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# Cloning and Expression of *pdxS* and *pdxT* genes from *Geobacillus* sp. H6a

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## Abstract

Pyridoxal 5′-phosphate (PLP), the biologically active form of vitamin B6, is an important coenzyme for metabolism of protein, carbohydrate, lipid, nervous system, hormone and heme synthesis. The pyridoxal 5′-phosphate synthase (PLP synthase) are involved in DXP-independent of *de novo* vitamin B6 biosynthesis pathway. This enzyme complex consists of two proteins (PdxS and PdxT) which encoded by *pdxS* and *pdxT* genes. PLP synthase synthesizes PLP from ribose 5′-phosphate, glyceraldehyde 3 -phosphate and glutamine. Thermophilic bacterium, *Geobacillus* sp. H6a (*Gh*) can produce vitamin B6 in PM and PMP forms, was isolated from hot springs at the North of Thailand. The *GhpdxS* and *GhpdxT* were amplified by touchdown PCR. The *GhpdxS* had a length of 885 base pairs (bp) encoding a protein of 294 amino acids (aa) and the *GhpdxT* had a length of 591 bp encoding a protein of 196 aa. The result of phylogenetic analysis confirmed that PdxS and PdxT protein from *Geobacillus* sp.H6a were closed group with genus *Geobacillus*. We inserted *GhpdxS* and *GhpdxT* genes into pET28a(+) vector at the site of *Nhe*I and *BamH*I, then transformed into *E.coli* BL21(DE3). Two of transformants were induced with 1mM IPTG at 25, 30 and 37°C for 0, 1, 3 and 6 hours. The recombinant PdxS and PdxT proteins with His-tag had molecular weight 39 kDa and 28 kDa, respectively. The soluble rGhpdxS and rGhpdxT showed the activities of synthase and glutaminase, respectively. The purification and characterization of both rGhpdxT and rGhpdxS will be further investigated.

**Keywords:** Cloning, Expression, *GhpdxS*, *GhpdxT*, PLP synthase, *Geobacillus* sp.H6a

## Introduction

Vitamin B6 is an essential component in the human diet. It functions as a coenzyme in many metabolisms, including protein, carbohydrate, and lipid. It is also important to the nervous system, hormones function, and synthesis of heme (Lau et al., 2006). Vitamin B6 has free form *i.e.* pyridoxine (PN), pyridoxal (PL) and pyridoxamine (PM) and the 5′ phosphorylated derivatives *i.e.* pyridoxine 5′-phosphate (PNP), pyridoxal 5′-phosphate (PLP) and pyridoxamine 5′ phosphate (PMP). The fungi, plants, archae, and most eubacteria are able to synthesize vitamin B6, but most animals and human, lack this ability and rely on the external supply of vitamin B6.
Biosynthesis pathways of vitamin B6 is known as *de novo* pathway and salvage pathway. The *de novo* biosynthesis pathway is found in plants, bacteria, fungi and parasite. There are 2 different pathways using and non-using deoxyxylose 5-phosphate

(DXP) as a precursor. The first pathway is a DXP dependent use 4-phospohydroxy-L-threonine (4HPT) and DXP as substrates to form PNP, the PdxJ and PdxA protein are involved (Mooney et al., 2009). The second pathway using ribose 5-phosphate or ribulose 5-phosphate, in combination with either glyceraldehyde 3-phosphate or dihydroxyacetone phosphate and glutamine as substrate, two proteins are involved by form complex with PdxS and PdxT to synthesize PLP. The PdxT protein acts as an amido transferase, which converts glutamine to glutamate as nitrogen source, and then PdxS protein condenses all of substrate to form PLP.

From a previous study, thermophilic bacterium *Geobacillus* sp. H6a (Gh), collected from a hot spring in the North of Thailand, produced extracellular vitamin B6 in PMP and PM forms (Anutrakunchai et al., 2010a). The expression analysis of *GhpdxS* and *GhpdxT* genes which encoded GhpdxS and GhpdxT protein to form PLP synthase, in DXP independent pathway from *Geobacillus* sp. H6a were studied (Anutrakunchai et al., 2010b). To more understand the function of PLP synthase, we cloned and expressed the GhpdxS and GhpdxT proteins from *Geobacillus* sp. H6a.

