

Development of an Assay
System for the Determination of
Transketolase and Transaldolase
Activities in Hyperthermophilic
Bacteria and Archaea

by

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Author's declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

Abstract

Only a few hyperthermophilic archaea are found to have a complete nonoxidative stage of pentose phosphate pathway (NOPPP), which indicates that most archaeal hyperthermophiles are partially missing pentose metabolizing enzymes. However, very limited research has been done in this interesting field. Although few hyperthermophilic enzymes in PPP have been studied in detail, the enzymatic activities reported previously were underestimated because assays were performed at temperatures much lower than 80° C. The commercially available auxiliary enzymes used in those assays were not thermostable, which limited assay temperature as well. The substrates used in those assays are extremely expensive, which is another factor limiting the study in this area. In this project, an inexpensive and accurate assay system was developed to overcome these limitations.

Genes encoding several auxiliary enzymes for PPP enzyme assays were cloned from selected hyperthermophiles and overexpressed in *E.coli* Rossetta2™ (DE3). These enzymes were glyceraldehydes-3-phosphate dehydrogenase from *Thermotoga maritima* (TmGAPDH), triosephosphate isomerase from *Pyrococcus furiosus* (PfTIM), glycerophosphate dehydrogenase from *Pyrococcus furiosus* (PfG3PDH) and *Aeropyrum pernixK1* (ApG1PDH), transketolase from *T. maritima* (TmTK), xylose isomerase from *T. maritima* (TmXI) and *Thermotoga neapolitana* (TpXI), and xylulose kinase from *T. maritima* (TmXuK). Their activities were determined under anaerobic conditions at 80°

C in both cell free extracts and for purified enzymes. The assay system contained the following parts: A) TmGAPDH, TmXI or TpXI, TmXuK (TmTK), PftIM, and PfG3PDH or ApG1PDH as auxiliary enzymes for TK (XuK) assay; B) TmGAPDH, PftIM, and PfG3PDH or ApG1PDH for transaldolase (TAL) assay. D-xylose, instead of the traditional assay substrate xylulose-5-phosphate (xylulose), was used as the substrate in this assay system for the determination of TK (XuK) activity.

The activities of XuK, TK, and TAL were also determined in several hyperthermophilic bacteria and archaea. All enzymes served as paradigms to prove the feasibility of using the new assay system for other hyperthermophilic PPP enzymes. The species of hyperthermophiles used in this study were *T. maritima*, *P. furiosus*, *Thermococcus guaymasensis*, *T. neapolitana*, *Thermotoga hypogea* and *T. petrophila*. Two different methods were tested for the TAL assay (part B), with either TmGAPDH or PftIM plus TmG3PDH as the auxiliary enzymes. Similar characteristics were obtained using both methods. The existence of TAL in all selected hyperthermophiles might indicate that the existence of the PPP is functioning among them.

The XuK assays in selected hyperthermophiles were successfully conducted using the new assay system (part A). *T. petrophila* showed the highest XuK activity (0.3 U/mg), indicating the feasibility of the assay system's application in the determination of hyperthermophilic PPP enzymes at high temperatures (80° C). TmTK activity may also be determined using the new assay system if the auxiliary enzyme XuK activity would be higher. Therefore, the new assay system developed in this project demonstrates dual functions for both XuK and TK assays in hyperthermophiles.

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List of Abbreviations

ApG1PDH:	glycerol-1-phosphate dehydrogenase of <i>Aeropyrum pernix K1</i>
CFE:	cell free extract
DHAP:	dihydroxyacetone-phosphate
DTT:	dithiothreitol
E4P:	erythrose-4-phosphate
F6P:	fructose-6-phosphate
GAP:	glyceraldehyde-3-phosphate
GAPDH:	glyceraldehyde-3-phosphate dehydrogenase
GPDH:	glycerol-phosphate dehydrogenase
LB broth:	Luria-Bertani broth
NAD⁺:	nicotinamide adenine dinucleotide
NADH:	nicotinamide adenine dinucleotide reduced
NADP:	nicotinamide adenine dinucleotide phosphate
NADPH:	nicotinamide adenine dinucleotide phosphate reduced
NOPPP:	non-oxidative stage of pentose phosphate pathway
OPPP:	oxidative stage of pentose phosphate pathway
Pi:	phosphate
PPP:	pentose phosphate pathway
PfTIM:	triosephosphate isomerase of <i>Pyrococcus furiosus</i>
PfG3PDH:	glycerol-3-phosphate dehydrogenase of <i>Pyrococcus furiosus</i>
Pur. :	Purified or pure
R5P:	ribose-5-phosphate
SDS-PAGE:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SDT:	sodium dithionite

TAL:	transaldolase
TIM:	triosephosphate isomerase
TK:	transketolase
TmGAPDH:	glyceraldehyde-3-phosphate dehydrogenase of <i>Thermotoga maritima</i>
TmTK:	transketolase of <i>Thermotoga maritima</i>
TmXI:	xylose isomerase of <i>Thermotoga maritima</i>
TmXuK:	xylulose kinase of <i>Thermotoga maritima</i>
TPP:	thiamine pyrophosphate
 TpXI:	xylose isomerase of <i>Thermotoga petrophila</i>
XI:	xylose isomerase
XuK:	xylulose kinase

1. Introduction

1.1 Hyperthermophiles and their enzymes

1.1.1 General characteristics of hyperthermophiles

Extremophilic microorganisms have been a focus of attention from many scientists for decades since they were discovered. The interests of studying these organisms are not only because of their metabolisms and biochemical adaptation to extremes in parameters, such as pH, temperatures, salt concentration, pressure, but also the potential applications of their enzymes. Based on growth temperatures, microorganisms can be divided into psychrophiles, mesophiles, thermophiles and hyperthermophiles (Lim 2003, Morozkina *et al.*, 2010).

Hyperthermophiles thrive at extremely high temperatures including natural and industrial environments, such as marine hyperthermal vents, geysers, hot springs, sun heated soils, litter, volcanoes, oil reservoirs and coal piles (Madigan *et al.*, 2000; Vieille and Zeikus, 2001; Lebedinsky *et al.*, 2007). *Methanopyrus kandler* strain 116, can grow at 122° C (Takai *et al.*, 2008). It is obvious that hyperthermophiles can sustain at very high temperatures. Moreover, they need high temperatures to maintain their basic metabolisms and proper functions (Charlier and Droogmans, 2005) and they generally cannot grow below 60° C (Stetter, 1996).

Hyperthermophiles possess thermostable enzymes, DNA modification mechanisms preventing damage by denaturation, special membrane and lipid structures (Albers *et al.*, 2001; Atomi, 2004; Hickey *et al.*, 2004). DNA and RNA of hyperthermophiles demonstrate high stability under such environments by the following main factors to resist denaturation: nucleic acid associated proteins, reverse gyrase, high salt concentration, unusual linear polyamines and efficient repair systems (Charlie *et al.*, 2005). Interestingly, reverse gyrase is found in all hyperthermophiles (Heine *et al.*, 2009).

1.1.2 Hyperthermophilic enzymes

Thermophilic and hyperthermophilic enzymes (also called thermozyms) are part of another enzyme category called extremozymes, which are evolved in extremophiles (Vieille *et al.*, 2001). Hyperthermophilic enzymes are characterized with increasing thermostability and higher reaction rate. Their optimal temperatures are generally above 90° C, compared to the enzymes isolated from mesophiles (maximum activity at 25-60 ° C) and those from psychrophiles (5-25° C) (Vieille *et al.*, 2001). It is speculated that one of the advantages of hyperthermophilic enzymes is expedition of the reaction range in industrial environments as biocatalysts. Other advantages of application of hyperthermophilic enzymes at high temperatures are less contaminations occurring, equilibrium towards desired product, and higher reacting velocity (Atomi *et al.*, 2005; Harris, 2009).

The promising properties of enzymes from hyperthermophiles confer ideal biocatalysts in industrial applications, such as ethanol production (Radianingtyas *et al.*, 2003), rare-sugar production (Short *et al.*, 1998; Coolbear, *et al.*, 1992), hydrogen production (Woodward *et al.*, 2002), and molecular work reagents (Atomi *et al.*, 2005).

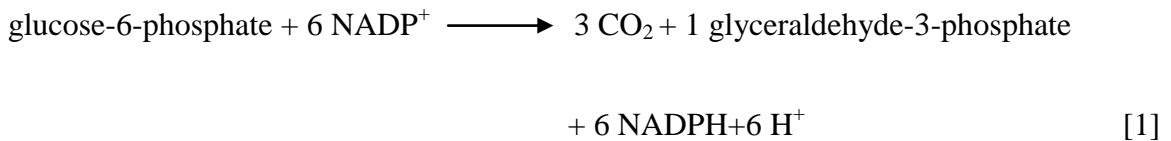
Although there are great phylogenetic differences between hyperthermophiles and other well-studied organisms, the same enzymes from them exhibit high homologies with mesophilic enzymes. They have 40%-85% similarity of amino acid sequences and catalytic functions, even their crystal structures exhibit great similarity from one another (Vieille *et al.*, 2001). From this point of view, it is possible to characterize hyperthermophilic enzymes by overexpressing their genes in *E. coli* or yeast strains, then determining their properties after the expression in host organisms.

1.2 Pentose metabolism in microorganisms

Pentose phosphate pathway (PPP) and the Calvin Cycle are main pathways in pentose metabolism in microorganisms. PPP is widely distributed among three domains of life. Calvin Cycle is found only in autotrophs that can form organic compounds from inorganic materials. Therefore, for the reactions in Calvin Cycle in most microbes, they are to be found in the chloroplast stroma instead of the cell cytoplasm (White, 2007). Both pathways have some identical reactions and enzymes so that some intermediates produced in the reaction sequence connect to other central metabolic pathways. Due to the subjects in this project, only PPP will be discussed in this thesis because all selected hyperthermophiles in this study are heterotrophs.

1.2.1 Pentose phosphate pathway

PPP is one of the catabolic central metabolic pathways. It has been found in most organisms. The overall reaction of PPP is the following (reaction 1).



The whole process of classic PPP found in eukaryotes and bacteria is illustrated in **Figure 1.1**.

There are two stages in PPP: oxidative stage (OPPP) and non-oxidative stage (NOPPP). Ribulose-5-phosphate is produced through decarboxylation by 6-phosphogluconate dehydrogenase. NOPPP starts from pentose phosphate. There are two parts in NOPPP: sugar isomerization and sugar rearrangement (Soderberg, 2005). Ribose-5-phosphate isomerase and ribulose-5-phosphate epimerase catalyze the reactions in the former part, which mainly converts pentose from one form to another form. Transketolase and transaldolase are involved in the latter part. Transketolase transfers a two carbon unit, while transaldolase transfers a three carbon unit from a ketose to an aldose (White, 2007), by which generates some important intermediates functioning as crucial precursors for biosynthesis, such as ribose-5-phosphate (R5P), glyceraldehyde-3-phosphate (GAP), fructose-6-phosphate (F6P) and erythrose-4-phosphate (E4P).

Pentose phosphates produced in PPP are the precursors to the ribose and deoxyribose in the nucleic acid biosynthesis. In addition, erythrose phosphate produced

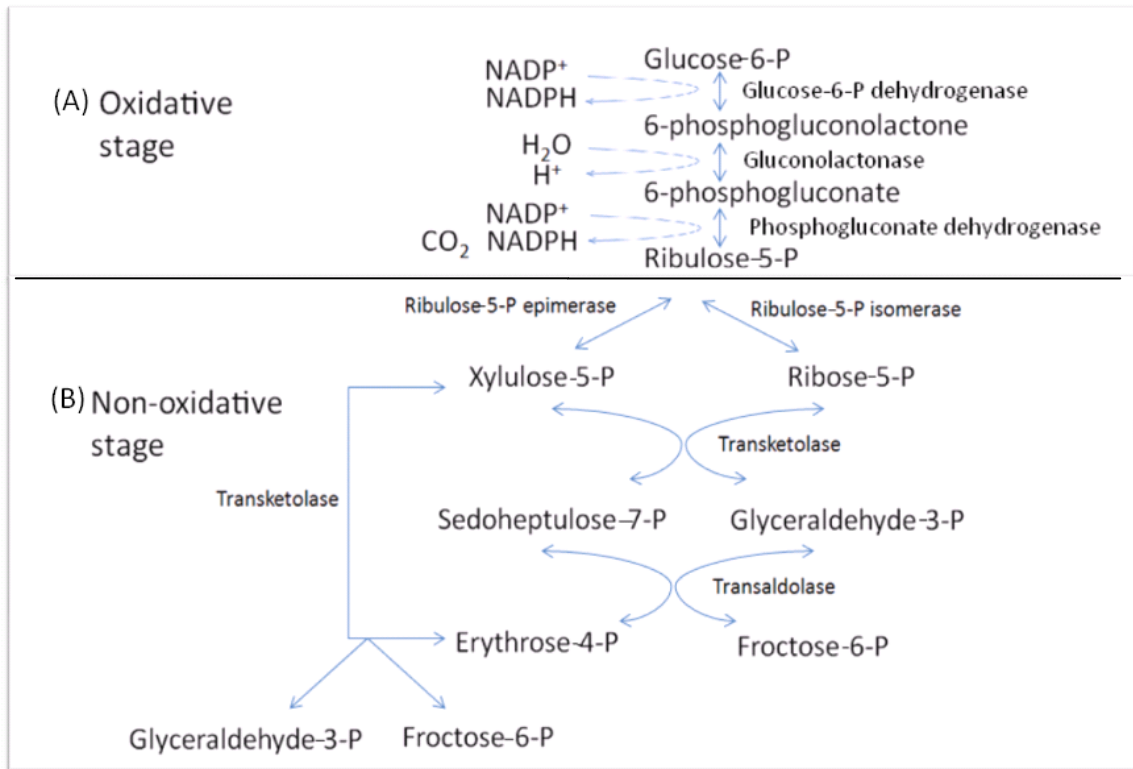


Figure 1.1 Pentose phosphate pathway. **(A)** Oxidative stage (OPPP). **(B)** Non-oxidative stage (NOPPP). See explanation in the text (page 4). (Modified from Soderberg, 2004)

in PPP is the precursor to all types of aromatic amino acids, such as phenylalanine, tyrosine, and tryptophan (White, 2007). Moreover, NADPH produced in PPP is a major source of electrons synthesized among several anabolic pathways. NADPH is an important electron donor involved in fatty acid synthesis, amino acid metabolism and so on. Furthermore, PPP shares some identical reactions with Calvin Cycle, by which many autotrophs can convert inorganic carbon source into organic compounds. Finally, D-glyceraldehyde-3-phosphate (GAP) and D-fructose-6-phosphate (F6P) produced in PPP can be oxidized into pyruvate by which connects to all of the central metabolic pathways and fermentations (Burg *et al.*, 2002).

1.2.2 Current studies of hyperthermophilic enzymes in PPP

Although PPP is such an important metabolic pathway for most of organisms, astonishingly, most of archaea whose genome sequences are available do not have the complete PPP (Soderberg, 2004; Brouns *et al.*, 2006; Grochowski *et al.*, 2005). It is suggested that certain substitute pathways may play a key role in pentose metabolisms in archaea and hyperthermophiles; however, no conclusive result has been obtained yet (Mitusi *et al.*, 2000; Rashid *et al.*, 2004; White *et al.*, 2004; Sato *et al.*, 2007; Werkern *et al.*, 2008). Another obstacle for exploring this research area is that little information has been obtained. Only three hyperthermophilic archaeal PPP enzymes' properties and molecular structures have been characterized: transaldolase in *M. jannaschii* (Soderberg and Alver, 2004), ribose-5-P isomerase in *Pyrococcus horikoshii* (Ishikawa *et al.*, 2002) and *Methanocaldococcus jannaschii* (Strange *et al.*, 2009). Their properties and

molecular structures are studied in detail. Insufficient research data obtained limit our understanding of the pentose metabolisms among hyperthermophiles.

Another drawback of the published data in this area is that the enzyme activities have been underestimated due to the lower assay temperatures ($< 80^{\circ} \text{ C}$) applied in those experiments. All the assay temperatures are below 80° C in published papers. Usually, it is believed that reaction velocity doubles if the reaction temperature increases 10° C . Therefore, the consequence of using lower temperatures (from 30° C to 70° C) in those assays is that the activities do not reflect what is achievable at higher temperature where these microbes live.

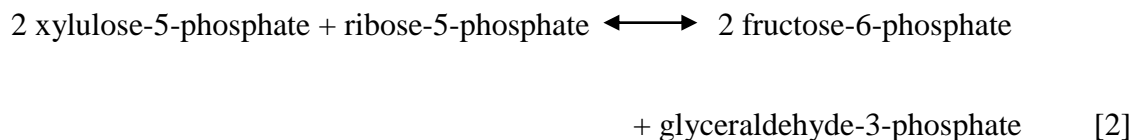
In addition, three limiting factors are also related to the lower estimation of the activities of those hyperthermophilic enzymes. The first is that the commercial auxiliary enzymes they used in assay mixture are mesophilic enzymes, which means they are not thermostable at high temperatures. The second is some key components in reaction mixture are not thermostable as well, such as ketose-phosphate used in the reaction mixture. Finally, some substrates used in the assay mixtures are rare-sugars. Rare-sugars (mainly from Sigma-Aldrich) are extremely expensive. All the factors listed above are barriers to further study of hyperthermophilic PPP enzymes.

In order to solve these problems, it is necessary to develop a consistent assay system at high temperature (80° C) to correct the previous assay discrepancy (Rehaber *et al.*, 1992; Kohlhoff *et al.*, 1996; Nishihara *et al.*, 1997; Soderberg and Alver, 2004). It would also be useful to find an economic approach for quantitative assays. If these limitations could

be overcome by developing a new assay system, a better understanding of the pentose metabolism in hyperthermophiles would be attainable.

1.3 Transketolase and transaldolase

Transketolase and transaldolase act as a bridge connecting PPP and glycolysis. They transfer a two- or a three-carbon unit from a ketose donor to an aldose acceptor to regenerate some key intermediates, which are important precursors for biosynthesis of building blocks in the cytoplasm. The overall reaction catalyzed by transketolase and transaldolase is the following (reaction 2). Thus, pentose produced in PPP can be completely converted into glycolytic intermediates.



1.3.1 Transketolase

Transketolase (TK, EC 2.2.1.1) is a thiamine pyrophosphate (TPP) dependant enzyme functioning in both PPP and Calvin Cycle. TPP serves as TK's key coenzyme in the catalytic process of sugar rearrangement. TK catalyzes two reversible reactions involved in transferring a two-carbon unit from a keto donor to an aldo acceptor. Fructose 6-phosphate and glyceraldehyde 3-phosphate produced during the reactions link PPP and glycolysis. The detailed mechanism of TK is illustrated in **Figure 1.2**.

