetted from beneath, and incubated at 30ºC for 30 min. The filter on the tray was frozen at –20ºC and thawed at room temperature. This procedure was repeated three times to destroy the spheroplasts. The filter or the gel of non-denaturing-PAGE was then floated in a solution containing 10 mM L-Phe, 0.4 mM NAD^+, 0.3 mM phenazine methsolfate, and 0.6 mM 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride in 0.1 M Tris-HCl buffer, pH 8.5. Colonies or bands of enzyme in the gel of non-denaturing-PAGE was developed a crimson color of formazan in a few minutes. Obviously, positive transformants were picked up from their original plates, and PheDH activity in the CFE of which were measured under the standard assay conditions.

Purification and PheDH Assay

B. subtiltis ISW1214 was grown aerobically in 2 liter of LB broth containing 0.25 μg/ml tetracycline at 37°C



Scheme 1. Reaction mechanism of PheDH

for 26 hours (OD_{610}>1.5). All procedures were carried out at 4°C unless otherwise stated. Cells were pelleted and suspended in buffer A (0.1 M potassium phosphate pH 7.9, 0.1 mM EDTA, 5 mM 2-mercaptoethanol) containing 1 mg/ml lysozyme. They were left at room temperature for 20 min, and were sonicated. Cell debris were removed and supernatant placed at 50°C for 10 min. After cooling on ice, the mixture was centrifuged and the supernatant was fractionated with ammonium sulfate (30-60% saturation) followed by centrifugation at 12,000×g for 30 min. The precipitate was dissolved in a small volume of buffer B (0.01 M potassium phosphate pH 7.9, 0.1 mM EDTA, 5 mM 2-mercaptoethanol). The resultant solution was dialyzed against buffer B. The dialysate was applied to a DEAE-Toyopearl column (4.5×17 cm). The column was equilibrated with buffer B and the elution was carried out using a buffer A containing 0.1 M NaCl. Active fractions were pooled, dialyzed and placed on a hydroxyapatite column (4.5×8 cm) which had been equilibrated with buffer B. The enzyme was eluted with a linear concentration gradient of NaCl (0.01-0.4 M) of buffer B. The fractions corresponding to PheDH activity were pooled and dialyzed against buffer B. The dialysate was concentrated by Amicon column and subjected to gel filtration chromatography using sephadex G-200 (1.5×85 cm) equilibrated with buffer B. The purified protein was characterized by means of SDS-PAGE and non-denaturing PAGE. Assay of PheDH activity were as reported earlier [3]. For K_m and V_{max} determination various concentrations of Phe and NAD^+ were employed. One unit of the enzyme activity in oxidative deamination reaction was defined as the amount of enzyme used to catalyze the formation of 1 μmol NADH/min. The protein level was determined spectrophotometrically or with a BioRad protein assay kit.

Results and Discussion

Construction and Transformation

Phenylalanine dehydrogenase genes have been cloned from different sources [12 (references therein)], and our various attempts to subclone the *B. sphaericus* *pdh* gene by conventional methods were successful. These included transferring the *pdh* gene to plasmids such as pUC19, pET16b, and pHY300PLK (Scheme 2) [13]. The palsmid pHYDH coding for the PheDH with the endogenous promoter of the gene was constructed as described in the experimental procedure (Figs. 1 and 2). Based on Takara Co. Ltd. note, transformation efficiency for plasmid of *S. subtilis* largely depends on the contents of plasmid dimmer. Therefore in order to increase frequency of transformation, the construct pHYDH was amplified in *E. coli* rec A^+ strain (C600) prior to transformation. *B. subtilis* ISW1214 was transformed with pHYDH by both chemical and electroporation procedures. Interestingly, transformation efficiency rate was higher by chemical method, compared to the electroporation protocol. Although researchers pointed out to different rates of transformants/μg DNA related to competent cells and plasmids [14]. On the other hand, to study the role of endogenous promoter in expression of target gene we transformed *E. coli* JM109, BL21(DE3), and *B. subtilis* ISW1214 with pUCDH, pETDH and pHYDH constructs, respectively.

