EFFICIENT HYDROGEN PRODUCTION USING ENZYMES OF THE PENTOSE PHOSPHATE CYCLE

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Abstract

Increase in the production of hydrogen from biomass-derived glucose and attainment of the maximum molar yield of H_2, can be achieved through the enzymes of the pentose phosphate cycle in conjunction with a hyperthermophilic hydrogenase. This process centers on three $NADP^+$ dependent enzymes, glucose-6 phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH) and hydrogenase from *Pyrococcus furiosus*. The dehydrogenases are currently obtained from mesophilic sources. However, in order to increase the rates and sustainability of hydrogen production, work has been carried out to isolate the genes for these enzymes from thermophilic sources. Success has been achieved through the isolation and cloning of both genes from the bacterial species *Thermotoga maritima*.

Individual characterization of both mesophilic enzymes has revealed that the first enzyme G6PDH, has a 20-fold greater turnover number than that of the second enzyme. However, the second enzyme 6PGDH, is far more thermostable, possessing a $t_{50\%}$ of 130 mins at 60°C. The maximum yield of hydrogen from glucose using only the oxidative portion of the pentose phosphate pathway (two moles per mole of glucose 6-phosphate) has been achieved using mesophilic sources of these enzymes in conjunction with a hyperthermophilic hydrogenase at 40°C. Additional monitoring of CO_2 evolution confirmed the stoichiometry of H_2 to CO_2 to be 2:1. Variation of the components and conditions of the system show that 6-PGDH is the rate-limiting step in the pathway and is required in over 20-fold greater amounts to prevent the build up of the intermediate 6-phosphogluconic acid.

Introduction

Significance and Background

The inevitable consumption of all our supplies of fossil fuels requires the development of alternative sources of energy for the future. Introduction of a hydrogen economy will gain greater importance due to the promise of using hydrogen over fossil fuels. These advantages include its limitless abundance and also its ability to burn without generating any toxic by-products, where the only by-product of hydrogen combustion is water.

At present hydrogen is widely used in petroleum refining and chemical processes, metal processing operation, in the electronics industry and more famously as rocket fuel. In addition, it is now gaining more acclaim as an alternative fuel for transportation (Leslie, 1997).

Hydrogen is currently produced by several methods, including steam/methane reforming (Wilson and Newall, 1970), dissociation of ammonia, and by-product streams from chemical manufacturing and petroleum reforming. However, despite all the promise of this potential new fuel, much controversy is being made about these methods of manufacturing hydrogen. This is due to the absence of a practical natural source of hydrogen. Therefore, it must be made by transforming some other energy source at a cost. Even electrolysis of sea water, a vast reserve of hydrogen, would require the use of electricity. Additionally, the manufacture of hydrogen from petroleum and methane eventually leads to the release of carbon dioxide, thus creating a cost to the environment. Consequently this has led to the discovery of hydrogen manufacture from renewable, less costly and non-polluting sources.

Alternative method of hydrogen production

The enzymatic conversion of cellulosic waste to H_2 via an *in vitro* enzymatic pathway (Woodward *et. al*., 1996; Woodward and Orr, 1998; Inoue *et al*., 1999; Woodward *et al*., 2000) involves the conversion of potential glucose sources such as cellulose by cellulases, and plant sap (i.e. sucrose) by invertase and glucose isomerase to glucose. Glucose, the sugar produced by photosynthesis, is also renewable, unlike fossil fuels such as oil. The glucose substrate is then oxidized and the cofactor, $NADP^+$ is simultaneously reduced. The presence of a pyridine-dependent-hydrogenase in this system (Egerer *et al*., 1982; Bryant and Adams, 1989), causes the regeneration and recycling of $NAD(P)^+$ with the concomitant production of molecular hydrogen.

Pentose Phosphate Pathway (PPP)

The pentose phosphate pathway is primarily an anabolic pathway that utilizes the 6 carbons of glucose to generate 5 carbon sugars and reducing equivalents (Fig. 1). However, this pathway does oxidize glucose and under certain conditions can completely oxidize glucose to CO_2 and water. The pentose phosphate pathway has both an oxidative and non-oxidative arm.

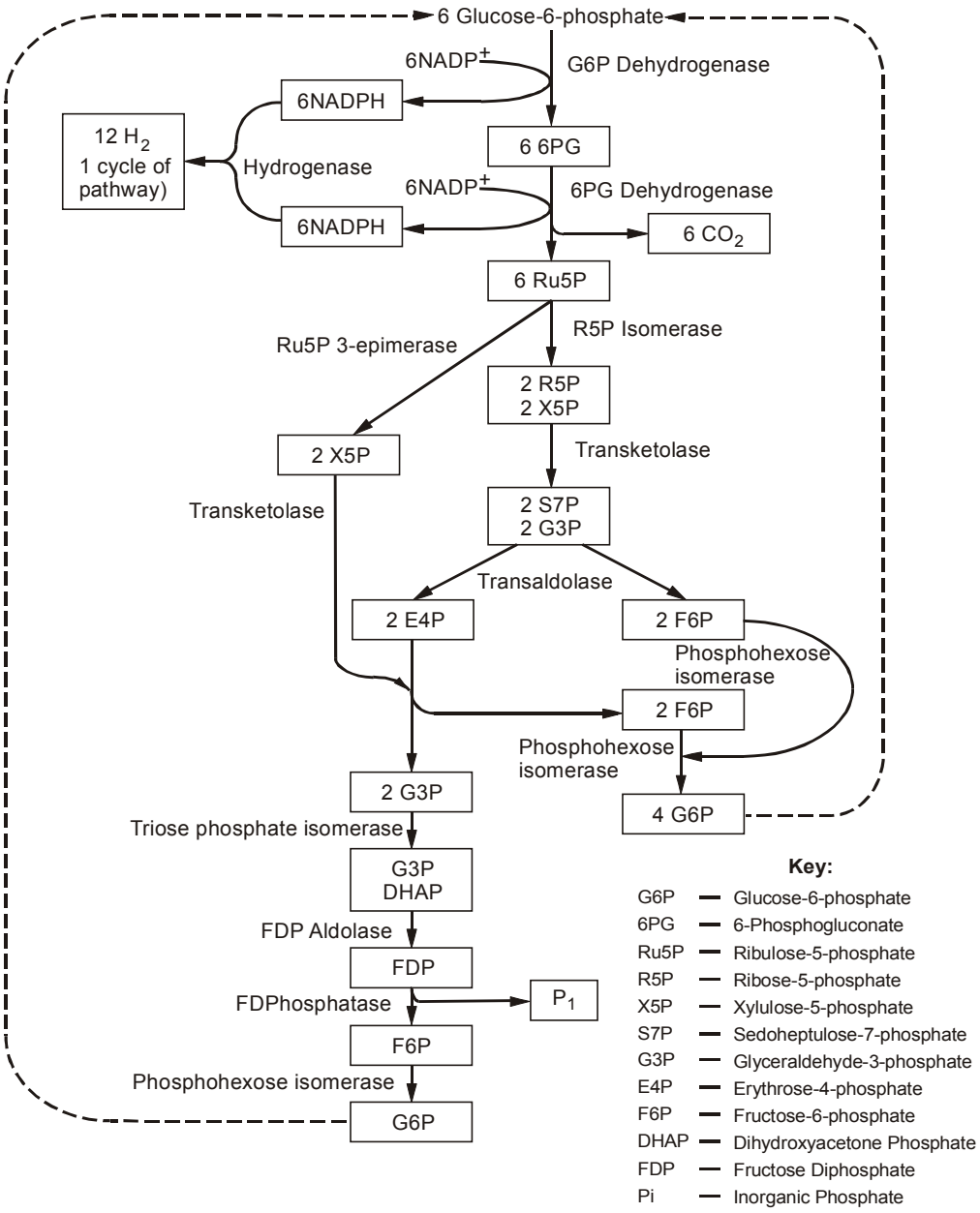

Fig. 1 The pentose phosphate pathway

Oxidative Branch of the Pentose Phosphate Pathway

The oxidation steps, utilizing glucose-6-phosphate (G6P) as the substrate, occur at the beginning of the pathway and are the reactions that generate NADPH. The reactions catalyzed by glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase generate two moles of NADPH for every mole of glucose-6-phosphate (G6P) that enters the PPP. The first step is rate-limiting and is essentially irreversible due to the spontaneous hydrolysis of the intermediate 6-

phosphogluconolactone into 6-phosphogluconic acid. It is regulated by the $NADP^+$/NADPH ratio through substrate competition.