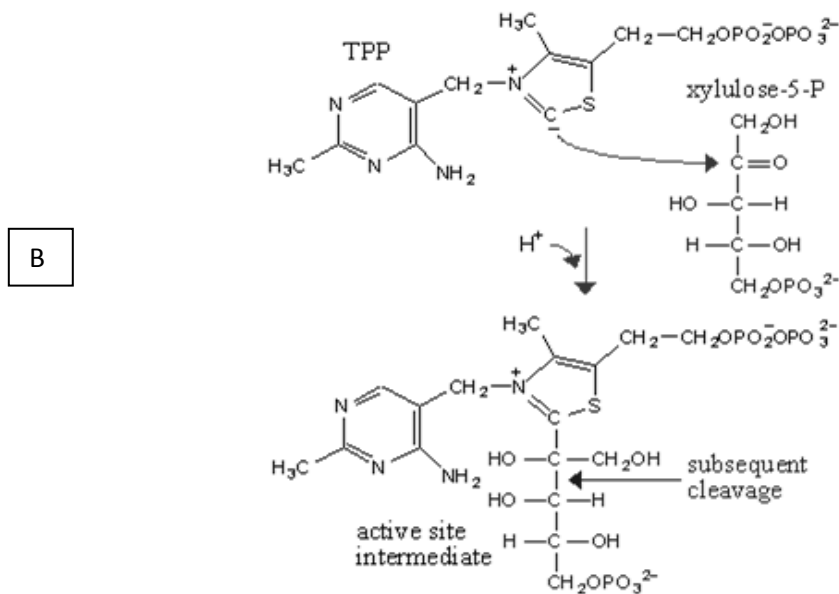
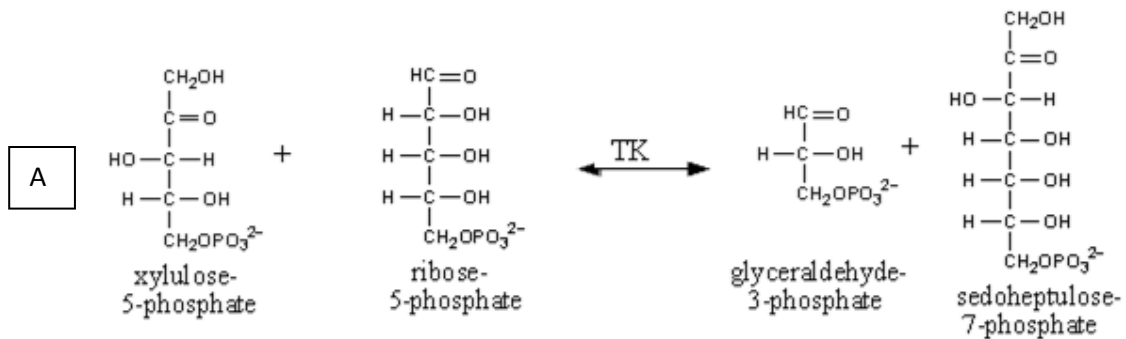


Figure 1.2 TPP involved in the transketolase catalysis (Modified from <http://nangluongsinhhoc2011.blogspot.com/2011/04/hinh-anh-nang-luong-sinh-hoc-2011.html>, 10pm, September 22, 2011). **(A)** The reversible reaction catalyzed by TK. **(B)** TPP involved a two-carbon unit transformation from xylulose-5-phosphate to ribose-5-phosphate.

The reaction catalyzed by TK is initiated by the thiazolium ring of TPP forming a covalent bond with the keto donor—xylulose-5-phosphate. The C-C bond between C₂ and C₃ is cleaved into GAP and a two-carbon unit, which is later condensed onto the aldo acceptor—ribose-5-phosphate to form sedoheptulose-7-phosphate (Berg *et al.*, 2002).

Most studies of transketolase are related to pathophysiology and clinical research. Very little has been done for transketolase in non-pathogenic microorganisms. For example, TK in *E.coli* has been studied in detail for several decades and the specific activity is reported to be 0.175 U/mg at 25° C and 1 U/mg at 37° C (Josephson and Fraenkel, 1969; Bayouml and Rosalkl, 1976). Immobilized *E. coli*'s transketolase was reported as a microreactor when using a microcapillary as the carrier material. Its V_{max} is 0.1± 0.02 mmol/min (4.6 U/mg) when using glyceraldehyde-3-phosphate as a substrate (Matosevic *et al.*, 2009). A 3D structure of *Saccharomyces cerevisiae* transketolase is solved in 2.0 Å resolutions (Lindqvist *et al.*, 1992; Nikkola *et al.*, 1994). However, no hyperthermophilic TK is characterized to date.

1.3.2 Transaldolase

Transaldolase (TAL, EC 2.2.1.2) is another key enzyme involved in PPP, which connects PPP with other glycolytic pathways. It rearranges a three-carbon unit from a ketose to an aldose. The detailed catalytic mechanism of TAL is illustrated in **Figure 1.3**.

The lysine residue in TAL forms a Schiff based intermediate with sedoheptulose-7-phosphate and the C-C bond between C₃ and C₄ is then cleaved. Therefore, the seven-

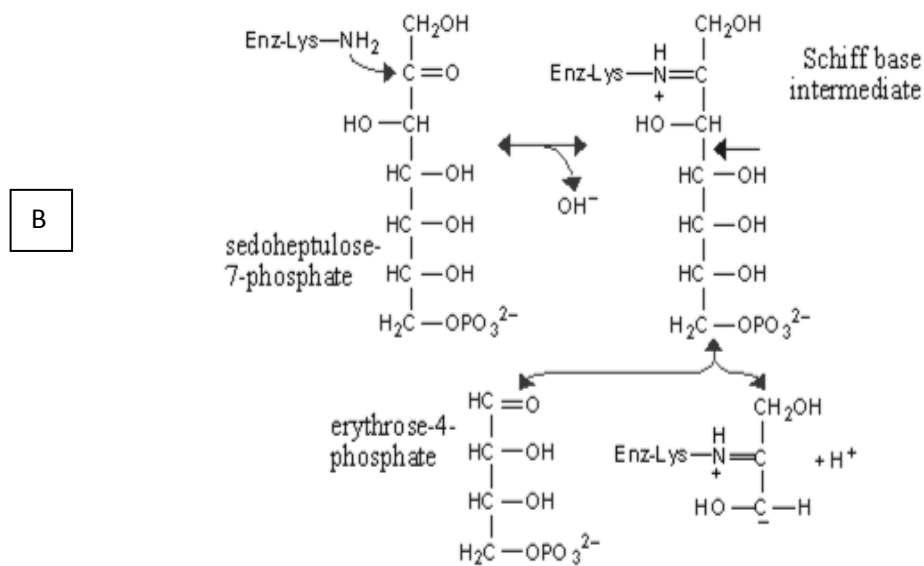
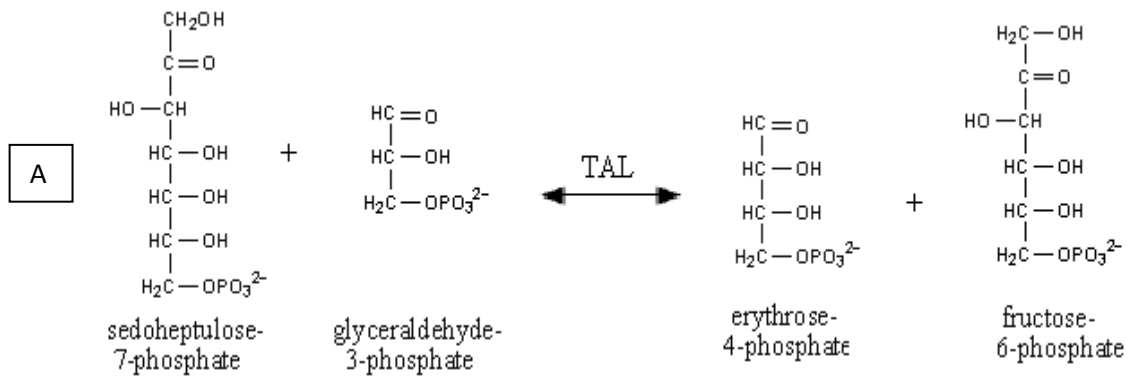


Figure 1.3 Catalytic mechanism of transaldolase (Modified from <http://nangluongsinhhoc2011.blogspot.com/2011/04/hinh-anh-nang-luong-sinh-hoc-2011.html>, 10pm, September 22, 2011). **(A)** The reversible reaction catalyzed by TAL. **(B)** TAL involved a three-carbon unit transformation from sedoheptulose-7-phosphate to glyceraldehyde-3-phosphate.

carbon compound forms an aldose sugar—E4P and a three-carbon unit. The latter one is condensed onto GAP to form F6P (Berg et al., 2002).

TAL is a ubiquitous enzyme, involved in sugar rearrangement in NOPPP (White, 2007). Together with another sugar rearrangement enzyme—TK, TAL regenerates F6P and GAP, both of which connect to other central metabolic pathways. TAL of *Methanocaldococcus jannaschii* (MJ0960) was cloned and overexpressed in *E.coli* by Soderberg and Alvin in 2004. This is the only recombinant hyperthermophilic TAL reported. Therefore, it is of significance to study other hyperthermophilic TALs.

1.4 Objectives

The purpose of this project is to develop a new assay system at high temperature (80° C) for characterizing hyperthermophilic PPP enzymes. The project has two parts: (i) preparation of auxiliary enzymes, and (ii) determination of activities of PPP enzymes in hyperthermophiles using the new assay system containing these auxiliary enzymes at high temperature.

1.4.1 Preparation of auxiliary enzymes

Genes encoding several auxiliary enzymes for pentose enzyme assays from selected hyperthermophiles were cloned and overexpressed in *E.coli* as the host organism. The recombinant enzymes would be purified for use as auxiliary enzymes in the assays for PPP enzymes. Their activities of the cell free extracts and the purified forms would be

determined under anaerobic conditions. It would be required for purified enzymes to have sufficient activity as helping enzymes.

1.4.2 Determination of activities of TK and TAL

Activities of TK and TAL would be determined from selected hyperthermophilic bacteria and archaea, which were *T. maritima*, *P. furiosus*, *T. guaymasensis*, *T. neapolitana*, *T. hypogea*, and *T. petrophila*.

1.4.3 Test of the new assay system

The overall tentative assay system under 80° C is illustrated in **Figure 1.4**:

To determine TK activity, the system would contain the following recombinant hyperthermophilic enzymes: xylose isomerase (XI), xylulose kinase (XuK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), triosephosphate isomerase (TIM), and glycerophosphate dehydrogenase (GPDH). The serial reactions would start from XI, which converted D-xylose to D-xylulose which would be then converted to D-xylulose-5-phosphate catalyzed by XuK in the presence of ATP. TK would catalyze the conversion of xylulose-5-phosphate and ribose-5-phosphate into glyceraldehyde-3-phosphate (GAP) and sedoheptulose-7-phosphate. GAP production would be determined by monitoring the absorbance change at 340 nm when GAPDH would catalyze the production of NADH using GAP as the substrate. Another monitoring method would be using both TIM and

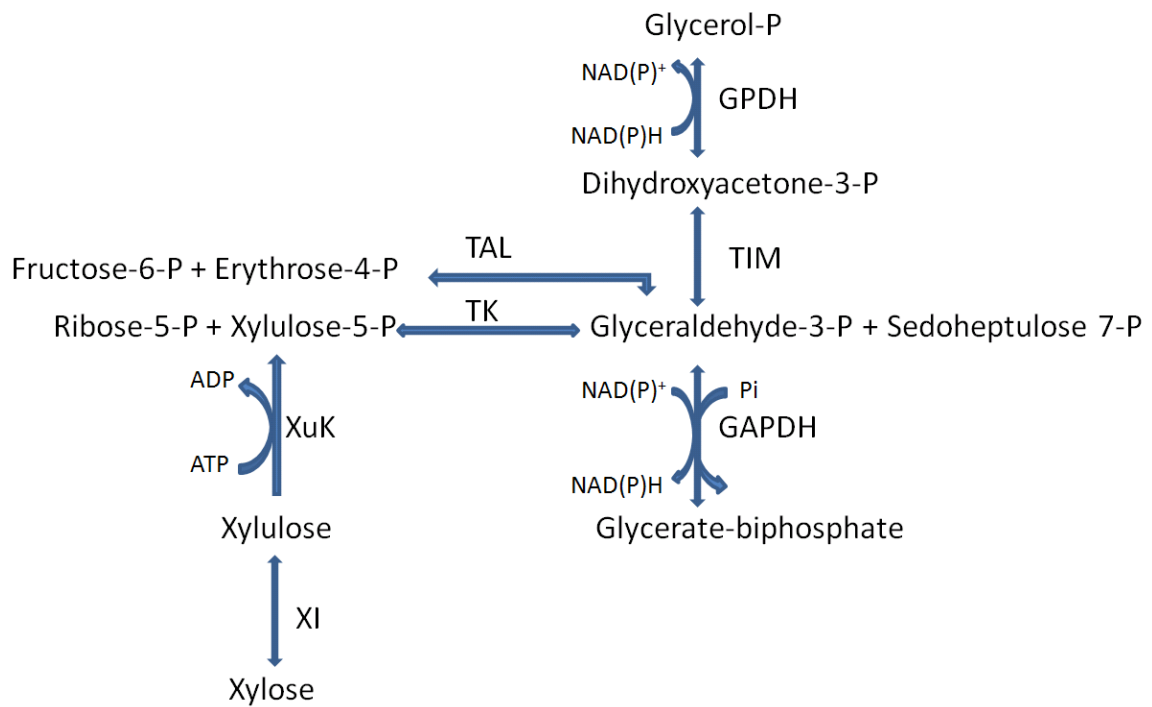


Figure 1.4 Tentative system for TK (or XuK) and TAL assays in hyperthermophiles. GPDH: glycerolphosphate dehydrogenase; TIM: trioesphosphate isomerase; GAPDH: glyceraldehyde dehydrogenase; TAL: transaldolase; TK: transketolase; XuK: xylulose kinase; XI: xylose isomerase. For TAL assay (part B), GPDH, TIM, and GAPDH served as the auxiliary enzymes; For XuK (TK) assay (part A), XI, TK (XuK), GAPDH, GPDH, and TIM served as the auxiliary enzymes.

GPDH. TIM would catalyze GAP to dihydroxyacetone phosphate (DHAP) that would be then reduced to glycerolphosphate by GPDH with the oxidation of NADH, whose absorbance change would be monitored at 340 nm. This assay system would also be used for the determination of XuK activity.

To determine TAL activity, the system would contain the following recombinant hyperthermophilic enzymes: GAPDH, TIM and GPDH. The serial reactions would be initiated by TAL that would use E4P and F6P as substrates. Once GAP was produced, it would be determined by monitoring the absorbance change at 340 nm using either GAPDH or both TIM and GPDH as described above.

2. Materials and Methods

2.1 Chemicals

All the antibiotics and IPTG were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada) or Fisher Scientific Canada (Ottawa, ON, Canada). All the fine chemicals (xylulose-5-phosphate disodium salt, erythrose-4-phosphate sodium hydrate, fructose-6-phosphate disodium salt, dihydroxacetone-phosphate, DL-glyceraldehydes-3-phosphate, D-ribose-5-phosphate disodium salt, beta-nicotinamide adenine dinucleotide [β -NAD⁺], and beta-nicotinamide adenine dinucleotide reduced [β -NADH]) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Commonly used chemicals were purchased from VWR international (Mississauga, ON, Canada) or Fisher Scientific (Ottawa, ON, Canada).

2.2 Strains of organisms

2.2.1 *E. coli* Strains for overexpressing recombinant auxiliary enzymes

Target genes from selected hyperthermophiles were cloned and ligated with pET30a. The resulted constructs were transformed into *E.coli* strain DH5 α following the instruction of the pET system manual (Novagen, Thorold, ON, Canada). After confirmation of the genes by sequencing (SickKids Hospital, Toronto, ON, Canada), each of the construct was transformed into the host strain *E.coli* RosettaTM 2(DE3) following the same protocol described above. The construct containing pET11a with the insert gene

encoding G1PDH was a gift from Yuji Kado (National Institute of Advanced Industrial Science and Technology, Osaka, Japan). After plating the transformed *E. coli* strain DH5 α on LB agar containing kanamycin (50 μ g/ml) and being incubated at 37° C overnight, a single colony was picked as an inoculum into 100 ml LB broth containing kanamycin (50 μ g/ml). When the cell density reached OD₆₀₀ between 0.6-0.8, cells were harvested by centrifugation and the construct was extracted using the QIAprep miniprep plamid extraction kit (Qiagen, Mississauga, ON, Canada). The construct was then transformed into *E.coli* RosettaTM 2(DE3) using the same transformation protocol described above.

Due to the uncertainty of each recombinant enzyme before overexpression and activity determination, same enzymes from different species were cloned in order to obtain sufficient activity as auxiliary enzymes for further experiments. The detailed gene sources from hyperthermophiles are listed in **Table 2.1**.

2.2.2 Selected hyperthermophiles

For the determination of TK and TAL activities, six species of hyperthermophiles were selected, which were *T. maritima* MSB8, *P. furiosus* DSM3638, *T. guaymasensis* DSM 11113, *T. neapolitana* DSM4359, *T. hypogea* DSM 11164, and *T. petrophila* RKU-1. All of the organisms were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany.

2.3 Growth methods

Table 2.1 Genes encoding auxiliary enzymes

Auxiliary enzyme	Gene source	NCBI Accession# and GI
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12)	<i>T. maritima</i> MSB8 (TM0688)*	CAA51205.1 GI: 939978
Glycero-1-phosphate dehydrogenase (G1PDH, EC 1.1.1.261)	<i>Aeropyrum pernix</i> K1(APE_0519.1)&	NP_147296.2 GI:118431088
Glycero-3-phosphate dehydrogenase (G3PDH, EC 1.2.1.59)	<i>P. furiosus</i> DSM3638 (PF1874)*	NP_579603.1 GI: 18978246
Triosephosphate isomerase (TIM, EC 5.3.1.1)	<i>P. furiosus</i> DSM3638 (PF1920)*	NP_579649.1 GI:18978292
Transketolase (TK, EC 2.2.1.1)	<i>T. maritima</i> MSB8 (TM1762) [#]	NP_229559.1 GI: 15644507
Xylose isomerase (XI, EC 5.3.1.5)	<i>T. maritima</i> MSB8 (TM1667) [#] <i>T. petrophila</i> RKU-1 (Tpet_1124) [#]	NP_229467.1 GI: 15644415 YP_0012447141 GI: 148270254
Xylulose kinase (XuK, EC 2.7.1.17)	<i>T. maritima</i> MSB8 (TM0116)*	NP_227932.1 GI: 15642891

*Genes were cloned in Ma's lab (Abdallah and Ma, unpublished data); # Genes were cloned in Ma's lab (Eram and Ma, unpublished data); &Gene was cloned by Nishihara *et al.* (Japan, 1999).

2.3.1 Growth and induction conditions of recombinant *E.coli* strains

Each *E.coli* strain RosettaTM 2(DE3) containing a recombinant target gene was routinely grown in 4L LB broth containing 50 µg/ml kanamycin sulfate and 50 µg/ml chloramphenicol at 30 ° C (for ApG1PDH, 100 µg/ml ampicillin and 50 µg/ml chloramphenicol were used in culture) with continuous shaking at 200 rpm until OD₆₀₀ reached 0.5-0.6. A final concentration of 0.5 mM IPTG was added into the culture and it was then further incubated at 30 ° C overnight. Cells were obtained by centrifugation at 6,000 × g at 4 ° C, and then stored at -80 ° C after being frozen in liquid nitrogen first.

2.3.2 Growth of selected hyperthermophiles

Six species of hyperthermophiles (described in section 2.2.2) were routinely grown under anaerobic conditions. Different sugar substrates (glucose or maltose, arabinose, and xylose) were used to determine if the pentose enzymes were inducible by pentose and hexose respectively. The detailed medium composition and growth conditions for each organism are described below:

Commonly used stock solutions for growing hyperthermophiles are 25% Na₂S₂O₃ (w/v), 15% Cysteine/HCl (w/v), 3% Na₂S (w/v), 15% sugar solutions (w/v), and vitamin solutions (**Table 2.2**), which were prepared separately and filtered into autoclaved bottles using a 0.2 µm syringe filter (Fisher Scientific, Ottawa, Canada). Due to the instability at autoclaving temperature, the reducing reagents and vitamin solution were added after the basic medium was autoclaved. Some hyperthermophiles need

Table 2.2 Vitamin solutions prepared for the growth of hyperthermophiles*

Vitamin	Solution 1 (mg/L)	Solution 2 (mg/L)
Biotin	2	4
Folic acid	2	4
Pyridoxine/HCl (VB₆)	10	10
Thiamine/HCl·2H₂O (VB₁)	5	10
Riboflavin	5	10
Nicotinic acid	5	10
D-Ca-pantothenate (VB₅)	5	10
Cobalamin (Vitamin B₁₂)	0.1	10
p-aminobenzoic acid	5	10
Lipoic acid	5	/
Niacin	/	10
Thioctic acid	/	10

* prepared with the protocol in Ma's lab. Vitamins were dissolved in deionized water and then filtered into autoclaved bottle. The stock solutions were stored at 4° C in darkness. Both solutions can be used for hyperthermophiles if needed.

elemental sulfur as electron acceptor during their growth. Element sulfur was steamed for 1 hour three times before adding into autoclaved medium.