Expression and Purification

The first step in gene expression is the transcription of DNA by RNA polymerase. A comparison of the RNA polymerase from the *E. coli* and *B. subtilis* did not revealed gross differences in the core enzyme but the vegetative sigma factors were of very different sizes: 43 kDa in *B. subtilis* and 70 kDa in *E. coli* [8,9]. Since then over 50 *B. subtilis* promoters, recognized by Sigma factors associated with vegetative growth, were sequenced and the consensus –35 and –10 sequences are identical to those from *E. coli* [15]. From detailed studies it would appear that recognition of a promoter in *B. subtilis* involves other structural features, but what these features are, is not clear [9].

The translation apparatus of *B. subtilis* differs significantly from that of *E. coli* [9]. The observation demonstrated the selectivity of *B. subtilis* ribosomes to recognize only homologous mRNA [15]. The S-D

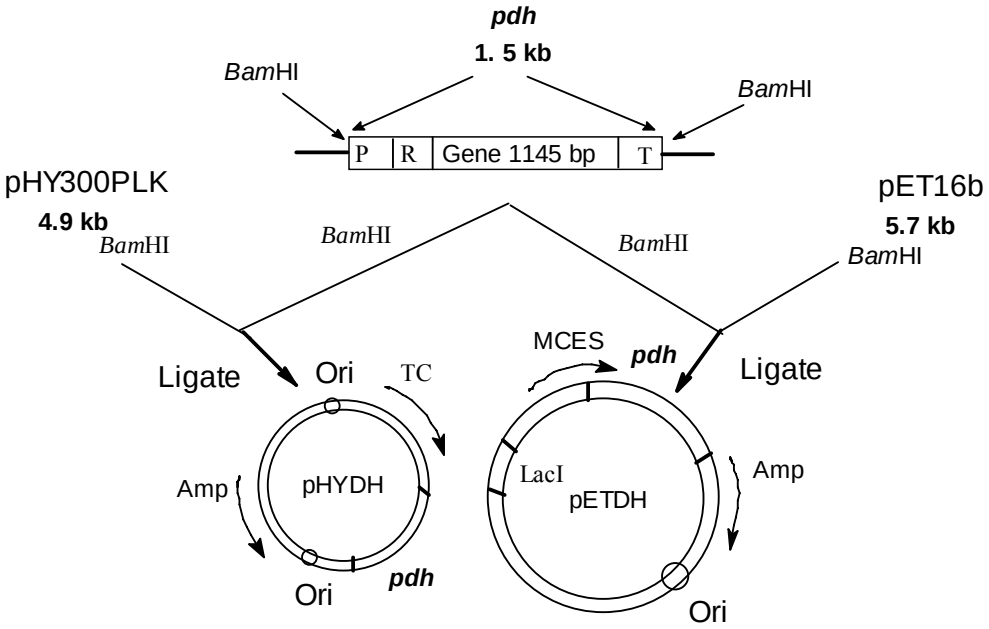
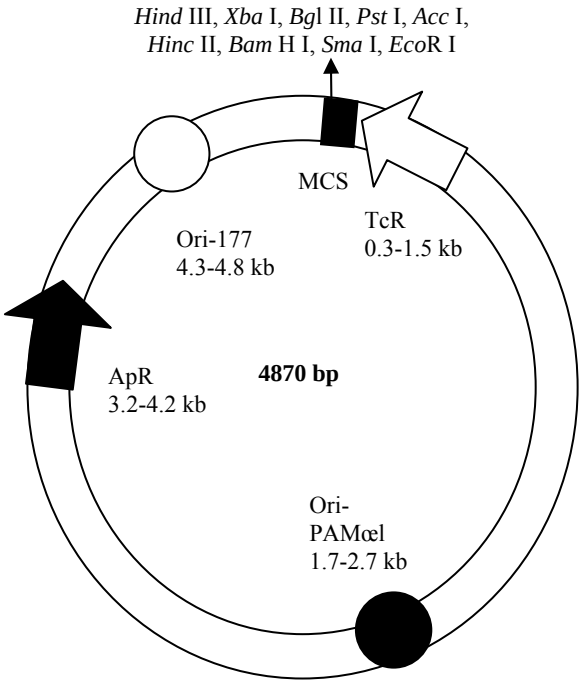