Non-oxidative Branch of the Pentose Phosphate Pathway

The non-oxidative reactions of the PPP are primarily designed to generate ribose-5-phosphate (R5P) for the synthesis of nucleotides and nucleic acids. Equally important reactions of the PPP are the conversion of dietary 5 carbon sugars into both 6- (fructose-6-phosphate) and 3- (glyceraldehyde-3-phosphate) carbon sugars, which can then be utilized by the pathways of glycolysis. The 6-carbon sugars can be recycled into the pathway in the form of G6P, generating more NADPH.

Thermostable Enzymes

With the exclusion of phylogenetic variations, thermostable enzymes are found to be very similar to their mesophilic counterparts in terms of their amino acid sequence (Vielle *et al*., 1995; Burdette *et al*., 1996) and three-dimensional structures and catalytic mechanisms (Fujinaga *et al*., 1993; Russel *et al*., 1997). In addition to enhanced stability at elevated temperatures, thermophilic enzymes also possess a greater resistance to chemical denaturation than their mesophilic counterparts (Lebbink *et al*., 1995).

Three Domains of Life

The evolutionary history of life is provided by sequence comparisons of small subunit rRNA due to its strictly conserved function and its non-transfer between species. Comparison of these sequences proposes a tripartite division of the living world into the following domains, Eukarya, Eubacteria and Archaea (Fig. 2) (Woese and Fox, 1977; Woese *et al*., 1990).

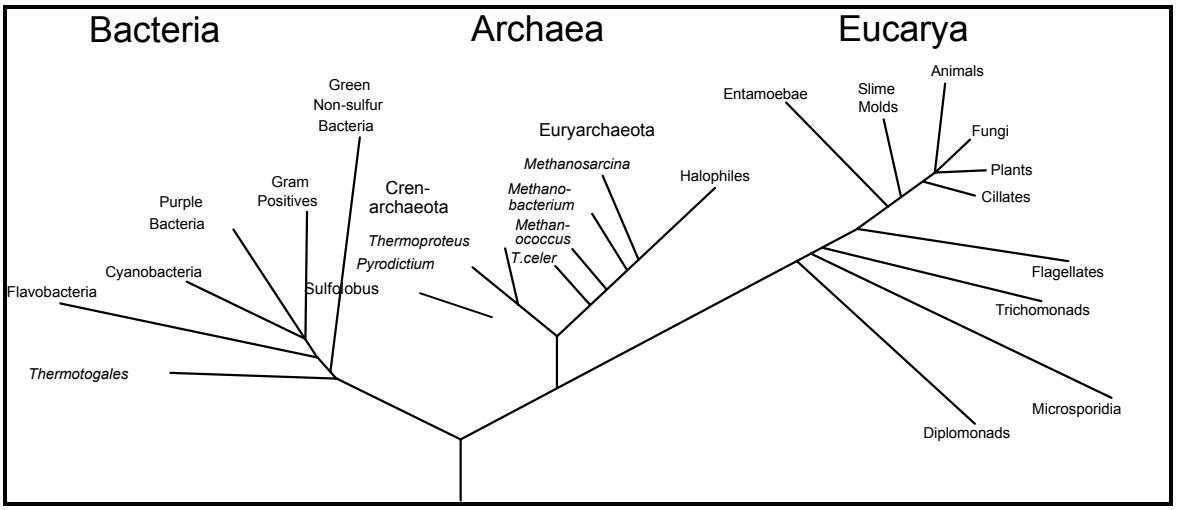

Fig. 2 The Universal phylogenetic tree, constructed from rRNA sequence comparisons, according to Woese *et al*. (1990).

Sulfolobus solfataricus

Sulfolobus solfataricus is an aerobic hyperthermophilic archaebacteria able to grow at 87^{o}C and pH 3.5 (Zillig et al., 1980). *Sulfolobus solfataricus* belongs to the kingdom of Crenarchaeota within the domain of Archaea (Fig. 2). Various strains of *Sulfolobus solfataricus* have been isolated from a number of geographically distant, aquatic and terrestrial solfatara fields (Brock *et al*., 1972; De Rosa *et al*., 1974; De Rosa *et al*., 1975; Zillig *et al*., 1980; Segerer and Stetter, 1991; Zillig *et al*., 1994). It is able to grow on various complex organic substrates, as well as simple sugars and amino acids (Brock et al., 1972; Segerer and Stetter, 1991). Its central metabolism is thought to occur via non-phosphorylated intermediates through a pathway called the non-phosphorylated Entner-Doudoroff (ED) pathway. However, further analysis by Selig *et al*. (1997) revealed that a variety of hyperthermophiles, and particularly the anaerobic archaea, were able to degrade sugar to pyruvate using modified Embden-Meyerhof (EM) and ED pathways or a combination of both.

Thermotoga maritima

Thermotoga maritima is an anaerobic hyperthermophilic eubacterium with an optimum growth temperature of 80^{o}C, that has been isolated from geothermally-heated sea floors in Italy and the Azores (Huber *et al*., 1986). *T. maritima* is able to grow on many simple and complex carbohydrates including glucose, sucrose, starch, cellulose and xylan (Huber *et al*., 1986; Huber *et al*., 1992).

T. maritima type strain, MSB8 was cloned, sequenced and assembled by The Institute for Genomic Research (TIGR) from a culture derived from a single cell provided by R. Huber (Nelson *et al*., 1999). Small subunit ribosomal RNA (SSU rRNA) phylogeny places this bacterium as one of the deepest and most slowly evolving lineages in the Eubacteria (Achenbach-Richter *et al*., 1987). In addition, conservation of gene order between *T. maritima* and the Archaea in many of the clustered regions suggests that lateral gene transfer may have occurred between thermophilic Eubacteria and Archaea. Complete genome sequencing revealed the presence of the enzymes constituting the pentose phosphate pathway. However, initial investigations did not find all the expected enzyme activities for this pathway (Nelson *et al*., 1999). Metabolism of sugar in *T. maritima* is thought to occur via the EM, ED and the pentose phosphate pathways (Nelson *et al*., 1999; Selig *et al*., 1997).

Oxidative pentose phosphate enzymes from T. maritima genome project

The gene coding for G6PDH is located at 1,170,072 – 1,168,555 bp at locus TM1155 and codes for a protein 496 amino acids in length (Fig. 3). At a remote location in the *T. maritima* genome of 459,482 – 460,888 bp, locus TM04308 is the gene coding for 6PGDH. This gene codes for a protein of length 469 aa (Fig. 3).