For culturing *T. maritima*, 1 L medium (modified from Huber et al., 1986) contained: 15 g NaCl, 2 g Na₂SO₄, 2 g MgCl₂·6H₂O, 0.5 g CaCl₂·2H₂O, 0.25 g NaHCO₃, 0.1 g K₂HPO₄, 0.05 g KBr, 0.02 g H₃BO₃, 0.02 g KI, 0.003 g Na₂WO₄·2H₂O, 0.002 g NiCl₂·6H₂O, 1 g yeast extract, 1 g tryptone, 4 g sugar, 5 g Na₂S₂O₃, 2.8 ml 15% Cysteine/HCl (w/v), 1 mg resazurin. The pH before autoclaving was adjusted to 6.8-7.0 with 2 M NaOH. The medium was autoclaving at 121° C for 30 minutes and the bottle was sealed with a rubber stopper and an aluminum cap after that. The complete medium was degassed and gassed with nitrogen after adding filtered sugar and Na₂S₂O₃ stock solutions. Degassed reducing reagent 15% Cysteine/HCl was added into the medium using a gas tight syringe. After adjusting pH to 6.8-7.0 again with filtered 1 M NaOH or 1 M HCl, the medium was incubated under 80° C until the color of the medium changed to colorless. Two percent fresh inoculum (v/v) was added into the bottle using a gas-tight syringe and the culture was incubated under 80° C for about 16 hours.

For culturing *P. furiosus*, 1 L medium (modified from Kengen et al., 1993) contained: 1.2 g NH₄Cl, 13.8 g NaCl, 3.5 g MgSO₄, 2.75 g MgCl₂· 6H₂O, 0.325 g KCl, 0.05 g NaBr, 0.05 g KI, 0.015 g H₃BO₃, 0.0075 g SrCl₂, 0.005 g citric acid, 4 g maltose, 1 g tryptone, 1 g yeast extract, 0.5 g CaCl₂·2H₂O, 0.5 g KH₂PO₄, 5.2 g HEPES or 6 g PIPES·1.5Na, 2.8 ml 15% cysteine/HCl (w/v), 8 ml 3% Na₂S·9H₂O (w/v), 0.5 mg resazurin, 10 ml trace mineral solution (Balch *et al.*, 1979) and 10 ml vitamin stock solution 2 (**Table 2.2**). The pH before autoclaving was adjusted to 7.0 with 2 M NaOH.

The medium was autoclaving at 121° C for 30 minutes and the bottle was sealed with a rubber stopper and an aluminum cap. The complete medium was degassed and gassed with nitrogen. The reducing reagents Cysteine/HCl and Na₂S were injected into the medium with gas-tight syringes. Then, the medium was incubated at 95° C until the color of the medium changed to clear yellow. Two percent fresh inoculum (v/v) was added into the bottle and incubated at 95° C for about 14-16 hours.

For culturing *T. guaymasensis*, 1 L medium (modified from Canganella *et al.*, 1998) contained: 0.25 g NH₄Cl, 1 g NaHCO₃, 18 g NaCl, 1.65 g MgSO₄, 3.9 g MgCl₂·6H₂O, 0.33 g KCl, 10 g yeast extract or trypticase soy broth, 0.14 g CaCl₂·2H₂O, 0.14 g KH₂PO₄, 2.8 ml 15% Cysteine/HCl (w/v), 8 ml 3% Na₂S· 9H₂O (w/v), 4 g dextrose, 5.2 g HEPES, 0.01 mg Na₂SeO₃, 0.01 mg NiCl₂·6H₂O, 3-5 g elemental sulfur, 1 mg resazurin, 10 ml trace mineral solution (Balch *et al.*, 1979) and 10 ml vitamin solution 1 (**Table 2.2**). The basic medium without reducing reagent and sulfur was adjusted to pH 7.0 with 2 M NaOH. The medium was autoclaving at 121° C for 30 minutes and the bottle was sealed with a rubber stopper and an aluminum cap after the steamed element sulfur was added. The complete medium was degassed and gassed with nitrogen after adding vitamin solution. Degassed reducing reagent Cysteine/HCl and Na₂S were injected into the medium with gas-tight syringes. Then, the medium was incubated at 88° C until the color of medium changed into clear yellow. Two percent fresh inoculum (v/v) was added into the bottle and the culture was incubated under 88° C for about 12 hours.

For culturing *T. neapolitana*, 1 L medium (modified from Childers *et al.*, 1992) contained: 20 g NaCl, 1.74 g KCl, 0.25 g NH₄Cl, 1.69 g MgSO₄, 0.2 g MgCl₂·6H₂O, 0.05 g CaCl₂·2H₂O, 0.14 g KH₂PO₄, 2.8 ml 15% Cysteine/HCl (w/v), 8 ml 3% Na₂S·9H₂O (w/v), 6 g PIPES·1.5Na, 0.5 g yeast extract (for rich medium only), 4 g sugar, 1 mg resazurin, 10 ml trace mineral solution (Balch *et al.*, 1979) and 10 ml vitamin solution 1 (**Table 2.2**). The basic medium without reducing reagents and sugar was adjusted to pH 7.0 with 2 M NaOH. The medium was autoclaved at 121° C for 30 minutes and the bottle was sealed with a rubber stopper and an aluminum cap. After adding 15% sugar stock solution (w/v) and vitamin solution, the complete medium was degassed and gassed under nitrogen. Degassed reducing reagents Cysteine/HCl and Na₂S were injected into the medium with gas-tight syringes. Then, the medium was incubated at 77° C until the color of medium changed from pink to colorless. Two percent fresh inoculum (v/v) was added into the bottle and incubated at 77° C for about 20-24 hours.

For culturing *T. hypogea*, 1 L medium (modified from Fardeau *et al.*, 1997) contained 2 g NaCl, 0.1 g KCl, 1 g NH₄Cl, 0.2 g MgCl₂·6H₂O, 0.1 g CaCl₂·2H₂O, 0.3 g K₂HPO₄, 0.3 g KH₂PO₄, 2.8 ml 15% Cysteine/HCl (w/v), 8 ml 3% Na₂S·9H₂O (w/v), 1 g yeast extract, 1 g trypticase soy broth, 4 g sugar, 0.5 mg resazurin, 10 ml trace mineral solution (Balch *et al.*, 1979). The basic medium without reductants and sugar was adjusted to pH 7.3-7.4 with 2 M NaOH. The medium was autoclaved at 121° C for 30 minutes and the bottle was sealed with a rubber stopper and an aluminum cap. After adding 15% sugar stock solution (w/v) and 20 ml 25% Na₂S₂O₃ stock solution, the whole medium was degassed and gassed with nitrogen. Degassed reducing reagents

Cysteine/HCl and Na₂S were injected into the medium with gas-tight syringes. After readjusting pH with filtered 1 M NaOH or 1 M HCl to pH 7.3, the medium was incubated under 70° C until the color of medium changed to colorless. Two percent fresh inoculum (v/v) was added into the bottle and incubated under 70° C for about 12-14 hours.

For culturing *T. petrophila*, 1 L medium (modified from Takahata *et al.*, 2001) contained: 1 g NH₄SO₄, 20 g NaCl, 3 g MgSO₄, 3 g MgCl₂·6H₂O, 0.3 g CaCl₂·2H₂O, 0.5 g KCl, 0.5 g KH₂PO₄, 0.05 g NaBr, 0.0025 g H₃BO₃, 0.02 g SrCl₂·6H₂O, 0.01 g sodium citrate, 1 g yeast extract, 1 g tryptone, 4 g sugar, 2.25 g HEPES, 2.8 ml 15% Cysteine/HCl (w/v), 0.6 mg resazurin, 10 ml trace mineral solution (Balch *et al.*, 1979) and 10 ml vitamin solution 2 (**Table 2.2**). The basic medium without reducing reagents and sugar was adjusted to pH 7.0 with 2 M NaOH. The medium was autoclaved at 121° C for 30 minutes and the bottle was sealed with a rubber stopper and an aluminum cap. After adding 15% sugar stock solution (w/v) and vitamin stock solution, the complete medium was degassed and gassed with nitrogen. Degassed reductants 15% cysteine/HCl and 3% Na₂S were injected into the medium with gas-tight syringes. Then, the medium was incubated under 80° C until the color of medium changed into colorless. The ratio of a 2% fresh inoculum (v/v) was added into the bottle and incubated at 80° C for about 15 hours.

The end products accumulated in the hyperthermophiles culture, such as H₂, H₂S, and some organic acids inhibit the growth of hyperthermophiles due to the sealed batch culture environment. Therefore, nutritious ingredients are not consumed too much and a

little biomass can be obtained (around 0.5 g per liter culture). The growth of each organism was monitored by direct cell count using a Petroff-Hausser counting chamber (1/400 mm², 0.02 mm deep) and a Nikon Eclipse E600 phase-contrast light microscope until the cell density reached 10⁹ per milliliter culture. Cells were harvested using centrifugation at 6,000 × g for 20 minutes at 4° C. After being frozen in liquid nitrogen, the cells were stored at -80° C.

2.4 Preparation of cell free extracts

2.4.1 Preparation of cell free extracts from recombinant *E. coli*

2.4.1.1 Method 1

About 1 g of frozen *E. coli* cells was used for the preparation of cell free extract under anaerobic conditions. All the solutions and vials were degassed and gassed with nitrogen before starting preparation. The frozen cells were thawed under degassing and gassing with nitrogen in a sealed serum bottle. Lysis buffer (50 mM Tris/HCl, 2 mM EDTA, 2 mM SDT, and 2 mM DTT pH 7.5) was added into the bottle at a ratio of 1:5 (w/v). A solution containing lysozyme, DNAase and MgCl₂ was also added into the vial, which resulted in the final concentration of 0.2 mg/ml of lysozyme, 0.01 mg/ml of DNase I, and 20 mM of MgCl₂, respectively. After incubation at room temperature for about 1 hour with shaking, the whole mixture was centrifuged at 8,000 rpm (6,740 × g) for 30 minutes at 4° C. The supernatant (cell free extract) was then transferred into vials that were degassed under vacuum and gassed with nitrogen with a gas-tight syringe. However,

relatively low percentage (30-50%) of lyzed cell was obtained using this method compared to the method 2 due to the lack of stir during incubation.

2.4.1.2 Method 2

The cell free extract was prepared aerobically. Lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 5 mM imidazole, 5% glycerol, pH 8.0) was added into the frozen cells (wet weight) at a ratio of 10:1 (v/w). Final concentrations of 0.4 mg/ml lysozyme, 0.02 mg/ml DNase I, and 20 mM MgCl₂ were also added into the mixture. After vortex mixing for 2 minutes, the mixture was incubated on ice for 30 minute and then was sonicated at 7 Volts (MicrosonTM ultrasonic cell disruptor, QSONICA LLC., Newtown, TC, U.S.A) for 15 minutes on an ice bath. The whole mixture was centrifuged at $47,810 \times g$ at 4° C for 1 hour. The supernatant was the cell free extract that was used for enzyme activity assays and enzyme purification.

2.4.2 Preparation of cell free extracts of hyperthermophiles

The whole process for the cell free extract preparation was conducted under strictly anaerobic conditions. The frozen cells (wet weight) of hyperthermophiles were thawed under nitrogen in a sealed serum bottle. Degassed lysis buffer (50 mM Tris/HCl, 2 mM SDT, 2 mM DTT, 20 mM MgCl₂) was added into the bottle at a ratio of 1:5 (w/v) using a gas-tight syringe. The final concentration of 0.2 mg/ml lysozyme and 0.01 mg/ml Dnase I were also added into the bottle. The whole mixture was incubated at room temperature for 1 hour with shaking at 200 rpm. The cell free extract was obtained after centrifugation at $6,869 \times g$ for 30min at 4° C. Finally, the cell free extract was carefully transferred into

degassed small bottles with a syringe and stored at -20° C for future use. About 40% of cells were lysed with this method in general.

2.5 Enzyme assay methods

2.5.1 Assay methods for the auxiliary enzymes

All the assays were performed under anaerobic conditions at 80° C. Different controls were designed for each enzyme assay in order to obtain accurate activity and correct the non-enzymatic absorbance change caused by other factors. Reaction mixture without target enzyme (substrates and helping enzymes only) was operated as one type of negative control; the reaction mixture containing *E. coli* cell free extract (prepared from *E.coli* with blank plasmid pET30a) was operated as another type of negative control. Both negative controls were routinely conducted for each enzyme assay. All the activities were calculated within the initial linear reaction range after deducting the values of the negative control. All the assays for each enzyme were replicated at least three or four times in order to obtain reproducible results. Protein concentration of each enzyme was determined by using the Bradford method and BSA as the protein standard (Bradford, 1979).

2.5.1.1 TmGAPDH

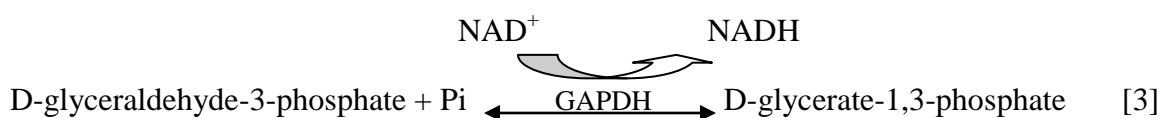
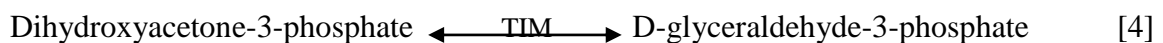
GAPDH catalyzes the following reaction (reaction 3).



The TmGAPDH assay was modified from the method of Rehabeer and Jaeni (1991). One milliliter assay mixture contained 50 mM EPPS (pH8.5), 5 mM EDTA, 20-40 mM KH_2AsO_4 , 5 mM glyceraldehyde-3-phosphate, 10 mM NAD^+ and 2-10 μl enzyme or cell free extract (3-30 μg protein). One-centimeter glass cuvettes (Starna Cells Inc., Atascadero, CA, U.S.A) containing EPPS, EDTA, arsenate were degassed and gassed with nitrogen. The cuvette was incubated at 80° C for 4 minutes. Other substrates and enzyme were added into the cuvette with gas-tight syringes in sequence, and then the reaction mixture was mixed by inverting several times. The absorbance at 340 nm was measured with the Genesys 10_{VIS} spectrometer (Spectronic Unicam, Rochester, NY, U.S.A) connected to a chart recorder. Different negative controls were also performed under the same assay conditions. One unit of TmGAPDH activity was expressed as the production of 1 μmol of NADH per minute ($\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$).

2.5.1.2 PFTIM

TIM catalyzes the reversible reaction between dihydroxyacetone-3-phosphate and D-glyceraldehyde-3-phosphate (reaction 4). D-glyceraldehyde-3-phosphate can be then converted to D-glycerate-1,3-phosphate, which is catalyzed by GAPDH (reaction 3).



The PFTIM assay method was modified from the method of Kohlhoff *et al.* (1996). One milliliter assay mixture contained 50 mM EPPS (pH8.5), 5 mM EDTA, 20 mM KH_2AsO_4 , 5 mM dihydroxyacetone-phosphate (DHAP), 10 mM NAD^+ , one unit of TmGAPDH and 1-5 μl PFTIM enzyme (0.3-1 μg protein) or cell free extract (1.5-20 μg protein). One-centimeter glass cuvettes containing EPPS, EDTA, and arsenate were degassed and gassed with nitrogen. The cuvette was incubated at 80°C for 4 minutes. Other substrates and TmGAPDH were added into the cuvette with gas-tight syringes. The mixture was incubated for another 30 seconds under 80°C and absorbance at 340 nm was measured immediately and recorded with a chart recorder. The PFTIM-containing cell free extract was added last and A_{340} was measured immediately. Different negative controls were also performed under the same assay conditions. One unit of PFTIM activity was expressed as the production of 1 μmol of NADH per minute ($\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$).

2.5.1.3 GPDH

GPDH catalyzes the conversion of dihydroxyacetone-3-phosphate to D-glycerol-phosphate (reaction 5).

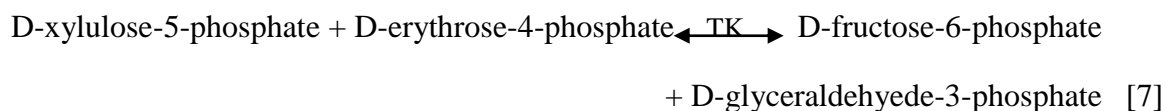
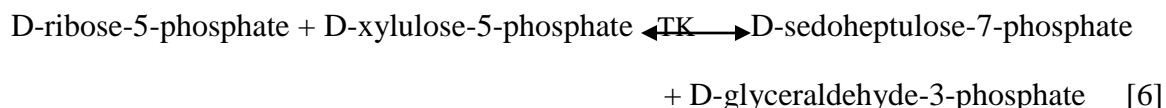


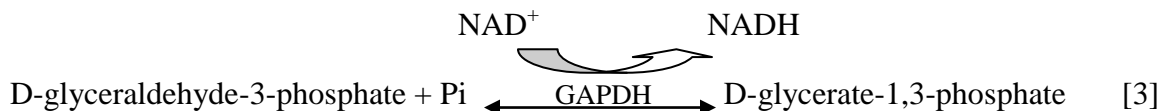
The assay was modified from the method of Nishihara and Koga (1997). Two types of assays were used based on the use of different substrates. One milliliter assay mixture for

oxidation direction contained 50 mM EPPS (pH8.5), 5 mM MgCl₂, 60 mM KCl, 2 mM DHAP, 0.3 mM NADH, 50-200 µl PfG3PDH or ApG1PDH cell free extract (50-150 µg protein) or 10-50 µl purified enzyme (20-100 µg protein). Another type of one milliliter reaction mixture with PfTIM as a helping enzyme (due to the lack of availability of key substrate DHAP) contained 50 mM EPPS, 5 mM MgCl₂, 60 mM KCl, 5 mM glyceraldehydes-3-phosphate, 0.5 U purified PfTIM, 0.3 mM NADH, 50-200 µl PfG3PDH or ApG1PDH cell free extract (100-300 µg protein) or 10-50 µl purified enzyme (30-130 µg protein). One centimeter glass cuvette containing the components EPPS, MgCl₂, KCl was degassed and gassed with nitrogen. The cuvette was incubated at 80° C for 4 minutes. Other substrates were injected into the cuvette as negative controls and the absorbance at 340 nm was measured using the spectrophotometer that was connected to a chart recorder for another 30 seconds or 1 minute. GPDH was added into the cuvette last, and A₃₄₀ was measured for about 3 minutes. Negative controls were also performed under the same assay conditions. One unit of GPDH activity was expressed by the oxidation of 1 µmol of NADH per minute ($\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$).

2.5.1.4 TmTK

TK catalyzes the following reactions (reactions 6 and 7) and D-glyceraldehyde-3-phosphate produced can be determined using GAPDH (reaction 3).





The TmTK assay was modified from the method of Bayoumi and Rosalki (2004). One milliliter reaction mixture contained 50 mM EPPS (pH 8.5), 5 mM MgCl₂, 0.5 mM TPP, 20 mM KH₂AsO₄, 5-10 mM NAD⁺, 2 mM ribose-5-phosphate, 0.4-1 mM xylulose 5-phosphate, GAPDH 1 unit, 10-30 μl TmTK cell free extract (40-120 μg protein) or 1-10 μl purified TmTK (4-40 μg protein). One centimeter glass cuvettes containing the components EPPS, MgCl₂, arsenate were degassed and gassed with nitrogen. The cuvette was incubated at 80° C for 4 min before the addition of TmTK and the cuvette was further incubated at the same condition for another 1 minute in order to activate the hyperthermophilic enzyme and denature the mesophilic proteins. Other substrates and TmGAPDH were injected into the cuvette and absorbance at 340 nm was measured immediately and recorded with a chart recorder. Negative controls were also performed under the same assay conditions. One unit of TmTK activity was expressed by the production of 1 μmol of NADH per minute (ε₃₄₀ = 6.22 mM⁻¹ cm⁻¹).

2.5.1.5 TmXI and TpXI

XI catalyzes the reversible conversion between D-xylose and D-xylulose (reaction 8).



The XI assay was modified from the method of Zhu *et al.* (1999). This assay had two stages: a reaction stage under anaerobic conditions at 80° C and a color development stage under aerobic conditions at room temperature. The one or two milliliter reaction

mixture contained 50 mM EPPS (pH 8.5), 2 mM MgCl₂, 2 mM MnCl₂, 10 mM D-xylose and a certain amount of TmXI- or TpXI-containing cell free extract or purified TpXI. Degassed serum bottles containing the components EPPS, MgCl₂, MnCl₂, and xylose were equilibrated at 80° C for 5 minutes. TmXI or TpXI was then added into the bottle and the reaction mixtures were incubated under the same condition. A volume of 100 µl aliquot was removed from each mixture every 5 minutes and the reaction was stopped by incubating the aliquot in an ice bath. The reaction mixture without enzyme was incubated under the same conditions as a negative control.