## Experimental and Methods

### Cloning *pdxS* and *pdxT* genes from *Geobacillus* sp.H6a

The *Geobacillus* sp. H6a genomic DNA was isolated using the High Pure PCR Template Preparation Kit (Roche, Germany). The *pdxS* and *pdxT* genes were amplified by touchdown PCR with specific primers containing *Nhe*I and *BamH*I recognition sites. The primers were: *pdxS*,5′-FpdxSNhe, ATTGCTAGCATGGCATTGACAGGT ACGGACC, 3′-RYaaDBam, TTTTGGATCCTTACCAGCCGCGTTCTTGCATC, and *pdxT*, 5′-YaaENhe, AAAGCTAGCATGAAAATAGGTGTACTTGGACTGC, 3′-YaaEBam, TTGGGATCCTTACTTGAGGCTTGACGCC, the underline show restriction sites of *Nhe*I and *BamH*I (Anutrakunchai *et al*., 2010b). PCR was performed in thermal cycler, for condition, denaturation as 95°C 1 min, annealing as 62°C 1 min for 3 cycles, 60°C 1 min for 4 cycles, 58°C 1 min for 23 cycles and extension at 72°C 2 min. PCR products were analyzed with 1.5 % agarose gel electrophoresis and stained with ethidium bromide. The DNA fragment was purified before and after digestion by Gel/PCR DNA fragments Extraction kit (Geneaid). The pET28a(+) vector was isolated using High-Speed Plasmid Mini Kit (Geneaid) and digested with *Nhe*I and *BamH*I. The digested pET28a(+) vector was purified by Gel/PCR DNA fragments Extraction kit (Geneaid).

### Expression of *pdxS* and *pdxT* in *E.coli* BL21(DE3)

The PCR products were ligated into expression vector pET28a(+) with *Nhe*I and *BamH*I sites by using 0.3 pmol digested gene, 0.03 pmol digested pET28a(+), 1× T4 buffer, 0.4 weiss Unit T4 DNA ligase, the reaction were incubated at 16°C for 16 h, yielding pET28a-*GhpdxS*/*GhpdxT*. The recombinant plasmid was introduced into *E.coli* BL21(DE3) by heat shock method. Alkaline lysis method and colony PCR were used to check transformants. The bacterial cells, harboring pET28a-*GhpdxS*/*GhpdxT* were grown at 37°C by inoculating 1 ml overnight culture into 100 ml LB medium containing 50 µg $ml^{-1}$ kanamycin, until the optical density ($OD_{600}$) reached 0.5. Expression of protein was induced by adding isopropyl-1-thio-D-galactopyranoside (IPTG) to a final concentration 1 mM and cell was collected at 30°C for 0, 1, 3 and 6

h. Cell pellets were resuspend with phosphate buffer saline (PBS) pH 7.4. The cells were sonicated and centrifuged at 10,000 g for 15 min at 4°C. Proteins in the supernatants and pellets were examined by SDS-PAGE.

**Homology search and multiple alignments**

Sequence similarity searches were performed on the deduced amino acids using BLASTX program on the nucleotide database (National Center for Biotechnology Information) with default parameters. The alignment was generated with the Clustal X 2.0.9 program (Larkin et al., 2007). The phylogenetic tree was visualized using the neighbor-joining method and TREEVIEW version 1.6.6 (Page, 1996). Bootstrap values were based on 1,000 trees generated with the program. The *GhpdxS* and *GhpdxT* nucleotide sequences were deposited into GenBank (accession nos. FJ497249 and FJ497250, respectively).

**Determination of the PdxT enzymatic activity**

The PdxT activity was assayed in two steps by measuring the formation of glutamate, which is converted to 2-oxoglutarate by glutamic dehydrogenase with $NADP^+$ as co-substrate. The reaction was assayed in 50 mM Tris-HCl (pH 8.5), 10 mM glutamine and 100 – 200 µl of crude enzyme PdxT in a total volume of 300 µl at 30 °C for 10 min. The reaction was stopped by boiling for 1 min and then added 50 mM Tris-HCl, pH 8, 1 mM EDTA, 500 µM $NADP^+$, and 7 units of glutamic dehydrogenase to final volume 1 ml and incubated for 90 min at 30 °C. Finally, the samples were centrifuged for 1 min at 14,000×*g*, and determined at 340 nm. The activity was calculated with the molar extinction coefficient of 6220 $M^{-1}$ $cm^{-1}$ of NADPH (Wrenger et al., 2005). One unit of enzyme was defined as the amount of the enzyme required to produce 1 nmol of NADPH per min at 30°C. Protein concentration was determined by the dye-binding assay, with bovine serum albumin as a standard (Bradford et al., 1976).