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  1 MKCSLGLEKC.PDDTLRCFPK.IEQPFGIVIF.GASGDLTKRK.LIPALNRLFE.AGILPERFFV
 61 GAARTKMDD.KKFRSRFDAN.PDFLEHCSYI.SVDYQDPESF.KQLKNTIETL.IKRIDSSNLV
121 FYLAVPPDLY IPILENLSKT GLNEKPARVV IEKPFGKDLE SARRLEDTLQ KYFQEDQIFR
181 IDHYLGKETV QNILVFRFAN FIFEEIWNNK FVDHVQITMA EDIGVEHRAG YFENVGLLRD
241 IFQNHMLQIL ALIAMEPPSS FNGENFRNER VKLLRSIRPF PVEELESWIV RGQYGRGVVN
301 GKEVPAYREE PGVAKDSNVE TFVAMKLFID NWRWSGVPFY LRSGKRLPKK ITEVAVVFKK
361 IPHSIFAGVP SDELEPNTIV FTLQPNEGIS LEFQVKRPCP GMFPQLLSMD FRYEDYFGVK
421 LPDAYERLLL DVILGDPTLF MRRDDLEVSW ELLDPVLKAW ENDPVRFSPY VYPAGTWGPR
481 EADLLIERDG RKWRKL

  1 MKSHIGLIGL AVMGQNLALN IARKGYKVSV YNRTAQRTEE FVKNRVTNEE IEPHYDIESF
 61 VKSLERPRKI ILMVKAGKPV DDTISQLLPH LEPGDLIIDG GNSHYMDTER RFKELSEKGI
121 LFLGMGVSGG EYGALHGPSL MPGGSREAYN LVEEILLEIA AKTEDGPCCT YVGERSAGHF
181 VKMVHNGIEY AIMQAIAEVY HIMRDVLSLS SEEMSSIFEE WNRGELSSFL VEITYKILRK
241 KDEETGKPMV DVILDKAEQK GTGKWTSQAA LDLGIPTPSI NLAVVERVIS HFKDIRTRLS
301 KLYNKRRSAT QGSEEFLRDL RNSLFFAMFM AFSQGMWLIA EASKEFGYGV SLSEVLRIWK
361 GGCIIRAKLI DTLRRYISNE NAYLLENEEV MNLLKGKIDS LKNILKASIE NEIPVPVLSS
421 SYNYFMSLTE ERLPANLIQA QRDFFGAHTF ERVDREGVFH INWEEGEIG
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Fig. 3. Amino acid sequences of G6PDH and 6PGDH from *T. martima*, respectively.

Aim

The overall aim is to increase the production of hydrogen from biomass-derived glucose and achieve the maximum molar yield of H_2 by employing the enzymes of the pentose phosphate pathway in conjunction with the hydrogenase from *Pyrococcus furiosus*. This will also require the future development of an immobilized enzyme bioreactor for efficient hydrogen production at high theoretical yields. If this could be achieved practically, this would represent a major innovation that would advance our abilities to develop an efficient and practical system for bio-hydrogen production. The main advantage over hydrogen production by fermentation is that close-to-theoretical yields of hydrogen from sugar would be possible.

The initial objective is to characterize the mesophilic enzymes of the oxidative branch of the pentose phosphate pathway, with a view to identifying the rate-limiting steps and optimizing the system. This process centers on three $NADP^+$ dependent enzymes, glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH) and hydrogenase (Fig. 4). The dehydrogenases are currently obtained from commercial mesophilic sources, from *Leuconostoc mesenteroides* and Torula yeast, respectively. However, in order to increase the rate of hydrogen production, it is necessary to isolate the genes coding for G6PDH and 6PGDH from *Thermotoga maritima*. The final objective is to express these genes as recombinant enzymes in a mesophilic host and incorporate them into an entirely hyperthermophilic *in vitro* enzymatic H_2 production system.

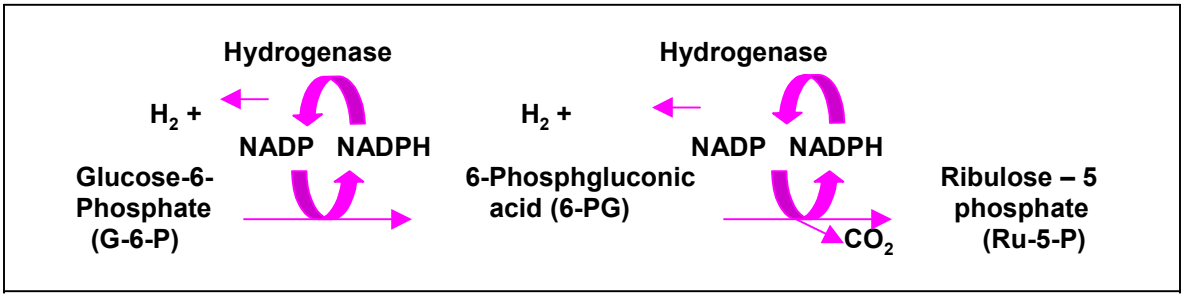


Fig. 4 *In vitro* enzymatic pathway to produce molecular hydrogen

Results

Characterization of the Mesophilic Oxidative Branch of the Pentose Phosphate Pathway

Characterization Studies

Temperature and pH optima

The optimal temperature and pH for the G6PDH from *Leuconostoc mesenteroides* are 30°C and pH 7.8, and for 6PGDH from Torula yeast are pH 7.6 and 50°C. The optimal pH of the two-enzyme pathway was also determined to be approximately pH 8 (Fig.5).

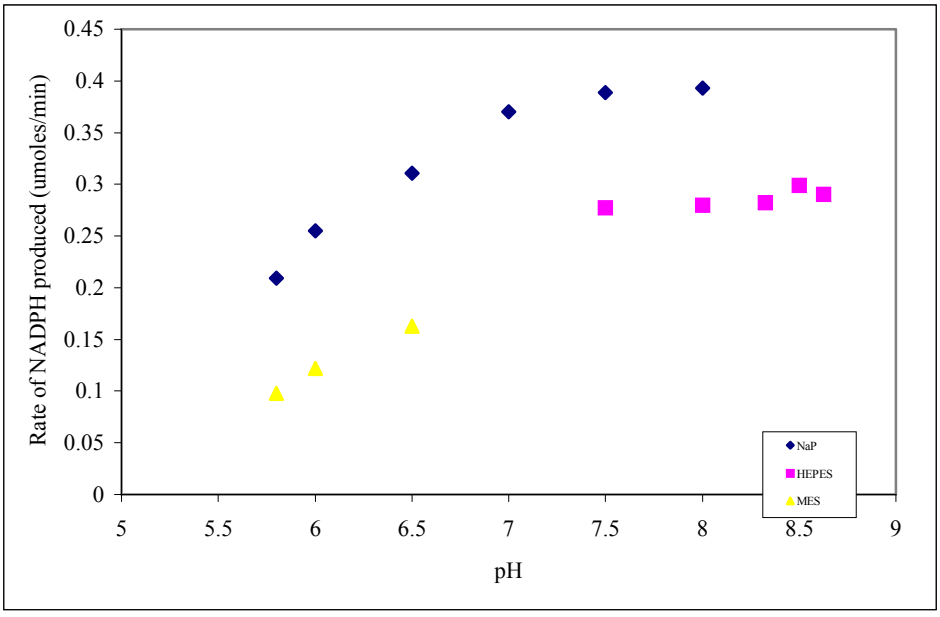


Figure 5. The effect of pH on the activity of the mesophilic oxidative branch of the pentose phosphate pathway.

Thermal inactivation

Thermal inactivation studies of the mesophilic enzymes (Fig. 6) have revealed that G6PDH and 6PGDH posses $t_{50\%}$ at 60°C of 2.88 min and 126.8 min, respectively. Co-incubation of both of the enzymes at a final concentration of 1 mg/mL reveals that the higher protein concentration of the 6PGDH enzyme has not influenced its thermal stability in comparison to the G6PDH enzyme.