The color development procedure is modified from that of Dische and Borenfreund (1951). The mixture contained 75 µl incubated reaction mixture, 1.25 ml 70% H₂SO₄ (v/v), 20 µl 1.5% cysteine/HCl (w/v), 20 µl 0.12% carbazole-ethanol (w/v). The mixture was incubated at room temperature for 40 minutes after vortex mixing, and the absorbance at 540 nm was measured. The negative control was identically treated.

The amount of xylulose production catalyzed by XIs in this reaction was calculated by using a xylulose standard curve obtained with 0-10 mM known D-xylulose under the same assay conditions. All the mixtures for color development were incubated at room temperature for 40 minute after vortex mixing, and the A₅₄₀ was measured. One unit of XI was expressed by 1 µmol of xylulose produced per minute.

2.5.1.6 TmXuK

According to the assay system designed for TmXuK measurement (**Figure 2.1**), one milliliter reaction mixture contained 50 mM EPPS (pH 8.5), 5 mM MgCl₂, 10 mM D-xylose, purified TpXI 1 unit, 2 mM ATP, 5 mM ribose 5-P, 5 mM NAD⁺, 0.5 mM TPP,

20 mM KH_2AsO_4 , 50 μl purified TmTK , purified TmGAPDH 2 unit, TmXuK cell free extract or purified TmXuK (50-100 μl). One centimeter glass cuvette containing the components of EPPS, MgCl_2 , D-xylose was degassed and gassed. All the freshly

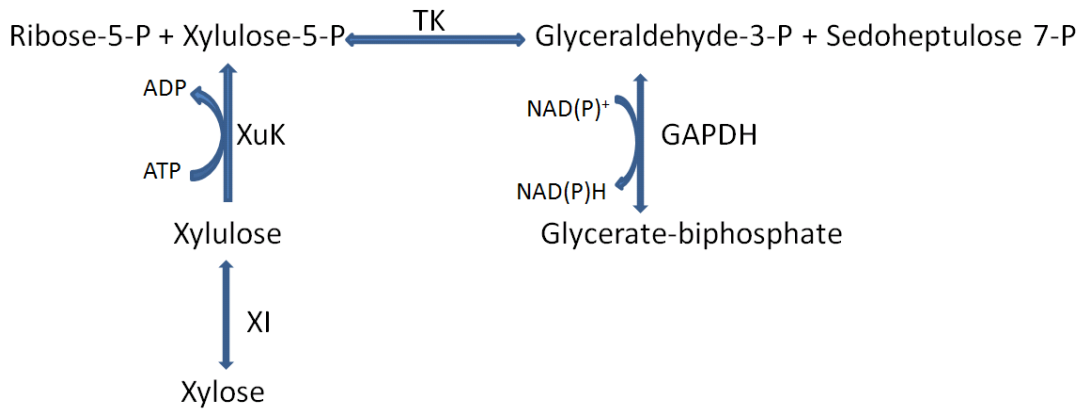


Figure 2.1 The assay system for XuK. XuK can be assayed with the help of three purified hyperthermophilic enzymes—TpXI, TmTK, and TmGAPDH. Xylose is firstly converted to xylulose by TpXI, and xylulose is further phosphorelated to xylulose-5-phosphate by XuK coupling with ATP to ADP production. TmTK can convert it plus ribose-5-phosphate into glyceraldehyde-3-phosphate once xylulose-5-phosphate is produced. Glyceraldehyde-3-phosphate is converted to glycerate-biphosphate coupling with NAD(P)H production, which can be monitored at 340 nm.

prepared chemicals were also degassed and gassed under nitrogen. The cuvette was then incubated at 80° C for 5 minutes after TpXI was added in order to have sufficient D-xylulose produced. All the auxiliary enzymes and substrates were also injected into the cuvette with gas-tight syringes and the whole mixture was incubated at the same conditions for another 40 seconds to 1 minute; meanwhile, A_{340} was monitored as a negative control. TmXuK cell free extract or purified TmXuK was added into the cuvette last, and absorbance at 340 nm was monitored using a spectrophotometer that was connected to a chart recorder. One unit of XuK activity was expressed by the production of 1 μmol of NADH per minute ($\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$).

2.5.2 Transketolase (TK) and transaldolase (TAL) assays in selected hyperthermophiles

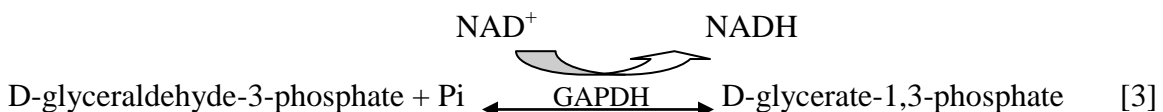
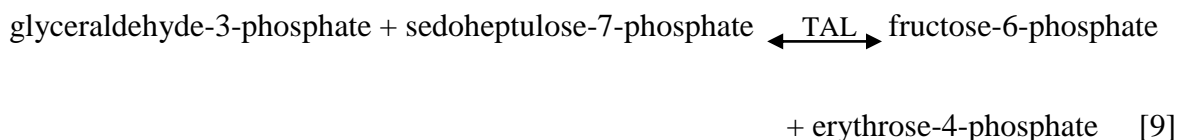
TK and TAL were selected as paradigms to demonstrate the possibility of the new assay system for other hyperthermophilic pentose enzymes. TmGAPDH can serve as an auxiliary enzyme for both TK and TAL assays. However, it might cause a negative effect or inhibit TK or TAL activity because of the arsenate used for assaying TmGAPDH activity. Thus, GPDH and TIM can also serve as alternate auxiliary enzymes in TK and TAL assays, for comparison.

2.5.2.1 TK assay

The TK assay for hyperthermophiles was conducted with the same methods described in section 2.5.1.4, except that cell free extracts directly prepared from hyperthermophiles was substituted for the recombinant TmTK.

2.5.2.2 TAL assay

TAL catalyzes the reversible reaction from fructose-6-phosphate and erythrose-4-phosphate to glyceraldehyde-3-phosphate and sedoheptulose-7-phosphate (reaction 9). GAP produced was determined using the GAPDH reaction (reaction 3).



The function of TmGAPDH can be substituted by PFTIM and PfG3PDH. TAL assays for hyperthermophiles were also conducted under anaerobic conditions. One milliliter reaction mixture contained 50 mM EPPS (pH 8.5), 5 mM MgCl₂, 20 mM KH₂AsO₄, 5 mM F6P, 0.25 mM E4P, 5 mM NAD⁺, 1 unit GAPDH (or 1 unit PFTIM and 0.5 unit PfG3PDH but without arsenate), and 100 µl cell free extract of hyperthermophiles (50-150 µg protein). One centimeter glass cuvette containing the components EPPS, MgCl₂, arsenate (or without arsenate) was degassed and gassed with nitrogen. The cuvette was incubated at 80° C for 4 min before that addition of other substrates and purified

TmGAPDH. The absorbance at 340 nm was measured immediately using a spectrophotometer that was connected to a chart recorder for another 30 seconds or 1 minute. The cell free extract of hyperthermophiles was added last, and the A_{340} was measured. Negative controls were also conducted under the same assay conditions. One unit of TAL activity was expressed as 1 μmol of NADH or NAD^+ produced per min ($\epsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$).

2.6 Purification of auxiliary enzymes

All the auxiliary enzymes were purified with a 20 ml gravity flow column (Bio-Rad, Mississauga, ON, Canada) packed with HisPur cobalt resin (Thermo-Fisher Scientific, Mississauga, ON, Canada) under aerobic conditions at 4° C. Cell free extracts were prepared using method 2 as described in section 2.4.1.2. After carefully removing the storage liquid, 6-7 ml cobalt resin was loaded onto the column and washed twice with 20 ml lysis buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 5 mM imidazole, 5% glycerol, pH 8.0). About 30 ml of cell free extract and the cobalt resin were mixed together while being rotated for 1 hour at 4° C. The whole mixture was then loaded into the column and the flowthrough was collected. After reloading the flowthrough onto the column, the flowthrough collected was labeled as fraction 1. The column was washed with 20 ml lysis buffer and the fraction collected was labeled as fraction 2. After that, the column was washed with 20 ml wash buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM imidazole, pH 8.0) two times and the flowthroughs collected were labeled as fraction 3 and fraction 4, respectively. All the fractions were kept at -20° C for downstream analysis. The column

was then washed with 10 ml elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 7.4) four times. All fractions were collected into 1.5 ml microtubes. Meanwhile, the protein concentration for each fraction was determined using the Bradford method in a 96-well plate as described in the Bio-Rad protein assay user manual (Bio-Rad, Mississauga, ON, Canada). The fractions containing higher protein concentration (dark blue color with Bradford method) were washed with protein dialysis buffer (50 mM Tris/HCl, 20 mM NaCl, 2 mM EDT and 2 mM DTT, pH 7.5) in order to remove the high concentration of salt and imidazole. An Amicon Ultra centrifugal concentrator (pore size of 30K molecular weight cut-off) containing purified protein samples was centrifuged at 3,000 × g for 10 minutes at 4° C and the concentrated protein was washed with 10 ml dialysis buffer under the same condition twice. The concentrated purified protein was droplized into liquid nitrogen and stored in a liquid nitrogen tank for further experiments.

2.7 SDS-PAGE analyses for protein purifications

Ten percent or 12.5 percent mini SDS gels were prepared with a Hoefer Dural Gel Caster SE 245 (Hoefer, Inc., San Francisco, CA, U.S.A) for analyzing protein samples. All the procedures for processing protein samples were strictly followed (Hoefer SE 250 mini-vertical SDS gel electrophoresis user manual). The volume of 20 µl protein samples were mixed with 4 µl 6× concentrated SDS loading buffer and boiled for 5 minutes. In order to observe the protein yield in the cell free extract or in the purified protein samples, and to observe the loss of proteins in flowthroughs, a 10 µl fixed volume of each boiled

protein sample was loaded onto the gel and run at 25 mA (for single gel) or 40 mA (for double gels) for about 1-1.5 hour (Hoefer mini-vertical SDS gel electrophoresis unit) with the BLUeye Prestained protein ladder (FroggaBio, Inc., Toronto, ON, Canada). After staining for 4 hours (or overnight) with the staining solution (0.3 mM Coomassie Blue R-250, 20% methanol [v/v], 10% glacial acetic acid [v/v]; Hoefer SDS gel electrophoresis user manual), the gel was destained with the destaining solution (20% methanol [v/v], 10% glacial acetic acid [v/v]; Hoefer SDS gel electrophoresis user manual). The gel was scanned with HP scanner (PrecisionScanner 5000C) and the scanned images were further saved as .bmp files. The molecular weight of each protein was calculated by using the standard curve of the commercial protein ladder plotted in a semi-log graph.

3. Results

3.1 Cells obtained

Cells of recombinant *E.coli* strains were obtained using the growth methods described in section 2.3.1. The amount of biomass of each cell type (wet weight) obtained from a four-liter culture after induction for 12-14 hours at 30° C is reported in **Table 3.1**.

Cells of selected hyperthermophiles were obtained with the growth methods described in section 2.3.2. The amount of biomass of each strain is illustrated in **Table 3.2**. Each cell type was measured from a 2-4 liter culture at late log phase under its specific growth conditions. The cell number was monitored with direct-counting, and increased from the initial 10^6 per milliliter culture to a final 10^9 per milliliter culture before harvest. *Thermotoga* species grew on all the monosaccharides supplied in this project, while *P.furiosus* and *T. guaymasensis* grew on maltose and glucose as the carbon source, respectively.

3.2 Auxiliary enzymes

All the assays were conducted under anaerobic conditions at 80° C. Negative controls for each enzyme assay were routinely conducted under the same assay conditions. All the activities were calculated during the linear range after deducting the absorbance change caused by the negative control.

Table 3.1 Available cell mass of recombinant *E.coli*.

<i>E.coli</i> strain carrying gene for	Wet weight (g)
TmGAPDH	10.1
ApG1PDH	8.9
PfG3PDH	9.8
PfTIM	12.3
TmTK	11.7
TmXI	10.5
TpXI	10.8
TmXuK	9.9

Table 3.2 Available cell mass of selected hyperthermophiles.

hyperthermophiles	Wet weight (g)		
	on D-glucose	On D-xylose	On D-arabinose
<i>T. neapolitana</i>	1.1	1.5	1.1
<i>T. hypogea</i>	2.2	3.4	1.6
<i>T. maritima</i>	1.3	2.3	2.1
<i>P. furiosus</i>	On maltose: 1.57		
<i>T. guaymasensis</i>	1.5	N/A	N/A
<i>T. petrophila</i>	1.17	1.2	1.36

N/A: not available. *P. furiosus* could only utilize maltose as its carbon source rather than the other three monosaccharides supplied in this project; *T. guaymasensis* could only utilize glucose but not xylose and arabinose.

3.2.1 TmGAPDH

The TmGAPDH activity was dependent on the presence of phosphate or arsenate as a substrate (**Figure 3.1**). The optimal concentration of phosphate or arsenate in the TmGAPDH assay was around 40 mM. A doubling of activity was observed when using arsenate as a substrate compared to phosphate. The activity was determined using the cell free extract from the overexpression host *E.coli* and its activity was dependent on the amount of protein (**Figure 3.2**). The increase of absorbance was obviously shown after adding different amount of cell free extract containing recombinant TmGAPDH, due to the formation of NADH. The absorbance change of negative control containing substrates only was shown during the first 30 seconds. The specific activity of TmGAPDH was 20-30 U/mg in the cell free extract when different amount of arsenate were present.

The TmGAPDH assay was also conducted after protein purification with the same method (**Figure 3.3**). Protein samples processed under aerobic conditions demonstrated sufficient activity, too. The specific activity of the cell free extract and the purified TmGAPDH was 22.4 U/mg and 88.2 U/mg, respectively.

All the TmGAPDH protein samples during the process of purification were kept for the downstream analysis using SDS-PAGE (**Figure 3.4**). A very heavy band of overexpressed TmGAPDH was shown in the lane of cell free extract sample. The same band was also detectable in flowthrough 1 and flowthrough 2, which indicated incomplete binding due to the high amount of overexpressed protein produced during the induction. Only a little target protein was washed out by the wash buffer (flowthrough 3 and flowthrough 4). There was a single band in the purified TmGAPDH sample after

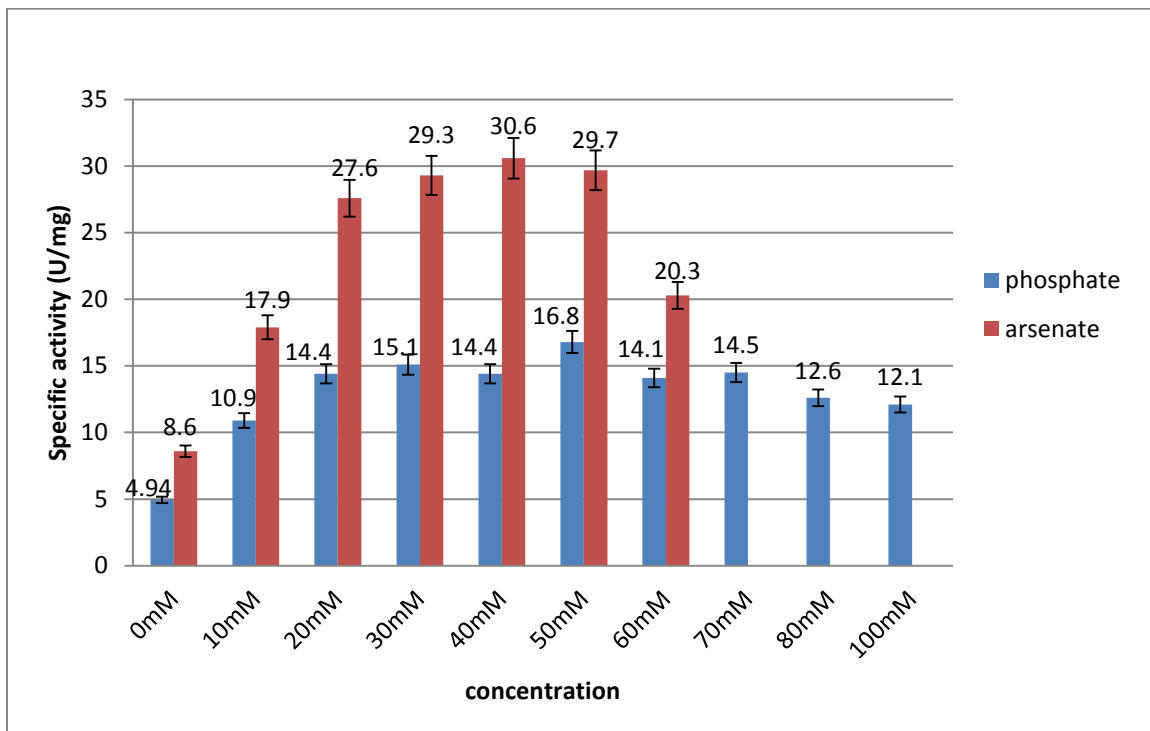


Figure 3.1 Comparison of TmGAPDH activities in the presence of arsenate and phosphate. The assays were conducted using the assay method described in section 2.5.1.1. The different concentrations of arsenate (red bar) or phosphate (blue bar) were used as the substrate for determining the specific activity of TmGAPDH. The specific activities obtained were labeled.

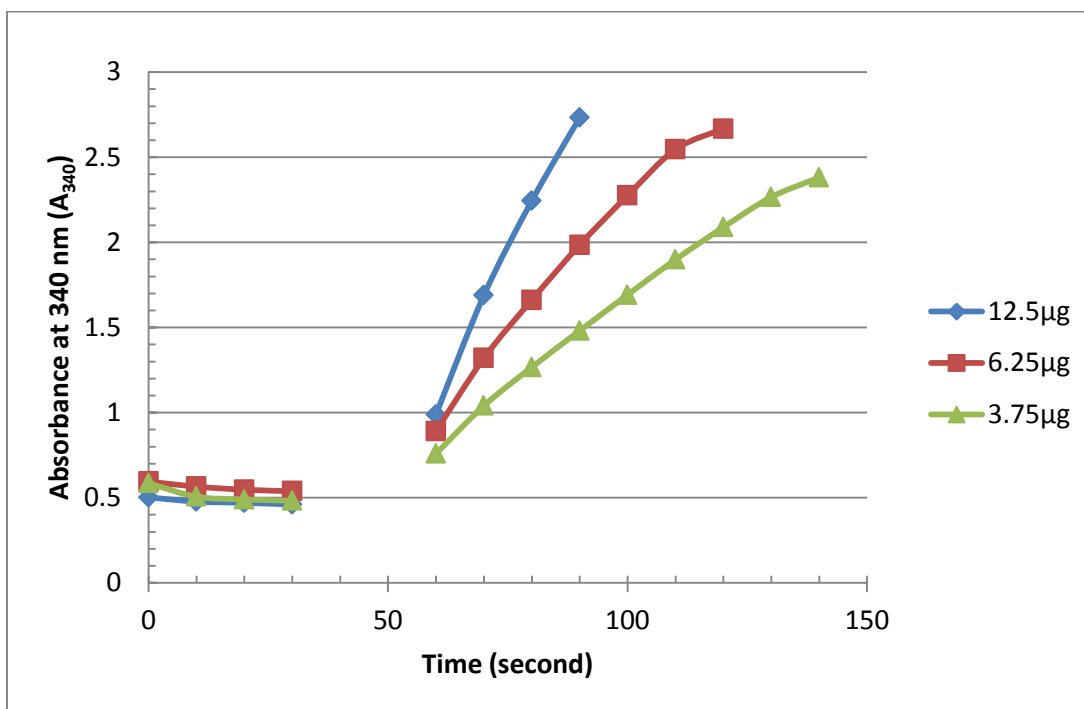


Figure 3.2 TmGAPDH activity in the cell free extract. The assays were conducted using the standard assay method described in section 2.5.1.1. Different amount of cell free extract protein was added in the assay samples (12.5 μg, blue diamond; 6.25 μg, red square; 3.75 μg, green triangle). The absorbance of negative controls of enzyme free samples was shown during the first 30 seconds. The specific activity was ranged from 27.5-31.5 U/mg.