Figure 1. Schematic of *B. sphaericus* phenylalanine dehydrogenase (*pdh*) gene and making expression construct. P, promoter; R, ribosome binding site; T, terminator; TC, tetracycline; MCES, multiple cloning site.



Scheme 2. Schematic map of pHY300PLK vector. The DNA (4870 bp) is a shuttle vector which pACYC177 of *E. coli* and DNA plasmid pAMAα1 of *Streptococcus faecalis*. It can be transformed both *E. coli* and *B. subtilis.* Since this DNA has ampicillin resistant gene can be expressed in *E. coli* , but only tetracycline resistant gene can be expressed in *B. subtilis*. The vector contains a single cleavage site for *Acc* I, *Bam*H I , *Bgl* II, *Eco*R I, *Hinc* II, *Hind* III, *Pst* I, *Sma* I, *Xba* I [13].

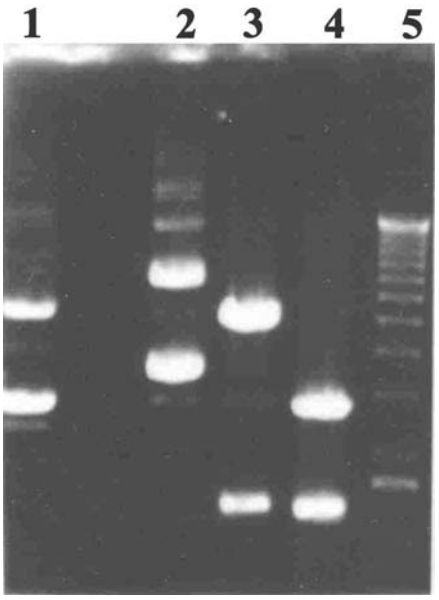


Figure 2. Agarose gel analysis of construct and vector: Lane 1, pHY300PLK uncut; Lane 2, pHYDH uncut; Lane 3, pHYDH *Bam*H I cut; Lane 4, pUCDH *Bam*H I cut; Lane 5, 1 kbp marker.

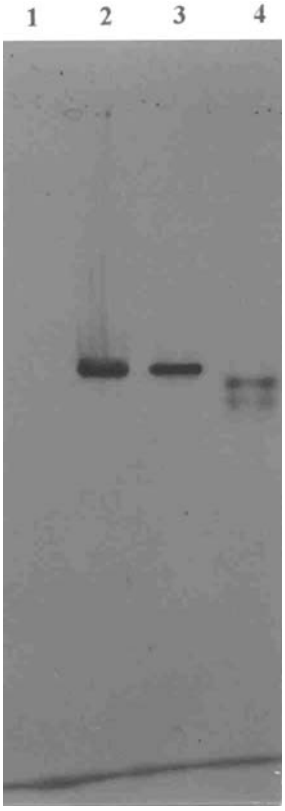


Figure 3. Non-denaturing-PAGE electrophoregram of cell free extract (CFE) in 8% gel activity stained with a solution (see text): Lanes 1, CFE of *E. coli* JM109/pHYDH, 2, *E. coli* BL21(DE3)/pETDH 3, *B. subtilis* ISW1214/pHYDH 4, PheDH of *S. ureae*.