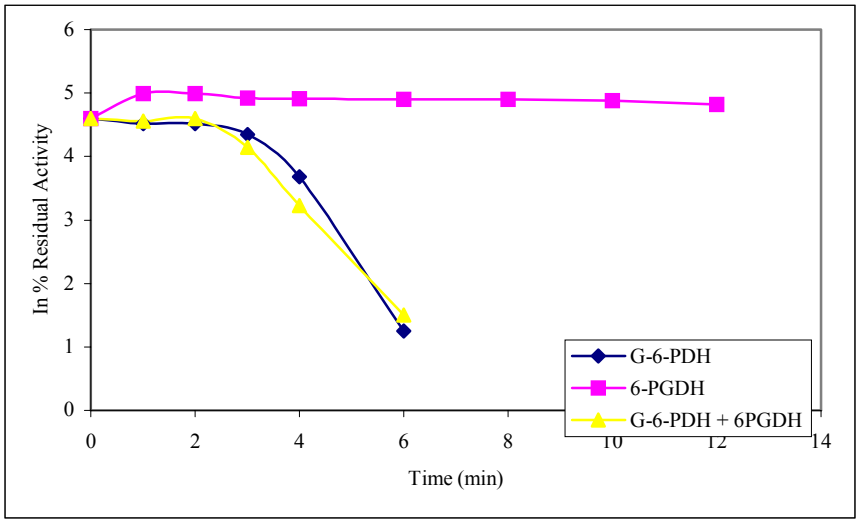


Figure 6. Thermal inactivation studies of 6PGDH and G6PDH at 60°C. Protein concentrations of incubation reactions were the following: 6PGDH at 2 mg/mL, G6PDH at 0.045mg/mL and mixture at 1 mg/mL.

Kinetic parameters

The two individual enzymes were characterized to determine their kinetic parameters (Table 1).

Table 1. Kinetic data for mesophilic pentose phosphate enzyme

Glucose 6-phosphate dehydrogenase enzyme from *Leuconstoc mesenteroides*			
Substrate	Km (mM)	Vmax (µmoles/min)	Turnover number (min^{-1})
glucose 6-phosphate	1.15	0.415	4×10^4
$NADP^+$	0.060	0.35	
6-Phosphogluconate dehydrogenase Type IV from Torula Yeast			
Substrate	Km (mM)	Vmax (µmoles/min)	Turnover number (min^{-1})
6-phosphogluconic acid	1.65	0.22	1.9×10^3
$NADP^+$	0.12	0.23	

Product inhibition

6-Phosphogluconic acid inhibition assays These were carried out at 40°C in 0.2 M sodium phosphate buffer, pH 7.5 using 0.1U of G6PDH, 1 mM of $NADP^+$ and 5 mM of Glucose-6-phosphate. No inhibition was observed at 10 mM concentrations of 6-phosphogluconic acid.

Ribulose-5-phosphate inhibition assays These were carried out at 40°C in 0.2 M sodium phosphate buffer, pH 7.5 using 0.1U of each enzyme, 1 mM of $NADP^+$ and 5 mM of Glucose-6-phosphate and 6-phosphogluonic acid, respectively (Table 2).

Table 2. Effect of Ru5P concentration on G6PDH and 6PGDH activity

Concentration of Ribulose-5-Phosphate (mM)	0	2.5	10
Glucose-6-phosphate dehydrogenase activity (Units)	2.02	2.1	1.754
6-Phosphogluconate dehydrogenase activity (Units)	0.41	0.39	0.28

In Vitro Hydrogen Production Studies

A continuous flow system was constructed to measure hydrogen and carbon dioxide production based on the system as shown and described in Woodward *et al*. (1996) and Greenbaum, (1984). A modification was made with the inclusion of a CO_2 analyzer in-line prior to the H_2 sensor. The maximum yield of hydrogen from glucose using only the oxidative portion of the pentose phosphate pathway (two moles per mole of glucose 6-phosphate) has been achieved using mesophilic sources of these enzymes at 1 unit concentrations in conjunction with 68 units of hyperthermophilic hydrogenase, at 40°C with 2.5 mM initial glucose 6-phosphate concentration.

Effect of pH

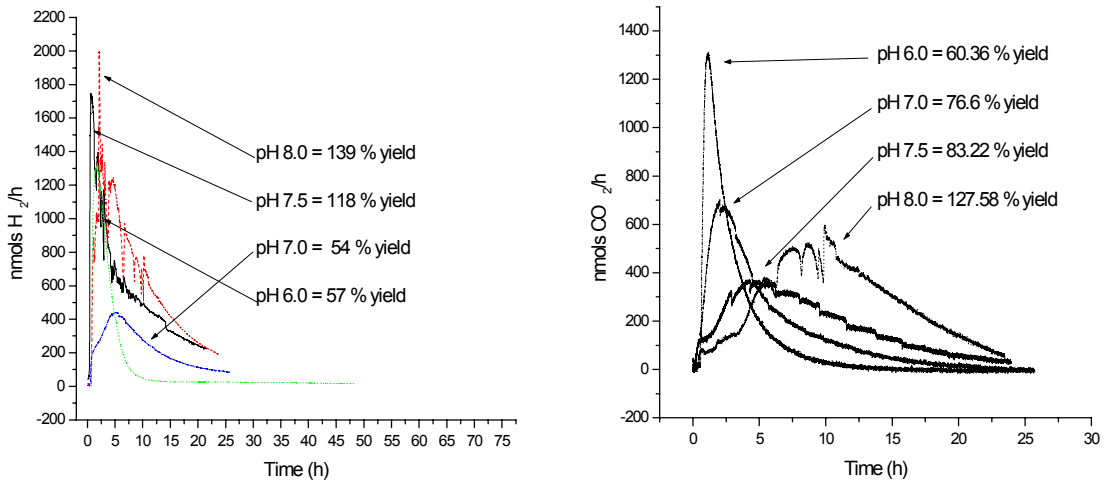


Fig. 7. Effect of pH on the rate and yield of H_2 and CO_2 production. Using 2.5 mM G6P initial concentration, 2.0 mM $NADP^+$, 1 Unit of each PPP enzyme, 68U Hydrogenase in 0.2 M Sodium phosphate buffer at 40°C.

Subjecting the complete hydrogen system to pH values of pH 7.5 and 8.0 have also resulted in the maximal percentage yields of H_2 and CO_2 from 2.5 mM G6P.

Effect of Temperature

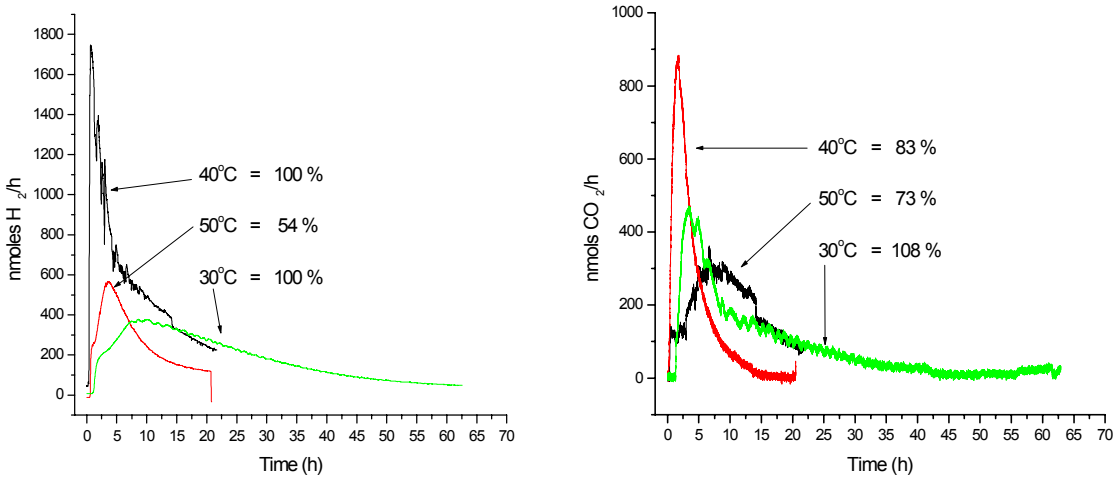


Fig. 8. Effect of temperature on the rate and yield of H_2 and CO_2 production. Using 2.5 mM G6P initial concentration, 2.0 mM $NADP^+$ 1 Unit of each PPP enzyme, 68 U Hydrogenase in 0.2 M Sodium phosphate buffer pH 7.5

Maximal theoretical yields of H_2 and CO_2 were achieved at 30 and 40°C. However, increasing the temperature to 50°C led to the decrease in the yields of these two gases.