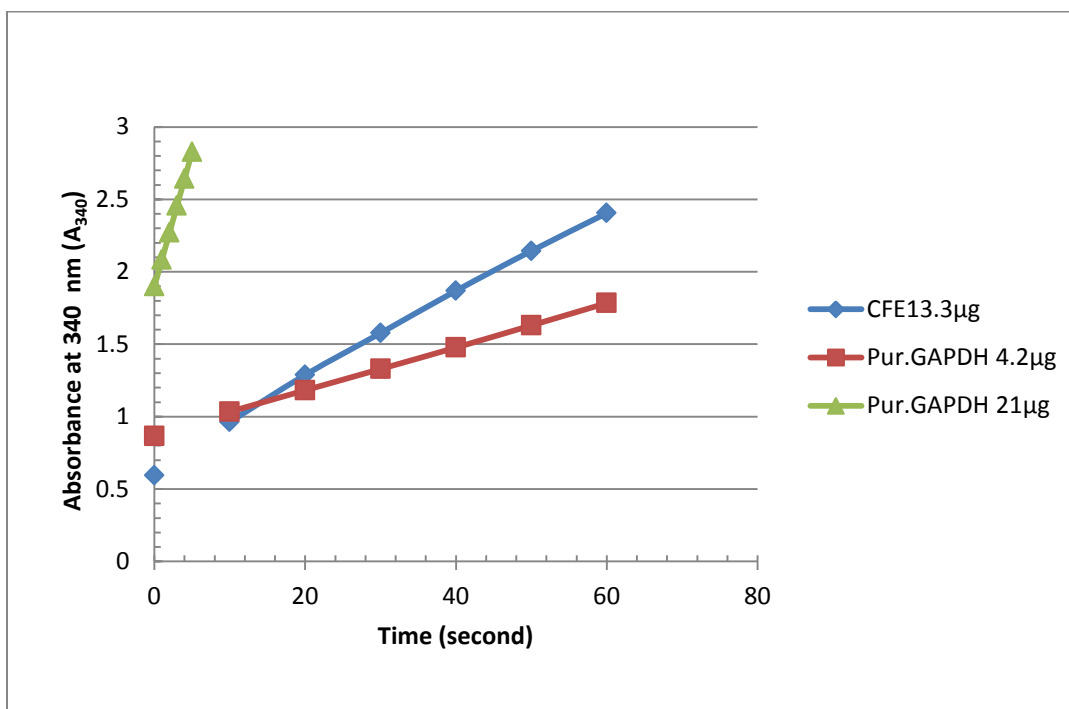


Figure 3.3 TmGAPDH activity after purification. The assays were conducted using the standard assay method described in section 2.5.1.1. Different protein samples were added into the assay mixtures (cell free extract 13.3 µg, blue diamond; purified TmGAPDH, 4.2 µg, red square; purified TmGAPDH 21 µg, green triangle). The specific activity was 22.4 U/mg, 35 U/mg, and 82.2 U/mg, respectively.

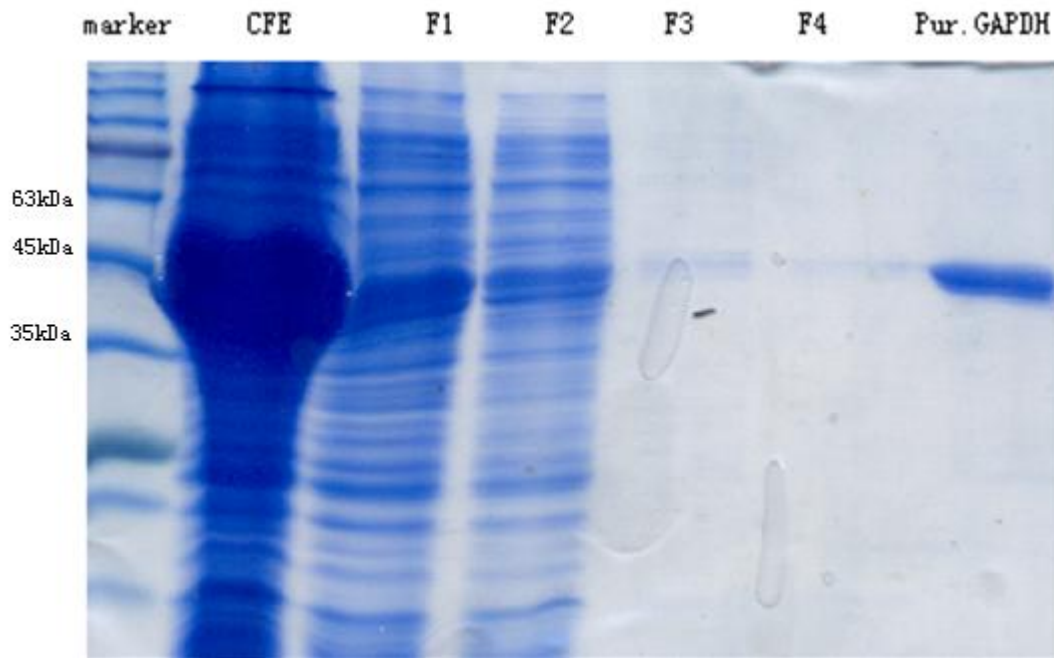


Figure 3.4 SDS-PAGE (12.5%) of TmGAPDH. Lane 1: marker; Lane 2: cell free extract, 110 μg ; Lane 3: flowthrough 1 (from cell free extract), 55 μg ; Lane 4: flowthrough 2 (washed with lysis buffer), 27 μg ; Lane 5: flowthrough 3 (washed with wash buffer), 0.3 μg ; Lane 6: flowthrough 4 (washed with wash buffer), 0.1 μg ; Lane 7: purified TmGAPDH, 5 μg .

elution, which indicated highly purified protein was obtained with the purification method. The molecular weight of TmGAPDH is about 45 kDa. However, it is different from the published paper (37 kDa; Wrab *et al.*, 1990) and the NCBI protein database (36.3 kDa).

3.2.2 PFTIM

PfTIM activity was determined using the cell free extract prepared using overexpressed recombinant protein in *E.coli* (**Figure 3.5**). Different negative controls (PfTIM only, TmGAPDH only, and substrates only) were shown in the graph. The negative controls of substrates only were shown during the first 30 seconds. The negative controls containing single enzyme were shown after 60 second. The sample of TIM only was served as the negative control for the calculation. Tremendous absorbance increasing was shown after adding both PfTIM and TmGAPDH compared to all negative controls. The specific activity of PfTIM was about 30 U/mg in the cell free extract.

PfTIM assays were also conducted using the same method after purification (**Figure 3.6**). Substrates only and purified TIM only were served as negative controls. Both cell free extract and purified enzyme prepared under aerobic condition demonstrated high activities. The specific activity was 22.5 U/mg in the cell free extract and 409 U/mg in the pure enzyme, respectively.

All the PfTIM protein samples during the process of purification were kept for the downstream analysis with a SDS-PAGE (**Figure 3.7**). There was a heavy band of PfTIM protein shown in the lane of the cell free extract sample. Thin target protein bands were

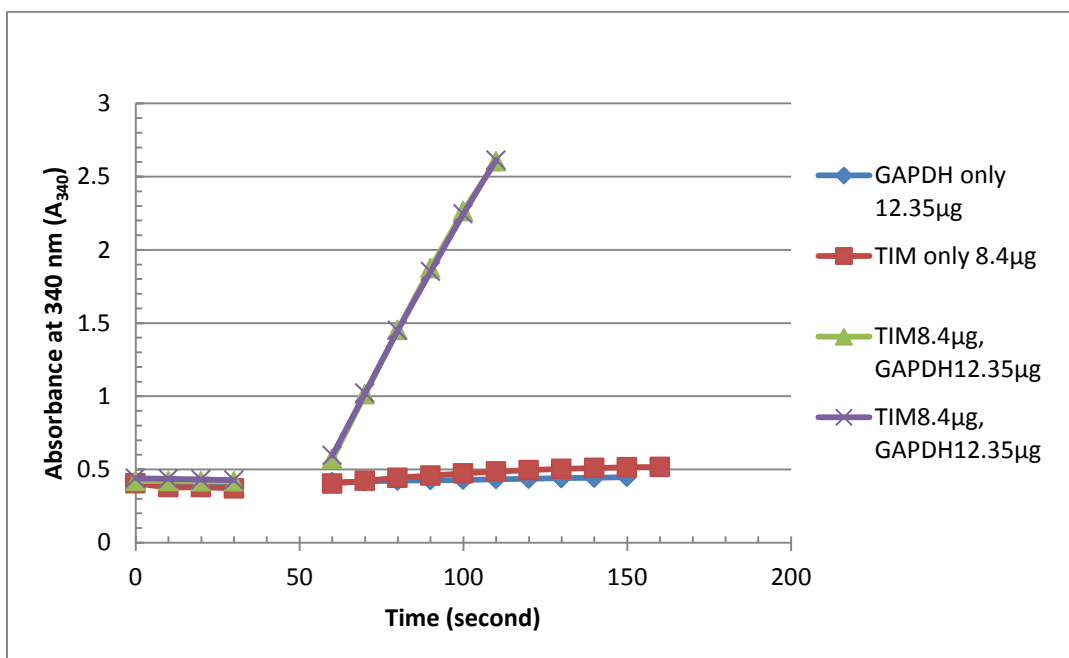


Figure 3.5 PfTIM activity in the cell free extract. The assays were conducted using the standard assay method described in section 2.5.1.2. Different negative controls were designed (GAPDH 12.35 μg only, blue diamond; TIM 8.4 μg only, red square; enzyme free, lines during the first 30 seconds). The assays were duplicated (green triangle and purple cross) when both TIM and GAPDH were present. The specific activity was 30 U/mg.

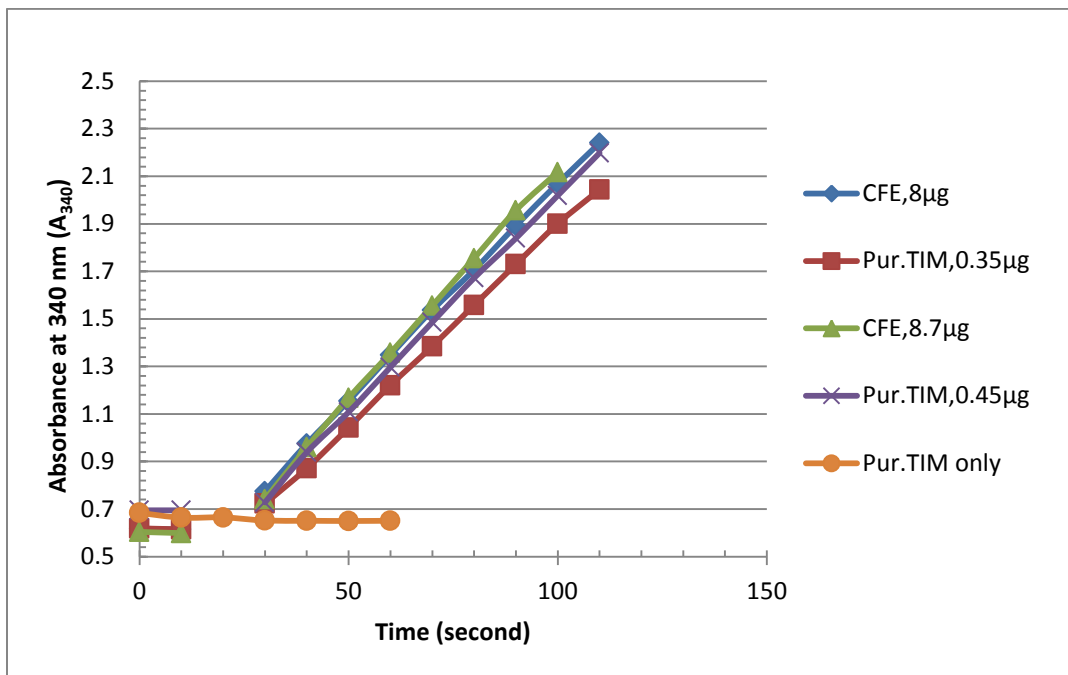


Figure 3.6 PFTIM activity after purification. The assays were conducted using the standard assay method described in section 2.5.1.2. Different protein samples were added into the assay mixtures (cell free extract 8 μg, blue diamond; cell free extract 8.7 μg, green triangle; purified TIM 0.35 μg with GAPDH, red square; purified TIM 0.45 μg with GAPDH, purple cross). Two negative controls were designed (substrates only, shown during the first 30 seconds; purified TIM only, orange dot). The specific activity was 22.5 U/mg in cell free extract and 409 U/mg in the purified PFTIM, respectively.

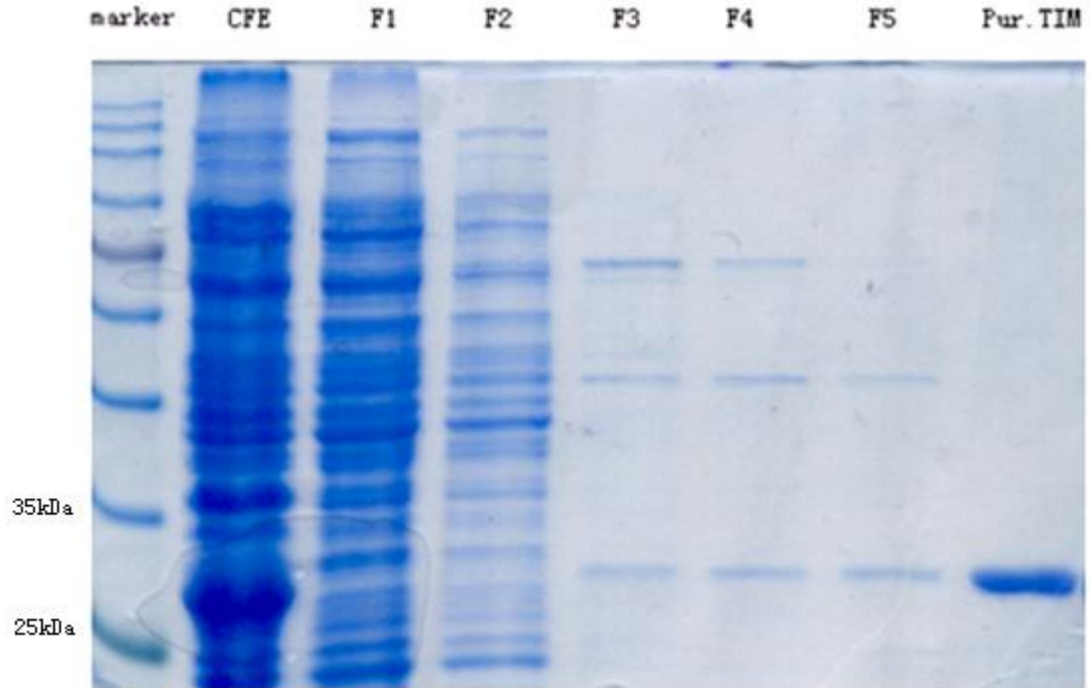


Figure 3.7 SDS-PAGE (12.5%) of PfTIM. Lane 1: marker; Lane 2: cell free extract, 55 μg ; Lane 3: flowthrough 1 (from cell free extract), 37 μg ; Lane 4: flowthrough 2 (washed with lysis buffer), 22 μg ; Lane 5: flowthrough 3 (washed with wash buffer), 0.8 μg ; Lane 6: flowthrough 4 (washed with wash buffer), 0.7 μg ; Lane 7: flowthrough 4 (washed with wash buffer), 0.1 μg ; Lane 8: purified PfTIM, 5 μg .

observable in all lanes loaded with flowthrough samples, which means not all PFTIM protien was bound with cobalt resin. There was a single protein band in the purified PFTIM sample, which indicates that highly purified target protein was obtained with the purification method. The molecular weight of PFTIM is about 30 kDa, which is larger than the estimated molecular weight of the same enzyme in NCBI protein database (24 kDa).

3.2.3 PfG3PDH

The PfG3PDH activity was determined in the cell free extract prepared from the overexpressed recombinant protein in *E.coli* (**Figure 3.8**). The activity was dependent on the amount of protein added into the reaction mixture. The negative control of PfG3PDH free was shown during the first 30 seconds. Obvious absorbance decreasing was shown after adding enzyme due to the oxidation of NADH. The specific activity was about 0.5 U/mg.

The PfG3PDH assay was also conducted after purification under aerobic conditions (**Figure 3.9**). Due to a lack of commercially produced DHAP, glyceraldehyde-3-P served as the substrate with the presence of purified PFTIM as the helping enzyme. Therefore, different negative controls were operated under the same assay conditions. The negative controls containing substrates and PFTIM were shown during the first minute (**Figure 3.9**). The negative controls containing single enzyme (PFTIM or PfG3PDH) were also shown in the graph (**Figure 3.9**). Obvious absorbance decreasing was detected when both G3PDH and TIM were present. The specific activity was

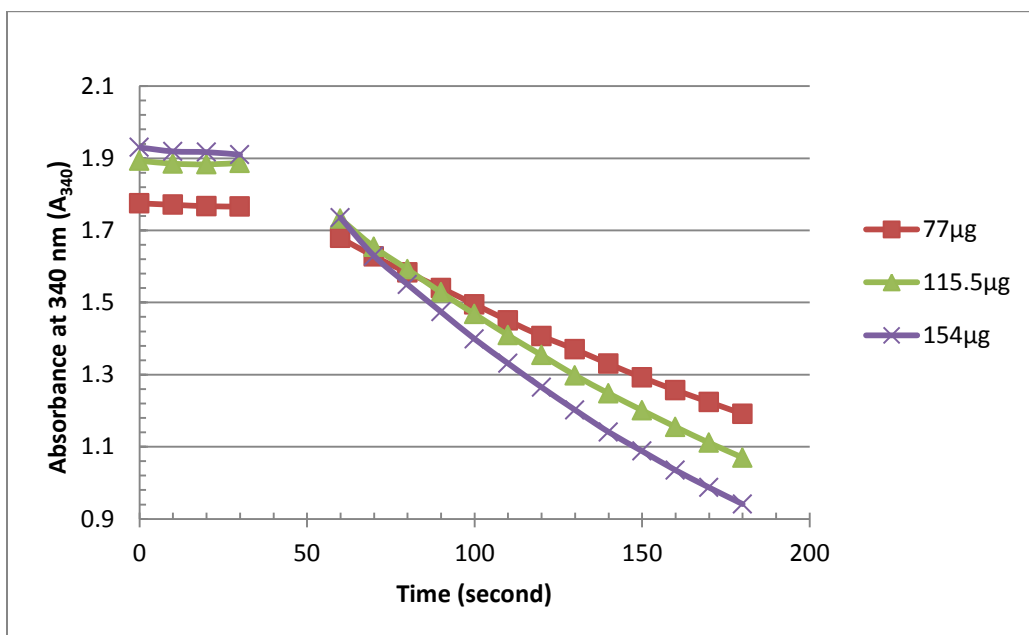


Figure 3.8 PfG3PDH activity in the cell free extract. The assay was conducted using the standard assay method described in section 2.5.1.3. Different amount of cell free extract protein was added (77 μg , red square; 115.5 μg , green triangle; 154 μg , purple cross). Negative controls of enzyme free sample were shown during the first 30 seconds. The specific activity was 0.5 U/mg.