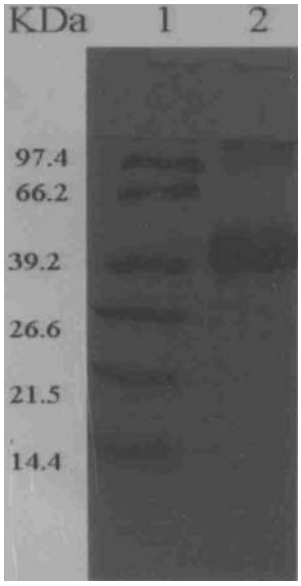


Figure 4. SDS-PAGE electrophoregram of purified phenylalanine dehydrogenase (PheDH) in 10% gel stained with Coomassie blue R-250: Lanes 1 protein marker, 2 purified PheDH.

sequence of *B. subtilis* and other Gram-positive organisms exhibits more complementary with 16S RNA than is observed with *E. coli* [15].

To examine whether the expression of the *pdh* gene depends on its native promoter, the enzyme activities were measured in the cell free extract of *E. coli* JM109/pHYDH, *E. coli* BL21/pETDH and *B. subtilis* ISW1214/pHYDH. *E. coli* JM109/pHYDH showed no activity in its cell free extract (Fig. 3). Although it has a plasmid that contained a *pdh* gene with its wild promoter and ribosome binding site (RBS). On the other hand, *B. subtilis* ISW1214/pHYDH produced PheDH enzyme. It means that the promoterless plasmid pHY300PLK contained *pdh* gene with its own promoter and RBS that expressed in *B. subtilis* ISW1214 cells. It seems that, the ability of *E. coli* BL21/pETDH to produce enzyme (6300U/L) related to the promoter of plasmid. In addition, IPTG induction has no effect on PheDH expression. Hence the *pdh* gene was transcribed from plasmid-based promoter system in *E. coli* BL21 (DE3) but at basal level.

The gene product of pHYDH, pETDH were determined by measurement of the oxidative activity of NADH as an indicator, and pHYDH product purified to near homogeneity with a final yield of 31%. The purification is summarized in Table 1.

The specific activity of enzyme for oxidative deamination of L-phenylalanine was 133.8 U/mg protein. Interestingly, *B. subtilis* ISW1214/pHYDH exhibited an PheDH activity (4700 U/L) that was over 100 times greater than that of the wild type *B. sphaericus* SCRC-R79a, (44 U/L). Nevertheless the highest expression level belonging to *E. coli* JM109/pBPDH1-DBL was about 7200 U/L [16].

Features of PheDH

The relative molecular mass (*Mr* 41 kDa) of the recombinant *B. subtilis* PheDH subunit calculated from its SDS-PAGE (Fig. 4) mobility was agreed with the value of wild type enzyme [17]. The substrate specificity of the recombinant PheDH enzyme was determined by the oxidative deamination reaction (the concentration of amino acids was 10 mM except for L-tyrosine was 0.3 mM). It was active toward L-phenylalanine (100%), L-tyrosine (74% relative to L-phe), L-norleucine (4.5%), L-methionine (3%), L-tryptophan (2%), L-valine (1.8%), and L-leucine (1%). The inert amino acids were D-phenylalanine, L-lysine, glycine, L-alanine, L-glutamic acid, L-asparagine, L-proline, L-serine and L-arginine. The K_m value for L-phenylalanine, L-tyrosine and NAD^+ were 0.24 mM, 0.48 mM and 0.19 mM respectively. The optimum pH of the recombinant enzyme was 11 for the oxidative deamination and 10.2 for the reductive amination. The features of recombinant PheDH enzyme were compatible with the wild type PheDH protein [17].