Effect of NADPH Concentration

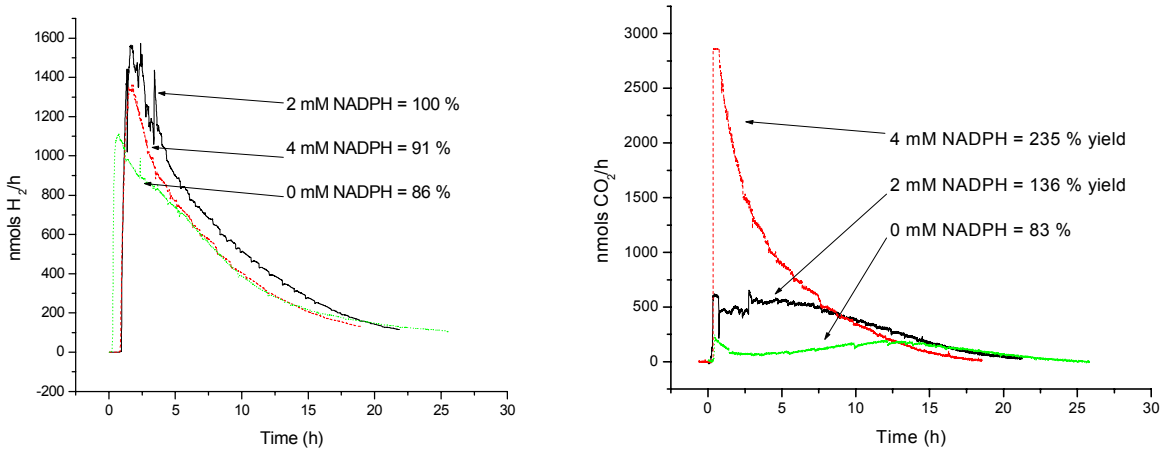


Fig. 9. Effect of NADPH on the rate and yield of H_2 and CO_2 production. Using 2.5 mM G6P initial concentration, 1 Unit of each PPP enzyme, 68 U Hydrogenase in 0.2 M Sodium phosphate buffer, pH 7.5 at 40°C

Supplementing the reaction with additional reduced cofactor led to the expected increase in H_2 and CO_2 production.

Adding increasing amounts of 6PGa from 0 – 10 mM, to the reaction system resulted in the reduction of H_2 and CO_2 that can be obtained from G6P at an initial concentration of 2.5 mM.

Effect of 6-Phosphogluconic Acid Concentration

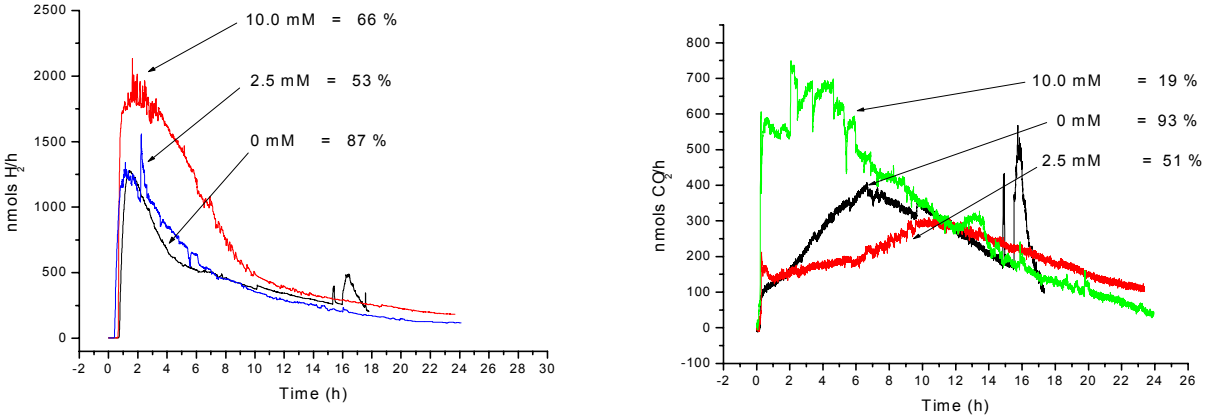


Fig.10. Effect of 6-phosphogluconic acid on rate & yield of H_2 and CO_2 production. Using 2.5 mM G6P initial concentration, 2.0 mM $NADP^+$ 1 Unit of each PPP enzyme, 68 U Hydrogenase in 0.2 M Sodium phosphate buffer, pH 7.5 at 40°C.

A 20-fold increase in the concentration of 6PGDH has brought about the increase in yields of H_2 and CO_2 from an initial concentration of 10 mM G6P.

Effect of Ratio of the Two Pentose Phosphate Enzymes

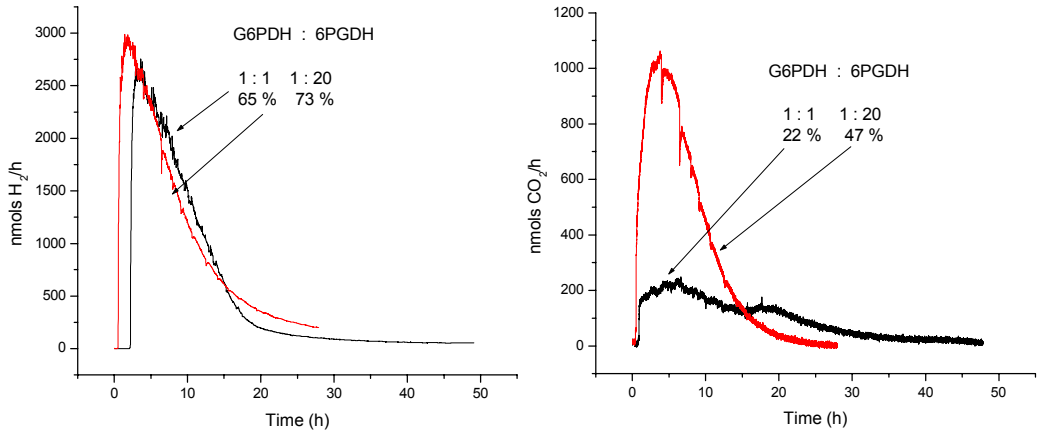


Fig. 11. Effect of enzyme ratio on the rate and yield of H_2 and CO_2 production. Using 10.0 mM G6P initial concentration, 2 mM $NADP^+$, 68 U Hydrogenase in 0.2 M Sodium phosphate buffer, pH 7.5 at 40°C.

Isolation of a Thermophilic Oxidative Branch of the Pentose Phosphate Pathway

Approach with Sulfolobus solfataricus

Growth of Sulfolobus solfataricus

The hyperthermophilic counterpart of this system was being established using enzymes isolated from the Archaeon *Sulfolobus solfataricus*. The culture was grown in ATCC medium #1304 at 70°C for approximately 2 days. Cultures were grown as 5 mL cultures statically in test tubes or as 500 mL cultures in specifically designed vessels that allow continuous aeration (Fig. 12). After this time the cells were harvested and cell pellets were stored at –20°C. Genomic DNA was isolated according to "Current Protocols in Molecular Biology" (1990).