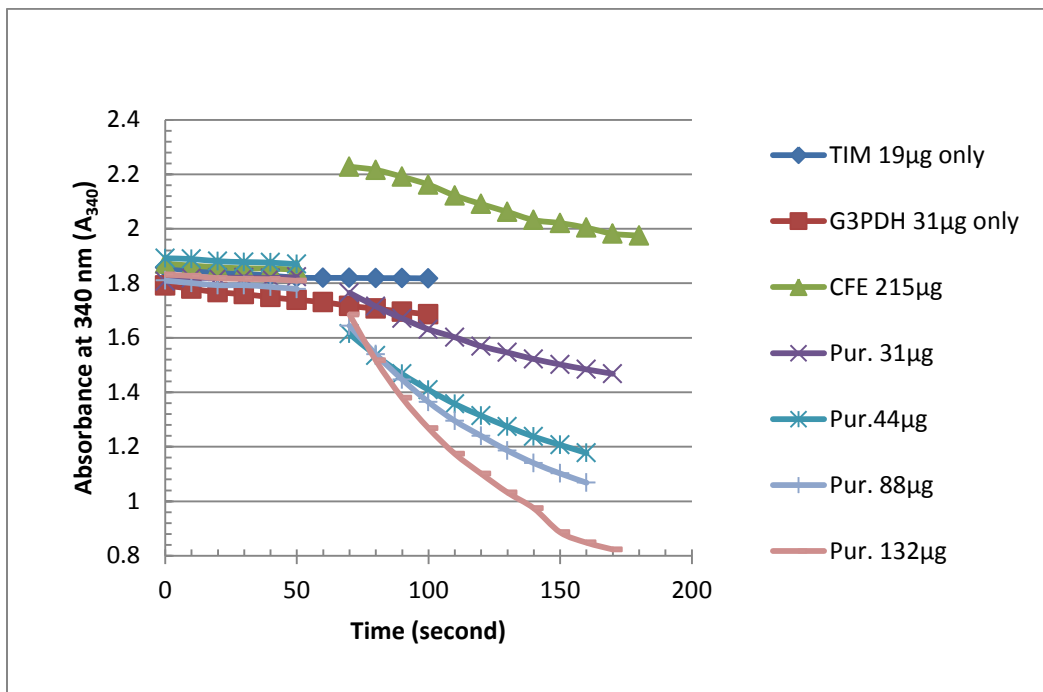


Figure 3.9 PfG3PDH activity after purification. The assay was conducted using the assay mixture described in section 2.5.1.3 with purified TIM as the auxiliary enzyme and glyceraldehyde-3-phosphate as the substrate. Three negative controls were designed (TIM only, blue diamond; G3PDH only, red square, and substrates and TIM only, were shown during the first 30 seconds). Different protein samples were assayed with the same method (cell free extract 215 µg, green triangle; different amount of purified G3PDH, the remaining curves). The specific activity was 0.2 U/mg in the cell free extract and 1.5 U/mg in the purified form, respectively.

0.2 U/mg in the cell free extract and 1.58 U/mg in the pure enzyme, respectively.

All the PfG3PDH protein samples during the process of purification were kept for further analysis with SDS-PAGE (**Figure 3.10**). There was a heavy band of PfG3PDH protein shown in the lane of cell free extract sample. Not fully binding PfG3PDH was shown in all the lanes loaded with flowthrough samples due to the detectable target protein band exhibited. There was a single protein band in the purified PfG3PDH sample, indicating that highly purified target protein was obtained with the purification method. The molecular weight of PfG3PDH is about 37 ± 1 kDa which is consistent with the estimated molecular weight of the same enzyme in NCBI protein database.

3.2.4 ApG1PDH

ApG1PDH activity was determined in the cell free extract prepared with the overexpressed recombinant protein in *E.coli* (**Figure 3.11**). There are two types of negative controls shown in the graph. The enzyme free negative controls were monitored during the first 30 seconds. The negative control using the cell free extract prepared with *E.coli* containing blank pET30a was conducted to exclude any potential absorbance changing caused by the mesophilic proteins from the host strain. Obvious absorbance decreasing was shown after adding ApG1PDH due to the oxidation of NADH. The activity was dependent on the amount of protein added into the reaction mixture. Its specific activity was 0.3 U/mg in the cell free extract.

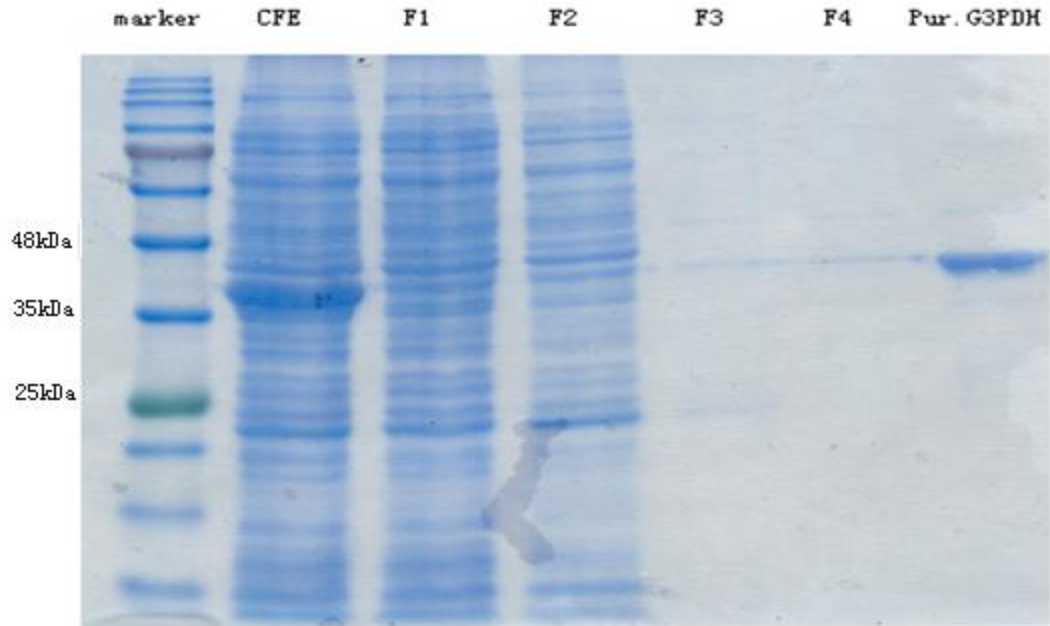


Figure 3.10 SDS-PAGE (12.5%) of PfG3PDH. Lane 1: marker; Lane 2: cell free extract, 36 μg ; Lane 3: flowthrough 1 (from cell free extract), 21 μg ; Lane 4: flowthrough 2 (washed with lysis buffer), 17 μg ; Lane 5: flowthrough 3 (washed with wash buffer), 0.2 μg ; Lane 6: flowthrough 4 (washed with wash buffer), 0.1 μg ; Lane 7: purified PfG3PDH, 5 μg .

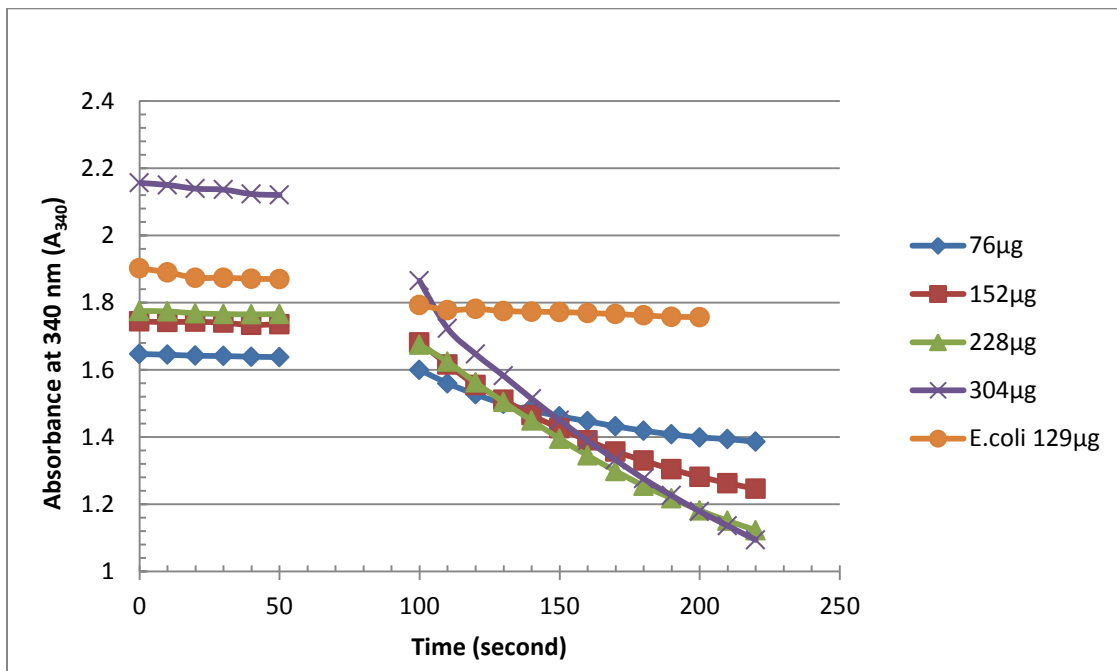


Figure 3.11 ApG1PDH activity in the cell free extract. The assay was conducted using the standard method described in section 2.5.1.3. Two negative controls were designed (cell free extract prepared from *E.coli* with blank pET30a 129 µg, orange dot; substrates only, shown during the first 60 seconds). Different amount of cell free extract were added into the assay mixture (76 µg, blue diamond; 152 µg, red square; 228 µg, green triangle; 304 µg, purple cross). The specific activity was 0.3 U/mg.

The deviation of negative controls from different samples was shown in the graph during the first minute. It was mainly caused by two factors: the amount of the cell free extract and NADH. Cell free extracts from recombinant *E.coli* caused turbidity after being denatured at 80° C; Absorbance was also very sensitive to the amount of NADH present in the mixture. Both of them lead to the absorbance deviation during the assay.

3.2.5 TmTK

The TmTK activity was determined using cell free extract prepared with the over expressed recombinant proteins in *E.coli* (**Figure 3.12**). The negative controls only containing substrates and TmTK were monitored in the first 30 seconds; the negative controls only containing single enzyme (TmGAPDH or TmTK) are shown in the graph; the negative control containing the cell free extract prepared from *E.coli* with blank pET30a and TmGAPDH is shown in the graph, too. Obvious absorbance increasing was observed if both TmTK and GAPDH were present. It was also shown that TmTK activity was dependent on the amount of protein. The more protein was added, the more activity was obtained. Xylulose-5-P was added into the reaction mixture at levels likely to be insufficient to saturated enzyme achieve sites, due to the limited amount purchased. This is the reason why the absorbance reached a plateau relatively early compared to the previous assays. The specific activity of TmTK in cell free extract was around 5U/mg.

All the TmTK protein samples during the process of purification were kept for further analysis with SDS-PAGE (**Figure 3.13**). There was a heavy band of TmTK protein shown in the lane of cell free extract sample. Thin target protein bands were shown in all

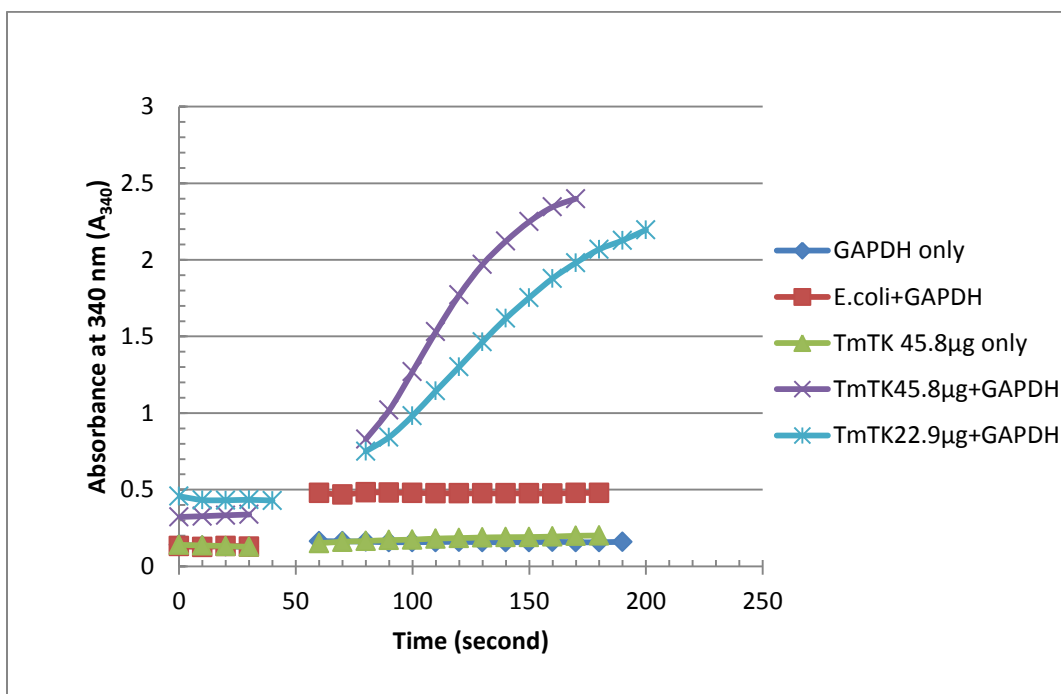


Figure 3.12 TmTK activity in the cell free extract. The assay was conducted using the standard method described in section 2.5.1.4. Negative controls were designed (GAPDH only, blue diamond; cell free extract prepared from *E.coli* with blank pET30a plus GAPDH, red square; TmTK 45.8 µg only, green triangle; substrates plus TmTK, shown during the first 30 seconds). Different amount of TmTK with GAPDH were also detected under the same assay conditions (TmTK 45.8 µg with GAPDH, purple cross; TmTK 22.9µg with GAPDH, light blue asterisk). The specific activity was about 5 U/mg.

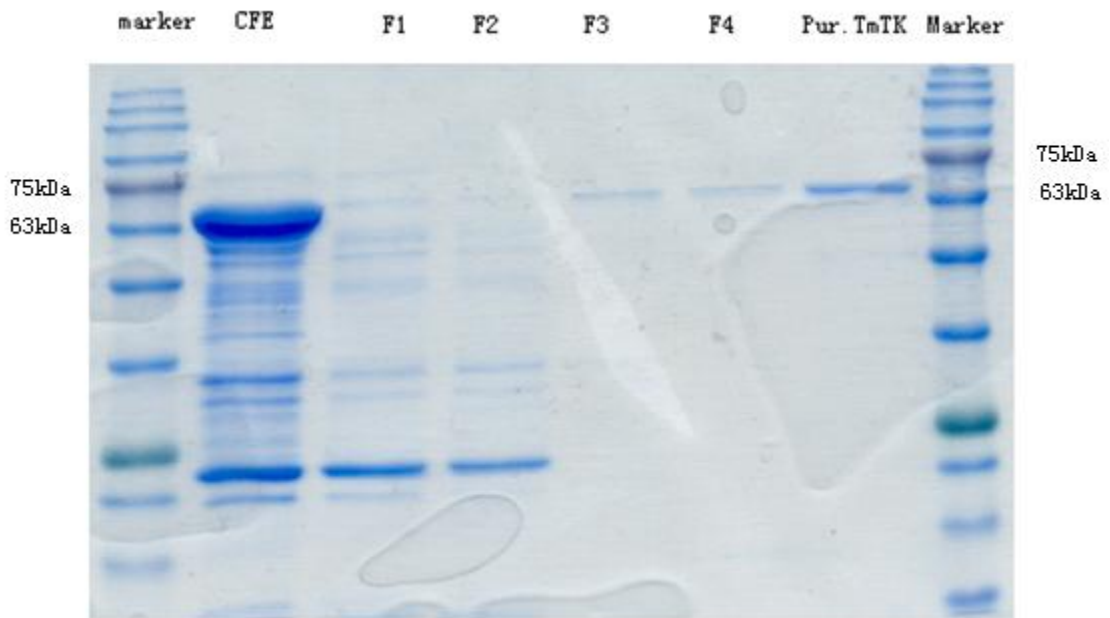


Figure 3.13 SDS-PAGE (12.5%) of TmTK. Lane 1: marker; Lane 2: cell free extract, 27 μg ; Lane 3: flowthrough 1 (from cell free extract), 10 μg ; Lane 4: flowthrough 2 (washed with lysis buffer), 8 μg ; Lane 5: flowthrough 3 (washed with wash buffer), 0.1 μg ; Lane 6: flowthrough 4 (washed with wash buffer), 0.1 μg ; Lane 7: purified TmTK, 2 μg ; Lane 8: marker.

the lanes of flowthrough samples, which revealed that the target protein was not fully bound with cobalt resin. There was a single protein band in the purified TmTK sample, indicating that highly purified target protein was obtained with the purification method. The molecular weight of TmTK is about 65 kDa.

3.2.6 TmXI and TpXI

TmXI and TpXI activity were determined in cell free extracts prepared with the overexpressed recombinant proteins in *E.coli*. A xylulose standard curve (**Figure 3.14**) was obtained with the method described in section 2.5.1.5. The concentration of 0-10 mM D-xylulose was added into the reaction mixture instead of D-xylose. Color formation at 540 nm was monitored at different time intervals. After analyzing all the data obtained, A_{540} at 40 minutes was plotted as the standard curve due to the linear range of color formation with 0-8 mM xylulose in the reaction mixture.

The amount of xylulose formation catalyzed by TmXI and TpXI cell free extracts (**Figure 3.15**) was calculated using the xylulose standard curve after deducting the absorbance of the enzyme free control. It was shown that the xylulose formation was protein dependent. TpXI also showed a higher ability of conversion from xylose to xylulose than TmXI did under the assay conditions. The specific activities of TmXI and TpXI cell free extracts were 3.8 U/mg and 6.5 U/mg, respectively.

Due to the higher activity exhibited in TpXI, it was purified. Purified TpXI activity was determined using the standard assay method described in section 2.5.1.5 (**Figure 3.16**). Xylulose formation by TpXI was also calculated with the standard curve after

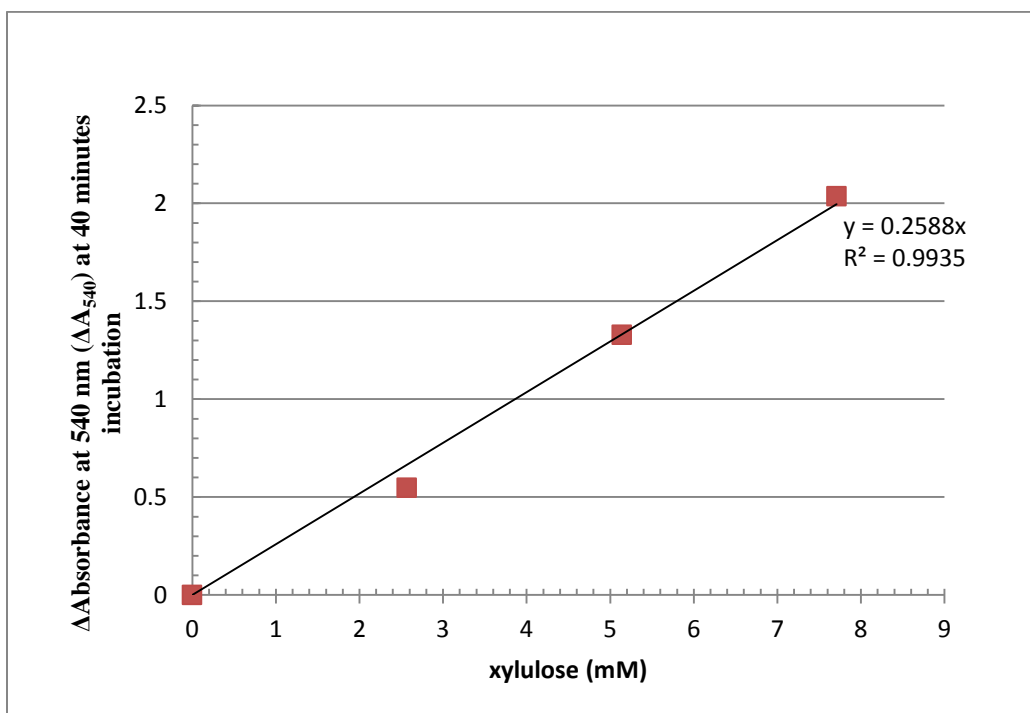


Figure 3.14 Standard curve for the determination of xylulose. The standard curve was obtained using the XI assay method described in section 2.5.1.5, with known amounts xylulose instead of xylose and in the absence of enzyme.