**Determination of the PdxS enzymatic activity**

The PdxS activity was assayed the formation of a Schiff's base between PLP and Tris buffer. The reaction was assayed in the presence of 0.5 mM ribose 5-phosphate, 1 mM glyceraldehyde 3-phosphate and 10 mM ammonium chloride, 500 µl of crude enzyme PdxS in a 50 mM Tris-HCl (pH 8.0) in total volume of 1 mL and then reaction was incubated for 30 min at 37°C. The samples were centrifuged for 2 min at 8,000 rpm. The increase of absorbance 414 nm was measured comparing with standard curve of PLP (Rachel et al., 2005). One unit of enzyme was defined as the amount of the enzyme required to produce 1 nmol of PLP per min at 37°C.

**Results and Discussion**

**GhpdxS and GhpdxT homologs**

The PCR product of *pdxS* gene from *Geobacillus* sp. H6a showed 885 base pairs with start codon TTG (Anutrakunchai et al., 2010b). This start codon (TTG) was found only 3% of start codon in *Escherichia coli* (Blattner et al., 1997). For suitable expression, we designed the forward primer (5′-FpdxSNhe) by using ATG as start codon and adding one nucleotide at 5′-end to make sure that restriction enzyme could completely attach with DNA strands in digestion step. The full length of *GhpdxT* has

591 base pairs. The deduced amino acid showed 294 and 196 amino acid residues for GhpdxS and GhpdxT, respectively. Comparison of the amino acid sequences of GhpdxS and GhpdxT with available amino acid sequences in a public database by Blast searching (GenBank, NCBI) showed that GhpdxS and GhpdxT were in the group of *Geobacillus* and *Bacillus* with high identity. The results of phylogenetic tree confirmed that GhpdxS and GhpdxT were closed group with *Geobacillus* (Figures 1 and 2).

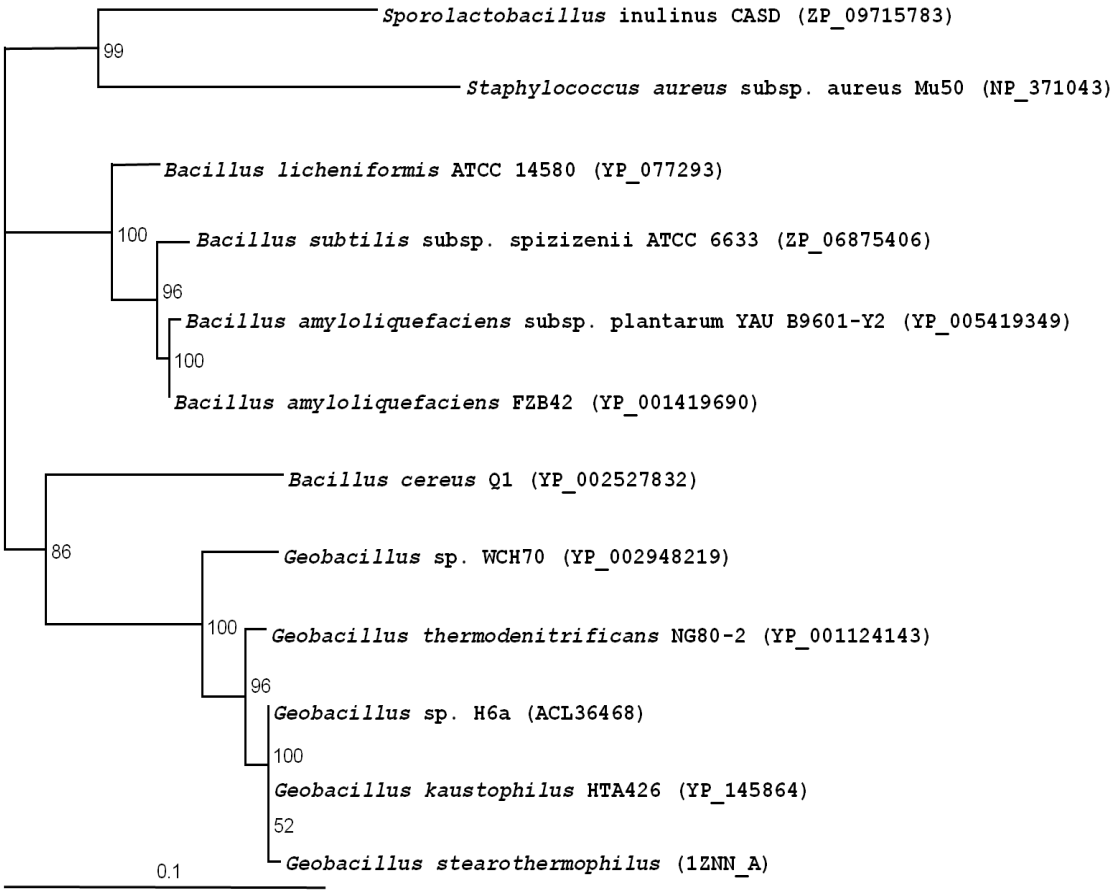


**Fig.1** Phylogenetics tree of PdxS protein of *Geobacillus* and *Bacillus* spp. The numbers indicate bootstrap values. The sequence of *Staphylococcus aureus* subsp. Mu50 and Sporolactobacillus inulinus CASD were used as the outgroup.