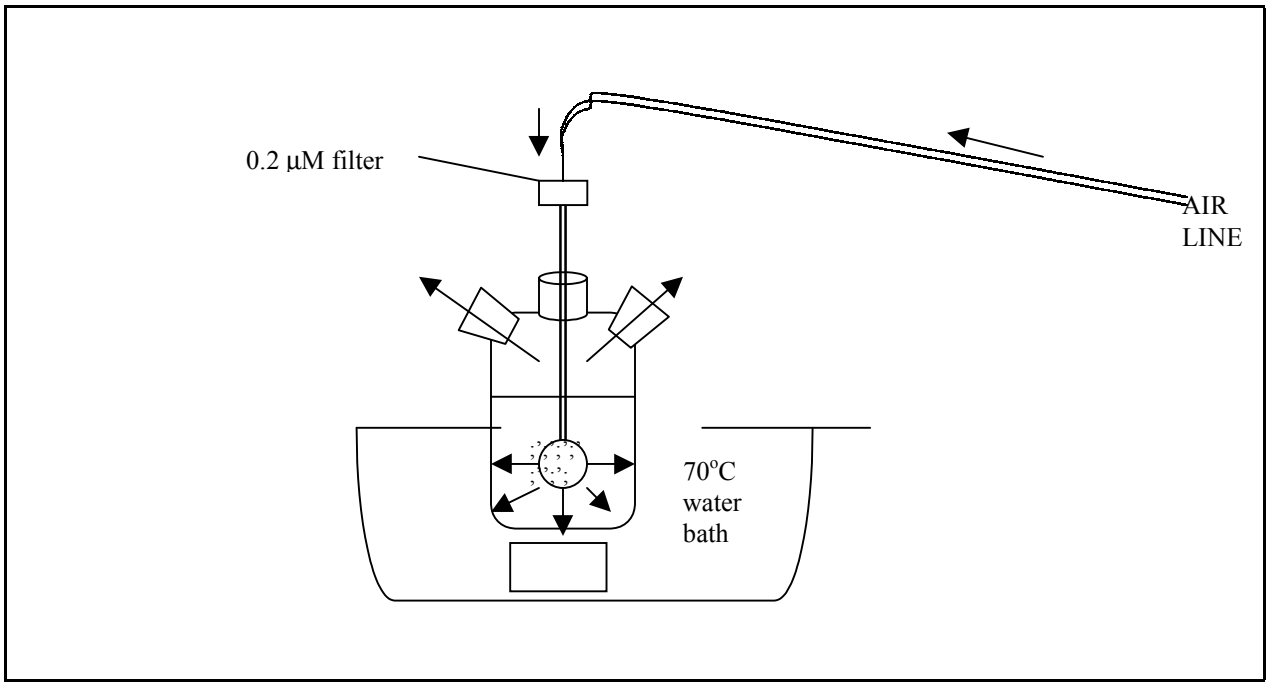


Fig. 12. Diagrammatic depiction of vessel used for large scale culturing of *S. solfataricus*.

Large Scale Growth of Sulfolobus solfataricus culture 500 mL of aerated cultures were grown in medium A - standard ATCC Medium (# 1304) with 0.1% Casamino acids and 0.2% yeast extract as the carbon sources - and in medium B, with the addition of 0.5% Glucose and 0.5% Casamino acids and 1.0% yeast extract (Table 3).

Cell breakage Cell pellets were lysed in detergent (0.1% Triton-X100), lysozyme and DNase at 37°C for 60 min

Cell Activity Assays These were carried out with lysed cell extracts at 70°C in sodium phosphate buffer, pH 7.5 with 1 mM $NADP^+$, 10 mM glucose, 1 mM glucose-6-phosphate and 2.5 mM 6-

phosphogluconic acid. The enzyme activities of glucose dehydrogenase, G6PDH and 6PGDH were determined, respectively (Table 4).

Table 3. *S. solfataricus* cell culture growth measured as absorbance at 600 nm

Time (hr)	Cell density – measured as absorbance at OD_{600}	
	Standard medium	Enhanced medium
0	0	0
7.5	0.035	0.009
8.5	0.03	0.008
24	0.19	0.15

Table 4. Determination of enzyme activities from *S. sulfolobus* cell extracts grown in standard and enhance media.

Enzyme activity	Medium type	Total Activity in 5 mL (Units)	Specific activity (U/mg of protein)	Specific activity (U/g of cells)
Glucose dehydrogenase	Standard	2.9	0.13	5.18
Glucose dehydrogenase	Enhanced	2.1	0.07	1.52
G6PDH	Standard	N.O.	-	-
G6PDH	Enhanced	0.4	0.013	0.29
6PGDH	Standard	N.O.	-	-
6PGDH	Enhanced	0.4	0.013	0.29

N.O. = no activity observed

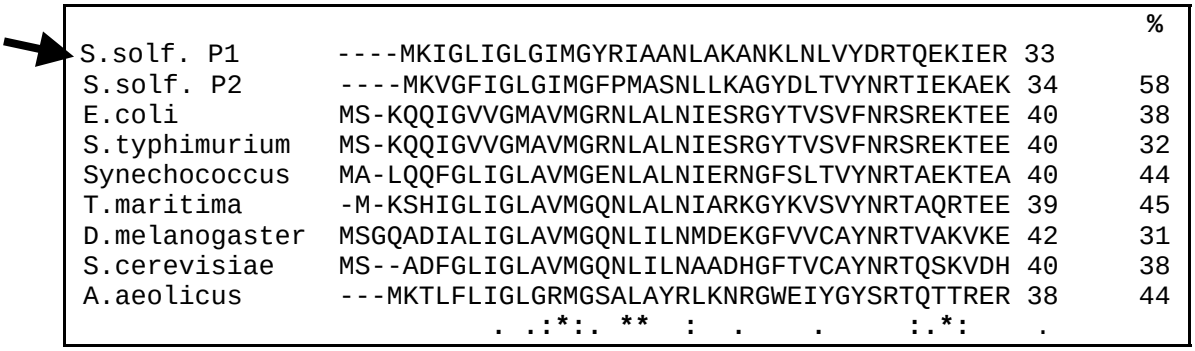
Semi-purification of 6PGDH

Purification of the 6PGDH from *S. solfataricus* cell pastes revealed a protein with approximate molecular weight of 48 kDa. The following N-terminal sequence was obtained for this protein – MKIGLIGLGIMGYRIAANLAKANKLNLVYDRTQE. This sequence has been found to align with a number of 6PGDHs from other species as shown in Fig. 13, compiled according to Thompson *et al*., (1994). This alignment also includes a putative 6PGDH from an alternative strain of *S. solfataricus*, P2, obtained from the P2 genome project (http://www.cbr.nrc.ca/magpie/sulfolobus/sulfolobus.html).

First PCR Approach

The first requirement was to create a suitably sized gene fragment to act as a probe for the 6PGDH gene in hybridization experiments with the *S. solfataricus* genome. An alignment of a variety of 6PGDHs from Eukarya and Eubacteria revealed a highly conserved region at approximately 175-200bp (Fig. 14). Therefore, on assuming the exact conservation of this

region in the 6PGDH from *S. solfataricus*, degenerate primers were designed with in the N-terminus and the highly conserved region that was expected to generate a PCR product of 125-175 bp in length. The PCR reaction was optimized in terms of template DNA concentration, primer concentration, $MgCl_2$ concentration and annealing temperature. Several PCR products were obtained at 3 mM $MgCl_2$ and 40^{o}C annealing temperature. However, subsequent cloning, sequencing and database searching of those products revealed that they only gave matches with catalase and a SocE regulatory protein. Another alignment of 6PGDHs including the recently released putative 6PGDH sequence from *S. solfataricus* strain P2 (http://www.cbr.nrc.ca/magpie/sulfolobus/sulfolobus.html), revealed that the putative 6PGDH from *S. solfataricus* strain P2 does not possess the highly conserved region possessed by all the other enzymes (Fig. 14). Thus, this indicates that if the P2 sequence is in fact a 6PGDH then the highly conserved may also be absent from the 6PGDH present in the P1 strain being used in this work.