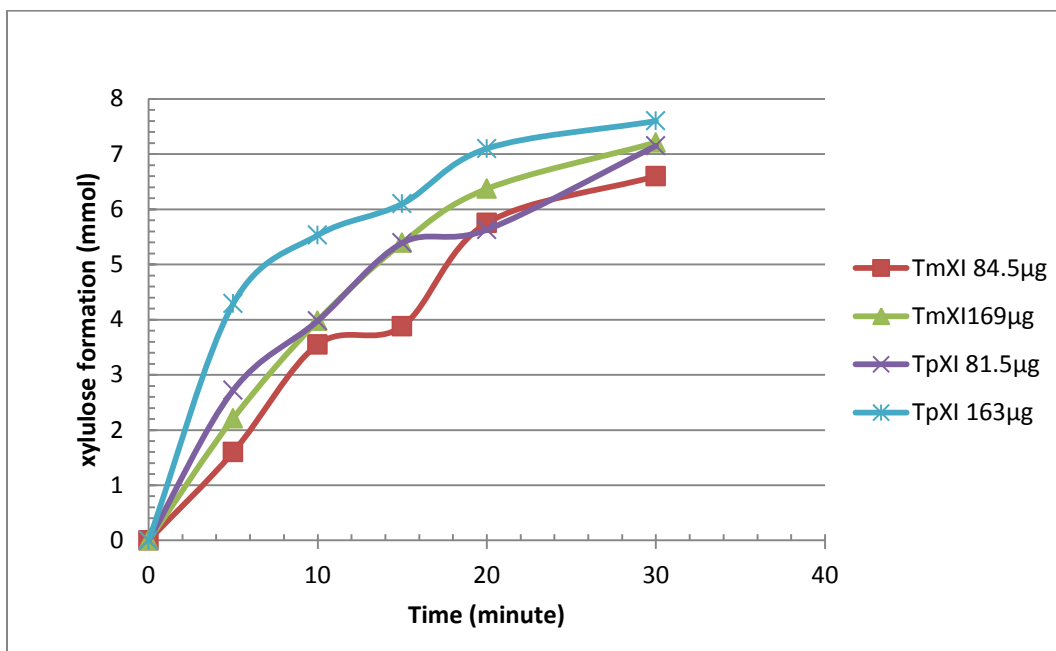


Figure 3.15 Xylulose formation catalyzed by the cell free extracts containing the overexpressed TmXI and TpXI. The assay was conducted with the method described in section 2.5.1.5. The absorbance after deducting negative control (enzyme free sample) was converted into the amount of xylulose by using the xylulose standard curve (**Figure 3.14**). Different amounts of TmXI or TpXI containing cell free extract was added into the assay mixture (TmXI 84.5 µg, red square; TmXI 169 µg, green triangle; TpXI 81.5 µg, purple cross; TpXI 163 µg, blue asterisk). The specific activity was 3.8 U/mg in TmXI cell free extract and 6.5 U/mg in TpXI cell free extract, respectively.

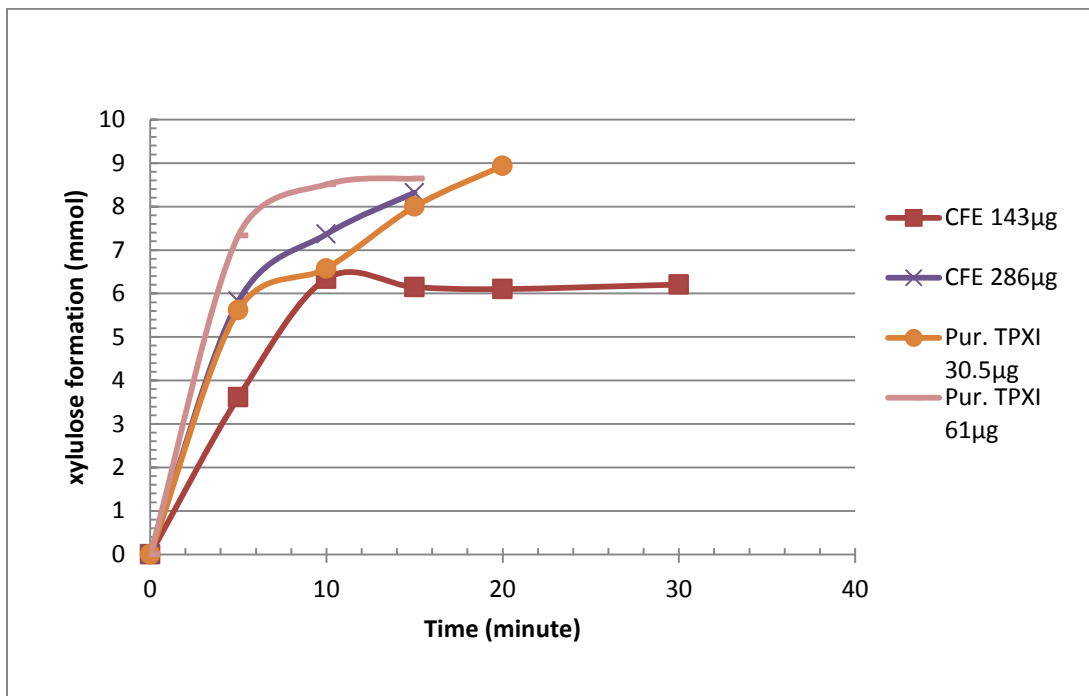


Figure 3.16 Xylulose formation catalyzed by purified TpXI. The assay was conducted with the same method described in section 2.5.1.5. The absorbance after deducting negative control (enzyme free sample) was converted into the amount of xylulose by using the xylulose standard curve (**Figure 3.14**). Different protein samples were assayed (TpXI cell free extract 143 µg, red square; TpXI cell free extract 286 µg, purple cross; purified TpXI 30.5 µg, orange dot; purified TpXI 61 µg, pink line). The specific activity was 4 U/mg in TpXI cell free extract and 36.8 U/mg in the purified form.

deducting the enzyme free negative control. The specific activities of TpXI in the cell free extract and of the pure enzyme were 4 U/mg and 36.8 U/mg, respectively.

All the TpXI protein samples during the process of purification were kept for the downstream analysis with a 12.5% SDS-PAGE (**Figure 3.17**). There was a heavy band of TpXI protein shown in the lane of the cell free extract sample. Thin target protein bands were visible in all the lanes of flowthrough samples, which meant the target protein was not fully bound with cobalt resin. There was a single protein band in the purified TpXI sample, which indicates that a highly purified target protein was obtained with the purification method. The molecular weight of TpXI is about 50 kDa, which is consistent with the estimated molecular weight from the same protein in NCBI protein database (50.8 kDa).

3.2.7 TmXuK

TmXuK activity was determined in the cell free extract or with purified TmXuK prepared from overexpressed recombinant protein in *E.coli* (**Figure 3.18**). Cell free extract was prepared under anaerobic conditions and incubated under 65° C or 70° C for 10 minutes, so that most mesophilic proteins from *E.coli* could be removed after centrifugation. The absorbance at 340 nm obtained in the assay was plotted. Different negative controls were designed for TmXuK assay (no xylose, no ATP or ADP, no TmXuK cell free extract, cell free extract prepared from *E.coli* containing blank pET30a) in order to obtain real activity of TmXuK. It was found that the absorbance increased after adding cell free extract no matter the presence of ATP, ADP or without ATP. However,

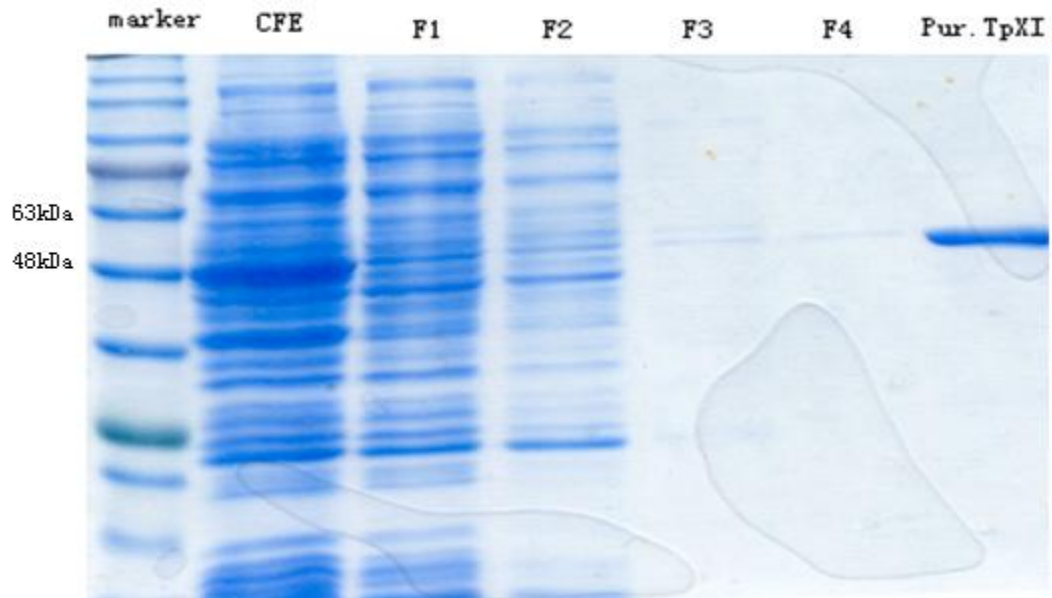


Figure 3.17 SDS-PAGE (12.5%) for TpXI. Lane 1: marker; Lane 2: cell free extract, 48 μg ; Lane 3: flowthrough 1 (from cell free extract), 24 μg ; Lane 4: flowthrough 2 (washed with lysis buffer), 11 μg ; Lane 5: flowthrough 3 (washed with wash buffer), 0.2 μg ; Lane 6: flowthrough 4 (washed with wash buffer), 0.1 μg ; Lane 7: purified TpXI, 2.5 μg .

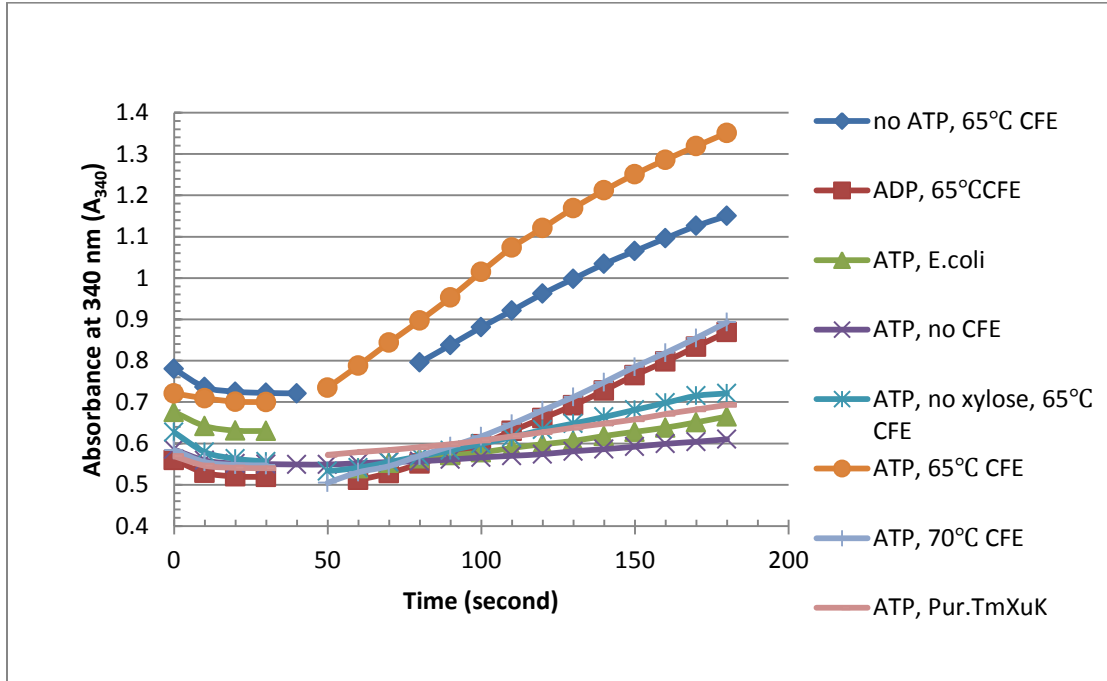


Figure 3.18 Determination of TmXuK activities of the cell free extract and purified enzyme. The assay was conducted using the same method described in section 2.5.1.6. TmXuK cell free extract was incubated at 65° C and 70° C for 10 minutes and was centrifuged before adding into the assay mixture. Different cell free samples with ATP or without ATP were assayed (65° C cell free extract but no ATP, blue diamond; 65° C cell free extract with ADP, red square; 65° C cell free extract with ATP, orange dot; 70° C cell free extract with ATP, light blue line; purified TmXuK with ATP, pink line). The remaining curves were all negative controls (no xylose, turquoise asterisk; no TmXuK cell free extract, purple cross; *E.coli* cell free extract with ATP, green triangle). The specific activity was 0.9 U/mg in 65° C cell free extract and 0.29 U/mg in 70° C cell free extract, respectively. However, there was no activity observed in purified TmXuK.

little absorbance change was observed in the cell free extract samples compared to the negative controls. The specific activity is about 0.2-0.9 U/mg in the cell free extract. However, purified TmXuK protein had no detectable activity.

All the TmXuK protein samples during the process of purification were kept for the downstream analysis with SDS-PAGE (**Figure 3.19**). There was a heavy band of TmXuK protein shown in the lane of the cell free extract sample. Thin bands were visible in all the flowthrough lanes, which meant untarget proteins existed in all samples. Several protein bands in the purified TmXuK sample were clearly evidence that the TmXuK protein was not purified with the method used. The molecular weight of TmXuK is about 63 kDa. However, it is not consistent with the estimated molecular weight from the same protein in NCBI protein database (54 kDa).

3.2.8 Activities of auxiliary enzymes after purification

All the activities of purified auxiliary enzymes were calculated, and results are summarized in **Table 3.3**. Specific activities of both the cell free extract and the purified form prepared under aerobic condition are listed. Total activities and activity recovery after purification were also calculated and shown in the table. The ApG1PDH was not purified with the HisPur cobalt column because the protein did not have polyhistidine-tag. The TmXI demonstrated lower activity than TpXI's, so it was not purified within this project. No purified TmTK activity could be tested due to the unavailable substrate—xylulose-5-P. Thus, there was no purified TmTK activity shown in the table.

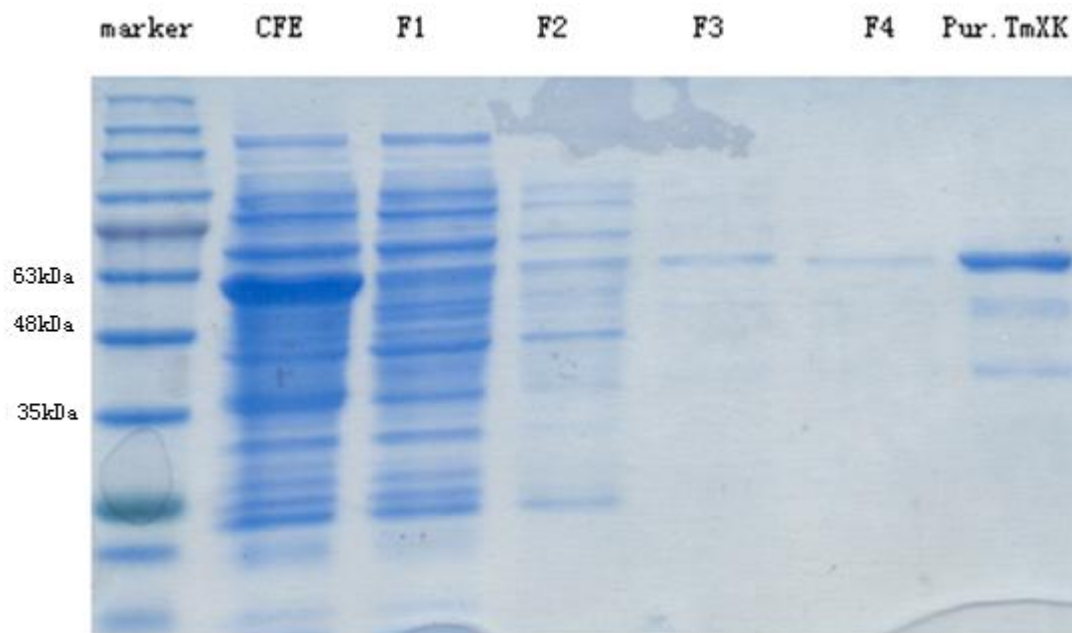


Figure 3.19 SDS-PAGE (12.5%) for TmXuK. Lane 1: marker; Lane 2: cell free extract, 101 μg ; Lane 3: flowthrough 1 (from cell free extract), 45 μg ; Lane 4: flowthrough 2 (washed with lysis buffer), 17 μg ; Lane 5: flowthrough 3 (washed with wash buffer), 0.1 μg ; Lane 6: flowthrough 4 (washed with wash buffer), 0.1 μg ; Lane 7: purified TmXuK, 5 μg .

Table 3.3 Activities of purified enzymes

Enzyme	Specific activity (U/mg)		Total activity (Unit)		Recovery (%)
	Cell free extract	Purified enzyme	Cell free extract	Purified enzyme	
TmGAPDH	22.4	82.2	5065	3452	68.2
PfTIM	22.5	409	3024	2658.5	87.9
PfG3PDH	0.2	1.47	10.4	9.72	93.5
ApG1PDH	0.1	ND	ND	ND	ND
TmXI	3.7	ND	ND	ND	ND
TpXI	5.05	36.8	694.4	561.2	80.8
TmTK	4.8	ND	ND	ND	ND
TmXuK	0.29	0	17.4	0	0

ND: not determined.

3.3 Assays for TK and TAL of hyperthermophiles

3.3.1 TmTK assay

TK activity was determined in the cell free extract prepared with overexpressed recombinant TmTK protein in *E.coli*. The TmTK activity was detected with the cell free extracts after heat treatment under 70° C or 80° C for 30 minutes in order to inactivate most mesophilic enzyme proteins. The assay method as described in section 2.5.1.4 was applied for the determination of TmTK activity. Comparison of TmTK activities at room temperature, 70° C treatment, and 80° C treatment are shown in **Figure 3.20**. The negative control (TmTK only) was shown little increasing absorbance. Another negative control (substrates and TmGAPDH only) was also shown in the first 30 seconds in the graph. The increase of absorbance was obvious when both TmTK and TmGAPDH were present. The specific activity of cell free extract after heat treatment under different conditions was 2.36 U/mg at room temperature, 3.38 U/mg at 70° C, and 3.79 U/mg at 80° C, respectively. Thus, recombinant TmTK demonstrated thermostability at such heat treatment conditions.

Due to the lack of availability of a commercial supply of xylulose-5-phosphate, TK activities in hyperthermophiles could not be determined within the time frame of this research project.

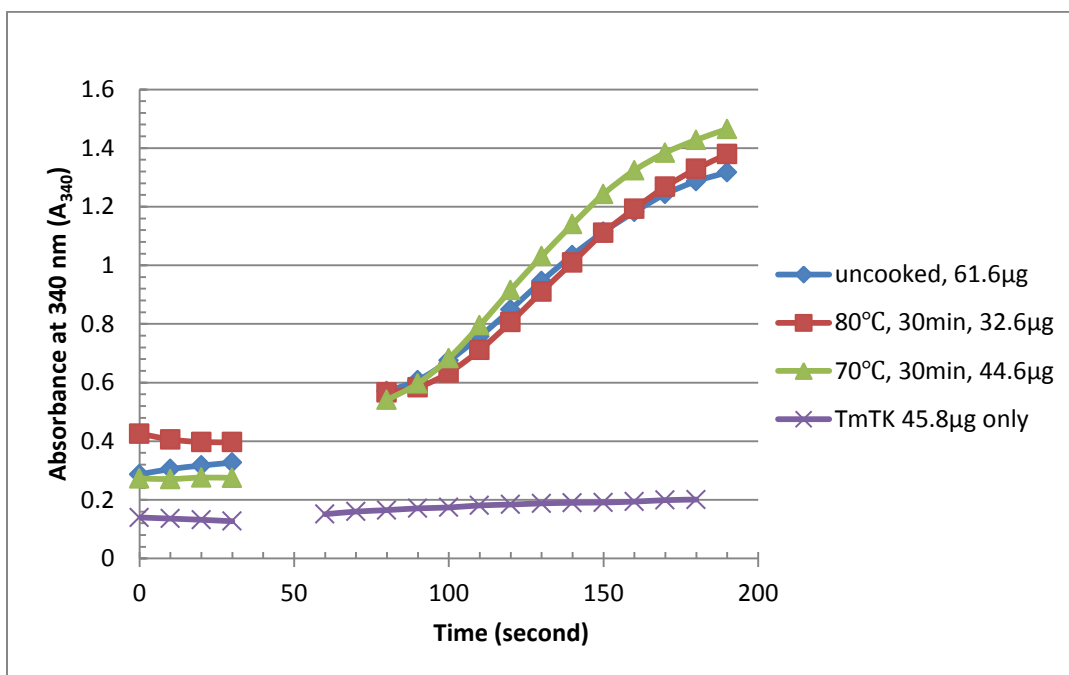


Figure 3.20 Comparison of TmTK activities after heat treatment. The assay was conducted using the standard method described in section 2.5.1.4. Recombinant TmTK cell free extract was prepared with heat treatment under 70° C and 80° C for 30 minutes in order to detect its thermostability. TmTK cell free extract after 70° C treatment was served as the negative control (purple cross). Different protein samples with TmGAPDH were assayed (uncooked cell free extract 61.6 µg, blue diamond; cell free extract after 70° C treatment 44.6 µg, green triangle; cell free extract after 80° C treatment 32.6 µg, red square). The specific activity was 2.36 U/mg in cell free extract, 3.38 U/mg in cell free extract with 70° C heat treatment, and 3.79 U/mg in cell free extract with 80°C heat treatment, respectively.