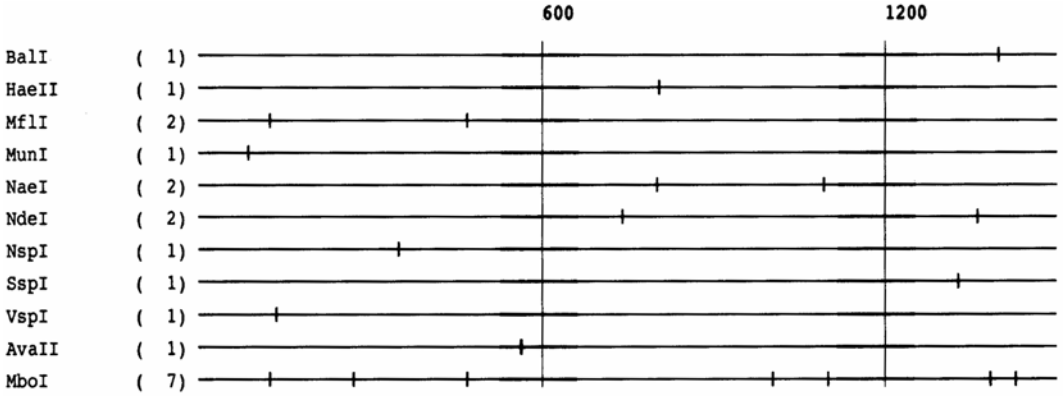


Figure 5. *Bacillus sphaericus* phenylalanine dehydrogenase gene (1500 bp) was cut with restriction type II (sequence position 1-1500).

Nucleotide Sequence Analysis

The complete nucleotide sequence of the 1500bp segment of DNA (*GenBank* M26661) containing the *pdh* gene of *B. sphaericus* was sequenced (not shown). The result of sequencing matched completely with the previous study [18]. Figure 5 shows the map of *pdh* gene that was treated with restriction enzyme type II. As we can judge, it doesn't have any site for *Bam*H I.

In order to isolate the complete *pdh* gene, the plasmid pBDH1DBL was cut with *Bam*H I. Computer programs studies has been defined, coding region extends for 1143 bp (381 amino acid codons) between coordinates 181 (ATG) and 1326 (TAA). The percentage of AT and GC untranslated regions were 59% and 40% respectively, compared with the coding region (56% for AT and 44% for GC). This supported the lack of any significant difference between the intergenic coding regions and structural genes. The 5′ untranslated region contained several TTG (-35) and ATTAAT (-10) sequences which are known to occur in Bacilial promoters [18,19]. There was also a potential ribosome binding site like those found in other Bacilial genes [20]. A GC-rich sequence that could serve as a transcriptional terminator was present at the 3′ end [18]. The codon usage of the pdh gene was closely related other Bacilial genes [21]. Although it has been pointed out that preferential use of rare codons in genes may constitute a translational barrier to expression in a heterologous hosts [22], however the observed high expression of the *pdh* gene in *E. coli* agreed with the view that gene expression is not modulated by codon usage [23]. Nevertheless the role of codon sequence usage is important and this seems to be related to genes with particularly low GC contents [24].

Briefly, The *pdh* gene of *B. sphaericus* has been cloned and expressed in *B. subtilis* cells with its own promoter successfully. Its endogenous promoter presented no effect on transcription of the target gene in *E. coli JM 109.* Furthermore a study to examine the expression of *pdh* gene as an infusion protein with spore coding genes of *B. subtilis* is presently under way.

Table 1. Summary of phenylalanine dehydrogenase Enzyme purification from recombinant *B. subtilis* ISW1214/pHYDH

Step	Activity (U)	Protein (mg)	S. activity (U/mg)	Yield (%)	Fold
Crude extract	9400	1266	7.42	100	1
Heat treatment	8920	671	13.29	94.9	1.79
$(NH_4)_2SO_4$	5721	448	12.77	60.86	1.72
DEAE-Toyopearl	5281	139	37.99	56.18	5.12
Hydroxyapatite	4124	48	85.9	43.87	11.57
Sephadex G-200	2944	22	133.8	31.31	18

Acknowledgements

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