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                                                                        %
S.solf. P1      ----MKIGLIGLGIMGYRIAANLAKANKLNLVYDRTQEKIER 33
S.solf. P2      ----MKVGFIGLGIMGFPMASNLLKAGYDLTVYNRTIEKAEK 34      58
E.coli          MS-KQQIGVVGMAVMGRNLALNIESRGYTVSVFNRSREKTEE 40      38
S.typhimurium   MS-KQQIGVVGMAVMGRNLALNIESRGYTVSVFNRSREKTEE 40      32
Synechococcus   MA-LQQFGLIGLAVMGENLALNIERNGFSLTVYNRTAEKTEA 40      44
T.maritima      -M-KSHIGLIGLAVMGQNLALNIARKGYKVSVYNRTAQRTEE 39      45
D.melanogaster  MSGQADIALIGLAVMGQNLILNMDEKGFVVCAYNRTVAKVKE 42      31
S.cerevisiae    MS--ADFGLIGLAVMGQNLILNAADHGFTVCAYNRTQSKVDH 40      38
A.aeolicus      ---MKTLFLIGLGRMGSALAYRLKNRGWEIYGYSRTQTTRER 38      44
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Fig. 13. N-terminal Alignment of 6-Phosphogluconate Dehydrogenases From All Three Domains of Life. Arrow indicates the N-terminal sequence of 6PGDH from *S. solfataricus*. Amino acid length and percentage identities to P1 N-terminal sequence are shown.

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E.coli            175  ADGAGHYV**KMVHNG**IEYGDMQLIAEA  200
S.typhimurium     174  ADGAGHYV**KMVHNG**IEYGDMQLIAEA  199
Synechococcus     175  PGGSGHYV**KMVHNG**IEYGDMQLIAEA  200
D.melanogaster    172  DGGAGHFV**KMVHNG**IEYGDMQLICEA  197
Homo sapien       176  DEGAGHFV**KMVHNG**IEYGDMQLICEA  201
S.cerevisiae      174  PAGAGHYV**KMVHNG**IEYGDMQLICEA  199
T.maritima        174  ERSAGHFV**KMVHNG**IEYAIMQAIAEV  199
A.aeolicus        164  SSGAGHFA**KMVHNG**IEYGIMEAIAEG  189
S. solf. P2       162  DVGSGQALKLCNQVVVALNMVSVVEG  187
                         .:*:  *: :: :    *  : *
```

Fig. 14. Alignment of 6PGDH "conserved region" From All Three Domains of Life

Second PCR Approach

Degenerate primers were designed immediately within the N-terminal sequence. A PCR fragment of expected approximate length of 100bp from genomic DNA was generated. This was intended for use as a probe in hybridization experiments. The tandem positioning of the 6PGDH

and the G6PDH genes in the genome of the extremophilic and ancient eubacteria *Aquifex aeolicus* (Deckert *et al*., 1998), it is expected that sequencing of the hybridized clone may reveal a glucose 6-phosphate dehydrogenase in its flanking regions. However, further searches of the DNA databases revealed the recent release of the sequence previously deemed to be the 6PGDH from *S. solfataricus* P2. However, it was now assigned the function of a 3-hydroxyisobutyrate dehydrogenase. Another alignment of the P1 N-terminal sequence with several 3-hydroxyisobutyrate dehydrogenases was compiled to compare the relative sequence identities (Fig. 15).