3.3.2 TAL assays

TAL activity was determined in cell free extracts prepared with hyperthermophile cells (**Figure 3.21**). TAL activities were detected with TmGAPDH or PftIM plus TmG3PDH as auxiliary enzymes. TAL activities were shown in all selected species. *T. petrophila* showed the highest activity among these strains (about 0.7 U/mg). However, little TAL activity was observed among three hyperthermophilic species: *T. hypogea*, *T. guaymasensis*, and *P. furiosus*. A similar pattern was found when using PftIM and Pfg3PDH as helping enzymes for determination of TAL activity. However, the assay with TmGAPDH showed a much higher result than with PftIM and Pfg3PDH, indicating that TmGAPDH offered a more sensitive function than Pfg3PDH did.

3.4 The new assay system for the determination of XuK activities in hyperthermophiles

XuK assays in hyperthermophiles were conducted using the designed assay system in order to prove its feasibility. The same method was used in XuK assays as described in section 2.5.1.6. The XuK activities in hyperthermophiles are illustrated in **Figure 3.22**. There were several negative controls designed: no hyperthermophilic cell free extract (*E.coli* CFE, about 150 µg), no xylose but using the cell free extract of *T.guaymasensis*, and no ATP but using the cell free extract from *P. furiosus*. There was no obvious absorbance change for the first two controls. However, hyperthermophilic cell free extracts could also increase absorbance even without ATP. This result was also consistent with the previous TmXuK assay when the same assay system was used, which may

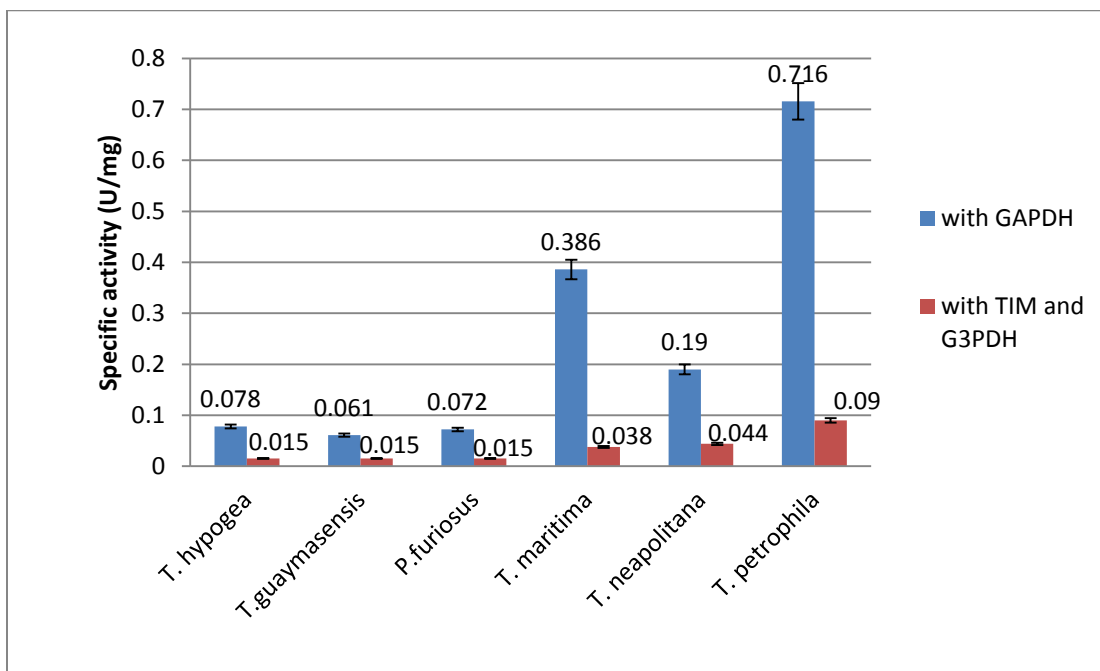


Figure 3.21 Specific activities of TAL of selected hyperthermophiles. The TAL assays were conducted using the standard methods described in section 2.5.2.2. The assays were determined with different auxiliary enzymes (with purified TmGAPDH, blue bar; with purified PFTIM and PfG3PDH, red bar). The specific activities obtained were also illustrated in the graph.

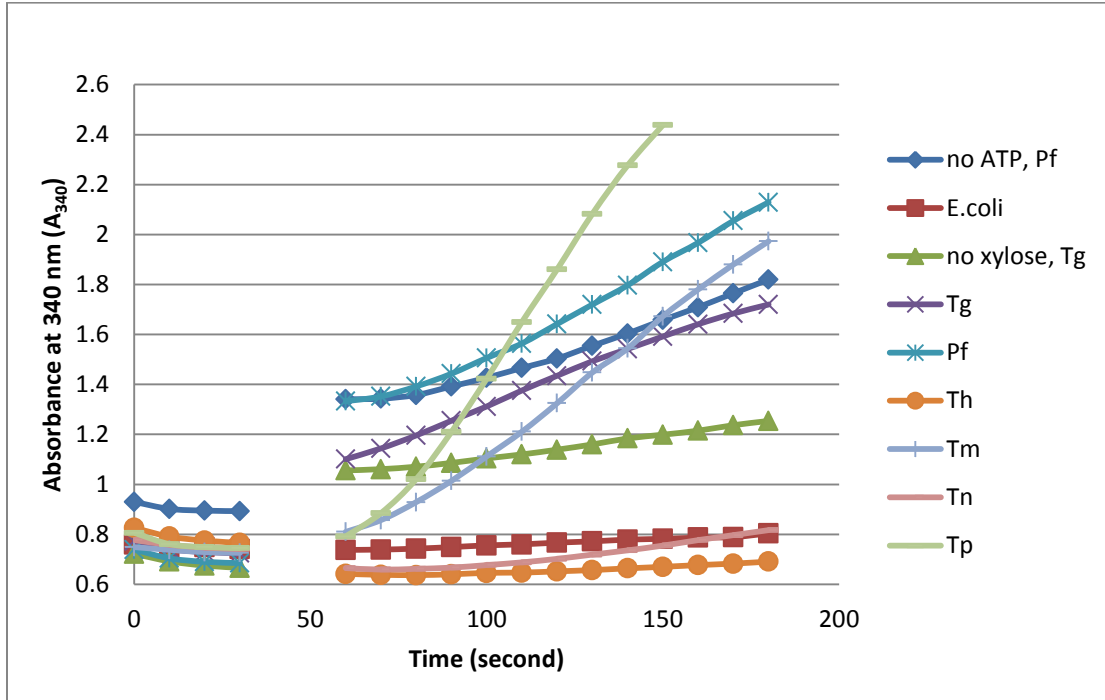


Figure 3.22 Assays for XuK of hyperthermophiles with the new assay system. The assay was conducted using the same method described in section 2.5.1.6. Different negative control were designed (*P. furiosus* cell free extract without ATP, blue diamond; *E.coli* cell free extract, red square; *T. guaymansensis* cell free extract without xylose, green triangle). The remaining curves were XuK assay among hyperthermohiles with their cell free extracts (*T. guaymansensis*, purple cross; *P. furiosus*, blue asterisk; *T. hypogea*, orange dot; *T. maritima*, light blue cross; *T. neapolitana*, pink line; *T. petrophila*, opaline green line). The specific activity was 0.06 U/mg in *T. guaymansensis*, 0.09 U/mg in *P. furiosus*, 0.14 U/mg in *T. maritima*, and 0.33 U/mg in *T. petrophila*, respectively. *T. hypogea* and *T. neapolitana* had no detectable XuK activity.

indicates there was still some energy source remaining in the cell free extracts. *T. petrophila* showed the highest XuK activity (0.3 U/mg) among these hyperthermophiles. No XuK activities could be detected in *T. neapolitana* and *T. hypogea*. Therefore, the successful XuK assays in hyperthermophiles provided a positive evidence for the feasibility of the new assay system.

4. Discussion and conclusion

4.1 The auxiliary enzymes

Most auxiliary enzymes required for this project were successfully obtained in a recombinant form that was active and could be purified using the methods described in section 2.6. This is the only report of the application of thermostable enzymes involved in determination of PPP enzymes' activities to date, compared to other commercially available mesophilic enzymes. These enzymes, which demonstrated to be thermostable, will confer a lot of benefits for potential applications.

Phosphate is a substrate of GAPDH and the phosphate can be substituted by arsenate that results in a higher activity, which was reported several decades ago (Ehring and Colowick, 1969). The comparison of TmGAPDH activity with phosphate and arsenate as a substrate confirmed the same conclusion for hyperthermophilic GAPDH (Zwickl *et al.*, 1990). Moreover, the GAPDH activity measured using the method described in this report was four times higher than previously reported (Rehaber and Janeicke, 1992). TmGAPDH activity increased four times after purification (from 22.4 U/mg to 82.2 U/mg), and the cell free extract can be utilized directly in the reaction mixture as a helping enzyme because of its high specific activity. TmGAPDH in the cell free extract showed a very thick band (around 45 kDa) on the SDS-PAGE, indicating a successful overexpression in *E.coli*. It was also found that TmGAPDH is not oxygen sensitive during the purification process, a significant feature for easy use.

There is a very high activity exhibited in both the cell free extract and purified form of PfTIM. PfTIM's activity increased twenty times after purification (above 400 U/mg), which had much higher specific activities compared to other TIMs reported previously (**Table 4.1**). Arsenate did not inhibit PfTIM's activity when TmGAPDH was used as the auxiliary enzyme in the previous experiment (Chen and Ma, unpublished results). The purified PfTIM had a molecular mass of about 30 kDa observed on the SDS-PAGE. It also showed stability under aerobic conditions during the process of purification, which showed a great feature for easy use.

The molecular weight of PfG3PDH was about 38 kDa showed on the SDS gel. PfG3PDH had little activity in the cell free extract. Its activity was only about 1.5 U/mg after purification, although the recovery of activity was quite high (93.5%). This result indicated that the recombinant PfG3PDH was not a fully active enzyme for being used as a helping enzyme for doing other enzyme assays.

ApG1PDH was not purified in this project. The ApG1PDH gene was ligated with pET11a, and then was overexpressed in *E.coli* strain Rossetta2™ (DE3). The disadvantage of this original design is that the target protein synthesized in *E.coli* did not have a polyhistidine-tag, which made the purification much more difficult because several different columns have to be used for ApG1PDH purification. It also showed low activities both in the cell free extract and in the purified form (Ishikawa *et al.*, 2002). Therefore, it is impractical and labor-intensive to obtain sufficient activity of ApG1PDH. So, it can not be used as a helping enzyme for other enzyme assays.

TmTK was the first and only recombinant transketolase obtained (Eram and Ma,

Table 4.1 Properties of TIM from different hyperthermophiles

Organisms	Cell free extract (U/mg)	Pure enzyme (U/mg)	Assay temperature	Molecular weight (kDa)	References
<i>Pyrococcus woessei</i>	2	3900	70° C	24	Kohlhoff, 1996
<i>Methanothermus fervidus</i>	1	2230	70° C	27.8	Kohlhoff, 1996
<i>Thermotoga neapolitana</i>	0.81	N/A	50° C	73 and 81	Yu, 1995
<i>P. furiousus</i>	22.5	409	80° C	28	This work

N/A: not available

unpublished data). As shown in SDS-PAGE, the molecular weight of TmTK was about 65 kDa, which was also consistent with the calculated molecular weight from the NCBI data base. The activity in the cell free extract was about 5 U/mg. However, the activity in the purified form cannot be determined due to the absence of a key substrate, xylulose-5-phosphate, which is not commercially available at this moment. In principle, TK activity could also be determined using the new assay system developed in this project. However, the recombinant TmXuK functioning as an auxiliary enzyme for TK assay lacked sufficient activity. Therefore, it is critical to obtain an efficient and highly active XuK from other hyperthermophiles for running this TK assay system.

Xylose isomerases (XIs) from different species of microorganisms have been studied for decades since they can enhance the sweetness of syrup (Brown et al., 1993; Eputing, 2004). XIs, mainly from *Thermotoga* species, have been reported in several papers (Brown et al., 1993; Viellie et al., 1995; Bandlish et al., 2002; Eputing, 2004). However, most research focused on the efficiency of conversion between glucose and fructose, not on pentose sugar conversion. The specific activities of published hyperthermophilic XIs are illustrated in **Table 4.2**. TpXI and TmXI had a molecular weight of about 48 kDa was determined by SDS-PAGE comparison to protein standards. TpXI exhibited 1.4 times higher activity than TmXI in the cell free extract so it was purified first for being used as a helping enzyme in other enzyme assays. Its specific activity increased seven times after purification (from 5.05 U/mg in the cell free extract to 36.8 U/mg in the purified enzyme), indicating it was an efficient catalyst for being used as a helping enzyme. More than seventy percent conversion from xylose to xylulose was observed under the assay

Table 4.2 Comparison of specific activities of purified hyperthermophilic XIs

Organism	Substrate	Assay temperature	Specific activity (U/mg)	Reference
<i>T. maritima</i> DSM3109	glucose	90° C	19.5	Brown <i>et al.</i> , 1993
	xylose		68.4	
<i>T. maritima</i>	glucose	80° C	8	Epting, 2004
<i>T. neapolitana</i>			9	
<i>T. maritima</i>	glucose	80° C	8	Bandlish <i>et al.</i> , 2002
<i>T. neapolitana</i> DSM5068			8.5	
<i>T. neapolitana</i> DSM5068	glucose	90° C	22.4	Vieille <i>et al.</i> , 1995
	xylose	98° C	75	
	fructose	98° C	28.5	
<i>T. petrophila</i> RKU-1	xylose	80° C	36.8	This work

conditions after incubation for 30 minutes. TpXI was found to be stable in the air and had high activity after purification. The final product in the reaction was xylulose, which was produced from xylose catalyzed by XI, possesses high market ability if it can be exploited through commercial manufacturing. The ideal result of pentose sugar conversion by TpXI in this work suggests XIs study may be a useful area for further applications.

The XuK-like gene from *T. maritima* was cloned and over expressed in *E.coli* in this study (Abdallah and Ma, unpublished data), which was the first one reported to date. A protein band of a molecular weight of about 63 kDa was shown on SDS-PAGE (**Figure 3.19**). A partially purified protein was obtained by using the current purification method described in section 2.6. However, only very little activity was found in the cell free extract and none occurred in purified protein. There are several possible reasons for the results. First, the XuK-like gene from *T. maritima* might not be the exact gene needed, even though it showed good yield with the overexpression system in the laboratory. Second, perhaps a nonfunctional protein was synthesized by the *E.coli* host organism with pET30a under the inducing condition. Moreover, arsenate for TmGAPDH in the reaction mixture may inhibit TmXuK activity so that very little absorbance change was observed when the assay was performed. Finally, TmXuK might be an oxygen-sensitive protein so it lost all activity after purification under aerobic conditions. However, all the assumptions for this protein need to be verified in the future. More effort is definitely needed for TmXuK in order to improve the functionality of the whole assay system.

Although some recombinant enzymes, such as TmGAPDH and PftIM, demonstrated promising activity in cell free extracts, it was the purified enzymes that would be

preferred in PPP enzymes assay for hyperthermophiles because of the interference caused by turbidity forming when mesophilic proteins were denatured at 80°C. Purified protein will not lead absorbance change by forming turbidity from denatured *E.coli* proteins, a situation that would minimize the interference during the assay process.

4.2 Assays for TAL and TK of hyperthermophiles

No TAL activity has been reported in hyperthermophilic cell free extracts. The only recombinant TAL in hyperthermophiles reported to date was from *M. jannaschii* (Sodeburg and Alver, 2004). TAL assays in this project were conducted successfully with cell free extracts of selected hyperthermophiles. Both methods with different auxiliary enzymes for TAL assays demonstrated the similar characteristics among different hyperthermophilic species. All selected species exhibited TAL activity, but *T. petrophilia* showed the highest TAL activity (0.7U/mg) among them (**Figure 3.15**). The presence of the pentose metabolizing enzymes detected in all the selected hyperthermophiles could be an indication of PPP functioning in these microorganisms.

Interestingly, TAL assays conducted with TmGAPDH showed several times higher activity than those with a combination of PfTIM and PfG3PDH. The sensitivity of auxiliary enzymes added into the TAL assay would be of great importance. Saturated helping enzymes should be added into the assay mixture; otherwise, lower activity would be detected as a consequence. TmGAPDH demonstrated a sufficient activity after purification, but PfG3PDH did not. Thus, TAL activity was under-estimated when PfTIM and PfG3PDH were used. Another fact was that arsenate did not inhibit

hyperthermophilic TAL activity when TmGAPDH was used as the helping enzyme. Furthermore, TmGAPDH demonstrated promising functionality in most cases. Improving G3PDH activity will be an alternative option if TIM and G3PDH are preferred for the TAL assay.

The key chemical, xylulose-5-phosphate for TK assays was no longer available from any manufacturer, so TK activities of other hyperthermophiles could not be detected without this compound. However, the newly developed assay system for the determination of TK activity in hyperthermophiles had been set up (see next section 4.3).

4.3 The new assay system

The feasibility of the new assay system was proved by conducting XuK assays in hyperthermophiles. The whole system (illustrated in section 2.5.1.6) was set up using three purified auxiliary enzymes: TmGAPDH, TpXI, and TmTK or XuK. It not only worked for TK assay, but also for XuK assay. If there would be sufficient activity of TK in the assay system, XuK activity could be detected by using TK as an auxiliary enzyme; on the other hand, if there would be sufficient activity of XuK, TK activity could be determined by using XuK as a helping enzyme in the assay system.

XuK assay in hyperthermophiles was conducted using the newly developed assay system described in section 2.5.1.6. *T. petrophila* demonstrated the highest XuK activity (0.3 U/mg) among these hyperthermophiles. In contrast, no XuK of *T. neapolitana* and *T. hypogea* could be detected. Although there was a XuK-like gene found to be present in

the genome sequence of *Thermotoga* species (NCBI gene database), the assay results showed not all *Thermotoga* species had XuK activity. Therefore, it would be better to clone the genes encoding XuK from *T. petrophila* and *T. maritima* because they had higher XuK activity when the assay was performed using the new system.

Although the feasibility of the new assay system was proved, it is still not fully completed due to the insufficient activity of the recombinant XuK obtained in our laboratory. New recombinant XuK needs to be obtained by cloning the gene(s) from other hyperthermophilic species in order to enhance the function of the assay system, which seemed to be the best one for detecting enzymes involved in pentose metabolism of hyperthermophiles at high temperatures. Moreover, the inexpensive chemicals used in this assay system provide an easy approach for future experimentation.

4.4 Recommendations for future work

There are three major directions for future experiments:

A) The new assay system for the determination of TK activity requires sufficient XuK as a helping enzyme. Different XuK genes from other hyperthermophilic species will be cloned and functionally overexpressed in *E. coli*. The one with the highest XuK activity will be chosen for being used in the TK assay system.

B) In case if none of the newly cloned and overexpressed XuK would have sufficient activity for being a helping enzyme, TK activities in selected hyperthermophiles will be determined using the assay method that requires xylulose-5-phosphate as the substrate,

which will be done only when xylulose-5-phosphate becomes available. The activity of the purified recombinant TmTK can also be determined using this method, before it can be used as a helping enzyme in the assay system for the determination of XuK activity. Sufficient recombinant TmTK will be prepared for such purpose.

C) TAL activities were determined only for those hyperthermophiles grown on hexose (glucose or maltose) in this study. To determine if the TAL activity would be inducible when the pentose sugar (xylose or arabinose) is used as growth substrate for the selected hyperthermophiles, more TAL assays need to be done for comparing the differences in activity for each of the selected organisms grown on various sugars.

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