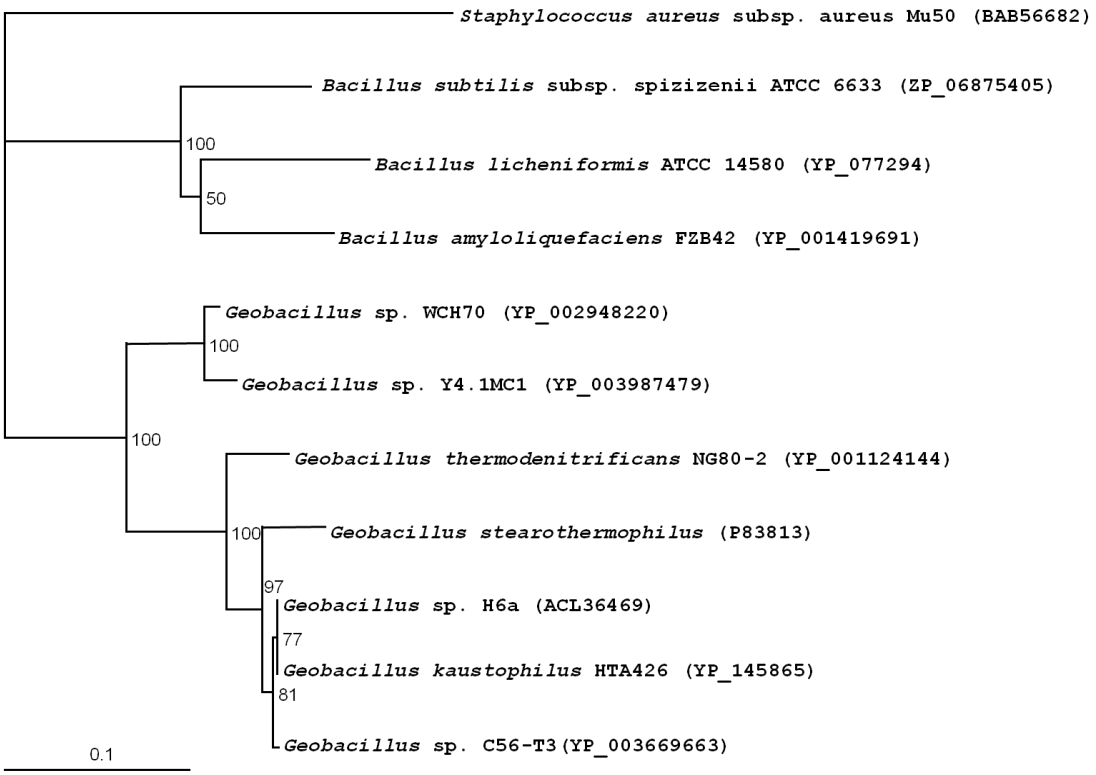


**Fig.2** Phylogenetics tree of PdxT protein from *Geobacillus* and *Bacillus* spp. The numbers indicate bootstrap values. The sequence of *Staphylococcus aureus* subsp. Mu50 was used as the outgroup.

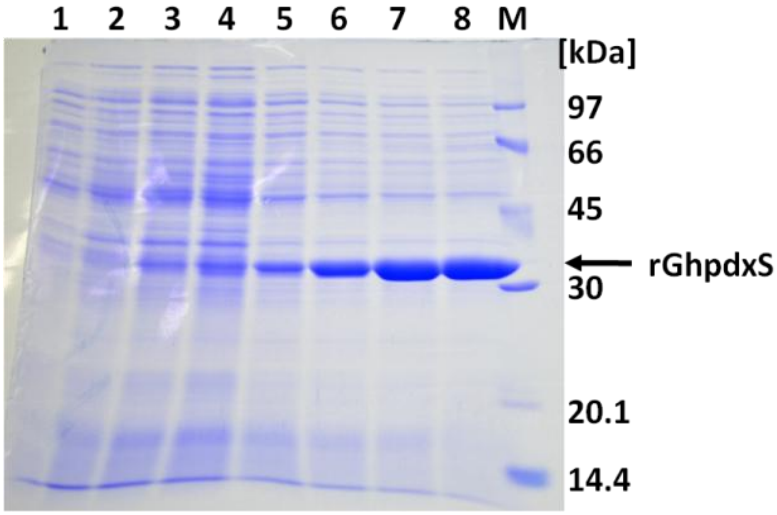


**Fig.3** SDS gel electrophoresis in 12% polyacrylamide gel of the crude extract of rGhpdxS obtained from induced and uninduced *E.coli* BL21 (DE3) pET28a(+)-*GhpdxS*. Lane 1-4: uninduced cultures at 0, 1, 3 and 6 h, respectively. Lane 5-8: induced cultures at 0, 1, 3 and 6 h, respectively. Lane M: protein marker.

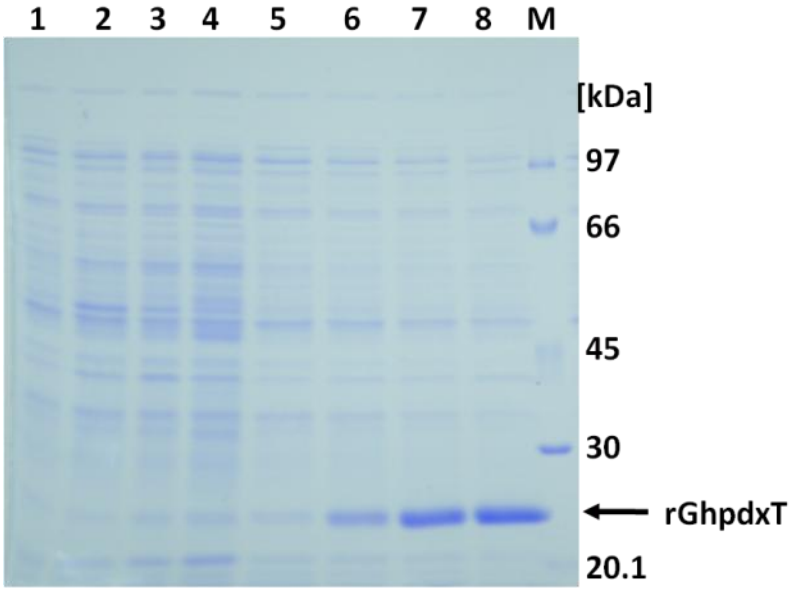


**Fig.4** SDS gel electrophoresis in 12% polyacrylamide gel of the crude extract of rGhpdxT obtained from induced and uninduced *E.coli* BL21 (DE3) pET28a(+)-*GhpdxT*. Lane 1-4: uninduced cultures at 0, 1, 3 and 6 h, respectively. Lane 5-8: induced cultures at 0, 1, 3 and 6 h, respectively. Lane M: protein marker.

**Expression of the recombinant $His_6$-tagged *GhpdxS* and *GhpdxT* in *E. coli* BL21 (DE3)**

The *GhpdxS* and *GhpdxT* were inserted into multiple cloning site of the pET28a (+), yielding pET28a(+)-*GhpdxS/GhpdxT* which contained a hexahistidine N-terminal tag. Recombinant expression of GhpdxS and GhpdxT were induced in *E.coli* BL21 (DE3) cells by adding of 1 mM IPTG at 25, 30 and 37°C. The level of expression of the highest at 30°C as shown in Figure 3 and 4. The expression of both recombinant GhpdxS (rGhpdxS) and GhpdxT (rGhpdxT) were found in pellet fraction at 25°C. The results of rGhpdxS and rGhpdxT expression showed-molecular mass 39 kDa and 28 kDa, respectively.

**rGhpdxT and rGhpdxS activities**

Analyze of rGhpdxT activity was done for 1, 3 and 14 days. The maximum rGhpdxT specific activity was 13.6 Units/mg protein at first day and then declined. The activity was remained 63.23% after kept at 4°C for 14 days. Analyze of rGhpdxS activity was done for 1 and 10 days. The maximum rGhpdxS specific activity was 0.12 Units/mg protein at 10 days. From this data, it is possible to be stable after kept at 4°C. However, both rGhpdxT and rGhpdxS activities were measured in form of crude enzyme. Many things in crude enzyme might have an effect on the activity measurement. Purification and characterization of both rGhpdxT and rGhpdxS will be further investigated.

## Conclusions

Both *pdxS* and *pdxT* genes were cloned from *Geobacillus* sp.H6a and expressed in *E.coli* BL21(DE3). Based on the sequence, both proteins were found to be in *Geobacillus* group. Both rGhpdxS and rGhpdxT showed enzyme activities.

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