```
                                                                    %
  S.solf.P1        -MK-IGLIGLGIMGYRIAANLAKANKLNLVYDRTQEKIER
38S.solf.P2        -MK-VGFIGLGIMGFPMASNLLKAGYDLTVYNRTIEKAEK 38
57E.coli           -MK-VGFIGLGIMGKPMSKNLLKAGYSLVVADRNPEAI—36
52L.lactis         MSK-IAFIGTGVMGAAMAGHLMDAGHDLIVYNRTKSKTD- 38
 41B.subtilis    MKKTIGFIGLGVMGKSMASHILNDGHPVLVYTRTKEKAE- 39   48      * :.:**
                          *:**  :: :: . .    *  *. .
```

Fig. 15. N-terminal alignment of 3-hydroxyisobutyrate dehydrogenases from all three domains of life. Amino acid length and percentage identities to N-terminal sequence are shown.

Approach with Thermotoga maritima

100 mL cultures were grown in standard ATCC medium (#2114) under 100% N_2 atmosphere for approximately 2 days at 70°C. After this period the cells were harvested and stored as cell pellets at –20°C. Genomic DNA isolation from *T. maritima* was carried out according to "Current Protocols in Molecular Biology" (1990)

Design of PCR Primers

PCR is employed to amplify the gene fragments from genomic DNA (Fig.16). Each set of primers allowed for the incorporation of the ribosome binding sites (Shine-Delgarno sequences) (Shine and Delgarno, 1974, 1975), which may aid future expression work (Table 4).

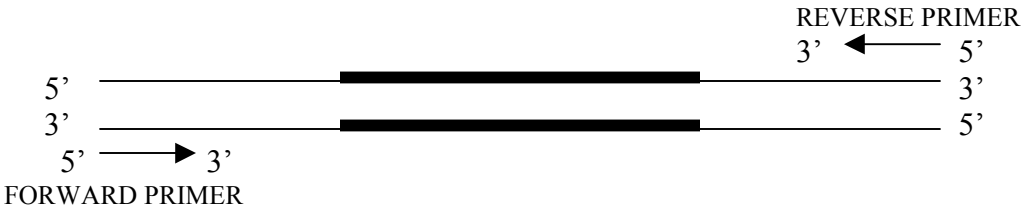

Fig. 16. Mechanism of PCR amplification

Table 4. Primer sets used to generate amplified genes of 6PGDH and G6PDH. Shine-Delgarno sequences are shown underlined.

Name	Sequence	Length (bp)	Melting temp. (Tm)
6PGDH-F	5' GCT TTA CAT CGA AGG TAA AGC TCT 3'	24	59.3
6PGDH-RA	5' GTA GAC CTT CGT TCT CAC GAG G 3'	22	62.1
G6PDH-F	5' CAA CGT ACC CAT CTT CGA GCA GG 3'	23	64.2
G6PDH-RA	5' GGC AGG TGA GAT CAT GAA GTG CAG TC 3'	26	66.4

PCR from T. maritima

Carried out using "hot-start" method to prevent non-specific binding, with 600 ng (template) gDNA, 1μM of primers, 1.5 - 2.5 mM $MgCl_2$ and at an annealing temperature of 55^{o}C, 1.5 Kb PCR fragments were obtained for both reactions, which is the desired size of each gene product.

Cloning & Transformation

The generated PCR fragments were directly ligated to the cloning vector pCR2.1 using standard TA cloning sites. The recombinant vectors were subsequently transformed into *Eschericia coli* strain TOP10F' (non-expression host). Colonies were selected by ampicillin resistance and blue/white screening (successful insertion into the multiple cloning site of pCR2.1 causes the interruption of the lacZ gene coding for the α peptide of β-galactosidase and results in the failure to convert substrates such as X-Gal to a colored product and produce blue colonies). Plasmid mini preps were employed to extract the recombinant plasmid DNA from selected cultures. Restriction digests were then carried out to excise the 1.5 Kb gene inserts using restriction enzyme EcoRI. This would confirm the successfulness of the cloning procedure. Both sets of vectors contained the 1.5 Kb inserts.

Sequencing

Partial sequence of the gene coding for G6PDH was obtained (Fig. 17). This sequence included the downstream primer and 1/3 of the gene sequence in the reverse orientation. Partial sequence was also obtained of the gene coding for 6PGDH (Fig. 18). This sequence included the upstream primer and 1/3 of the gene sequence in the correct orientation.

Confirmatory restriction digest

To confirm the initial restriction digest and sequence data, both gene-containing vectors were digested with EcoRI and HindIII to produce fragments of specific sizes (Table 5).

All the expected fragments were obtained on digestion, thus confirming the presence of the gene inserts.

Fig. 17. Partial gene sequence of 6PGDH in vector pCR2.1. Sequenced using T7 promoter primer. EcoRI site is underlined. Forward PCR primer (6PGDH-F) is in bold and gene start codon is underlined and in bold.

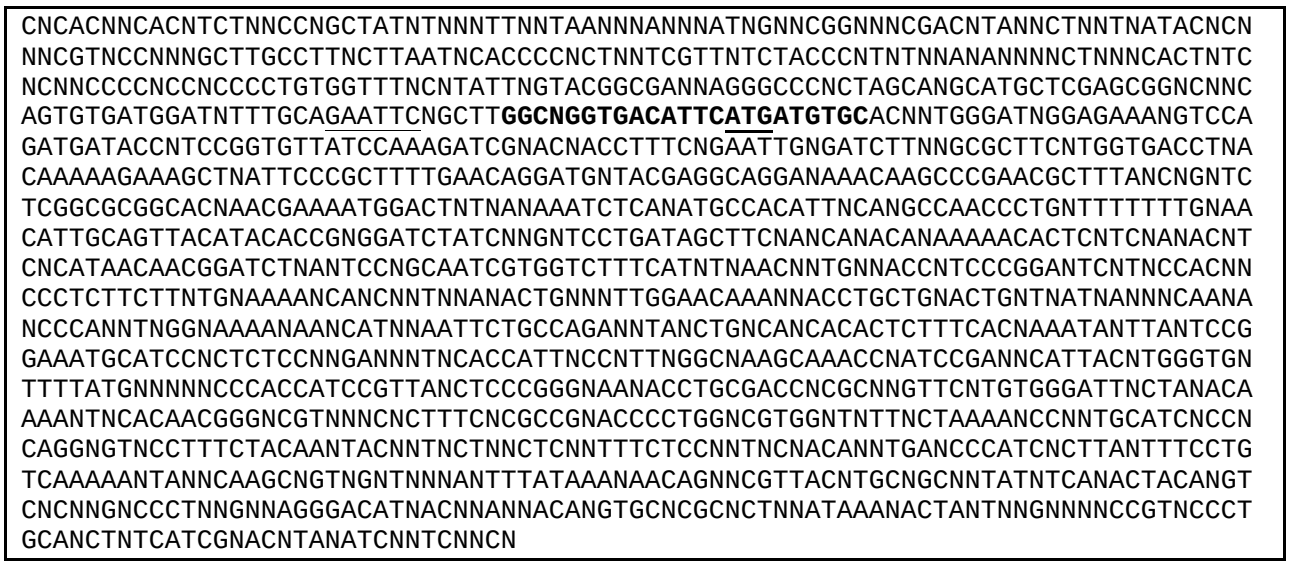

Fig. 18. Partial gene sequence of G6PDH in vector pCR2.1 in reverse orientation. Sequenced using M13 reverse primer. EcoRI site is underlined. Forward PCR primer (6PGDH-F) is in bold and gene start codon is underlined and in bold.

Table 5. Restriction digestion of cloned PCR gene fragments to confirm the inclusion of the complete fragments.

Recombinant vector	Expected fragment sizes
pCR2.1 + G-6-PDH	3.9, 824 and 683 bp
pCR2.1 + 6-PGDH	3.9, 1231, 184 bp

Growth of non-expression host

Cultures of *E. coli* strain TOP 10F' transformed with the recombinant vector pCR2.1 containing their respective PPP gene, were grown. The inclusion of ribosome binding sites upstream of the gene inserts would allow for expression to take place. 250 mL cultures were grown in Luria broth media with 100μg/mL ampicillin at 37°C until they reached a cell density of OD_{600} = 0.5. 0.4 mM of the inducing agent IPTG was then added and the cells were grown for a further 20 hours. Cell breakage was carried out with detergent 0.1% Triton X-100, DNase and lysozyme and followed by a 25-min heat step at 80°C. The resulting cell extracts were then analysed for PPP enzyme activity (Table 6).

Table 6. PPP enzyme activities from non-expression transformed *E. coli* cultures.

Sample	Weight of cells (g)	Volume (mL)	Total Activity (U)	Total Protein (mg)	Specific Activity	
					Per mg of protein	Per g of cells
G6PDH	0.9	12	2.4	48	0.05	2.6
6PGDH	0.7	12	4.8	72	0.067	6.86

As can be seen from the level of enzymic activity for G6PDH and 6PGDH, the expression levels are very low and will require further cloning into an expression vector.

PCR to introduce restriction sites for cloning into an expression vector

Primers were designed to introduce suitable restriction sites on either side of the PPP genes to facilitate their cloning in the correct orientation into the cloning vector pET-15b (Table 7). The genes were initially analyzed using a restriction enzyme analysis program, (http://darwin.bio.geneseo.edu/~yin/WebGene/RE.html) to determine all the restriction sites that do not cut within the gene sequence.

Restriction digestion of PPP genes and pET-15b.

Following PCR, the completed reactions were precipitated with ethanol in order to denature the DNA polymerase. The reactions were then resuspended in 100 μL of nanopure water. Restriction digestion of the PCR fragments and also the expression vector pET-15b were then carried out using the respective restriction enzymes at 37°C for 4 – 16 h. All the digested fragments were

separated by agarose gel electrophoresis in order to prevent re-ligation in subsequent steps. All the restricted fragments were purified to remove the agarose.

Table 7. Primers including the site mutations used to incorporate the desired restrictions sites.

	Restriction site	Restriction Site Sequence	Primer sequence
G6PDH Upstream	Nde I	CATATG	5' GGC AGG TGA GCA TAT GAA GTG CAG TC 3'
G6PDH Downstream	Bam H I	GGATCC	5' CGA CTA CAA AAT CAA GGA TCC CAT C 3'
6PGDH Upstream	Nco I	CCATGG	5 ' GGT GAC GCC CAT GGA ATC TC 3'
6PGDH Downstream	Bam H I	GGATCC	5' CTC ACG GGA TCC ACT TCG 3'

Ligation and transformation of PPP genes and pET-15b

The purified PCR and vector fragments were ligated together. The resulting recombinant vectors were transformed into *E.coli* strain JM109. Colonies were selected by ampicillin resistance. No colonies were obtained for the 6PGDH gene insert indicating the failure of the ligation reaction. Plasmid mini preps were employed to extract the recombinant plasmid DNA from cultures grown from single selected colonies. Restriction digests were then carried out to excise the 1.5 Kb gene inserts using restriction enzymes indicated in Table 9. This would confirm the success of the cloning procedure. However, none of the plasmids from the selected colonies appear to posses the 1.5 Kb gene inserts.

Discussion

Characterization of the Mesophilic Oxidative Branch of the Pentose Phosphate Pathway

Assay characterization

The rate of turnover of the final products, H_2 and CO_2, shows that 6PGDH is the rate-limiting step in the pathway. In terms of the life-time of the mesophilic pathway, the first enzyme G6PDH is rate-limiting and posses a $t_{50\%}$ of only 3 min at 60^{o}C. However, the $t_{50\%}$ possessed by both of the mesophilic enzymes is far inferior to that of the hydrogenase from *P. furiosus*, which has a $t_{50\%}$ of 21 h at 80^{o}C (Bryant and Adams, 1989). The pH optima of the mesophilic pathway enzymes are in agreement with that of the hydrogenase and the components of the H_2 production system.

In vitro hydrogen production studies

Monitoring of the system at different temperatures (Fig. 7) revealed that the optimal temperature for this set of enzymes is 40°C. 100% of the expected yield was achieved at this temperature as with an experiment carried out at 30°C. However, that maximal rate of H_2 and CO_2 production was decreased by approximately 78% at 30°C. Raising the temperature to 50°C produced a slightly higher rate but only resulted in approximately half the maximal yield of H_2 and 73% of the CO_2 that could be expected. The ratio of H_2:CO_2 obtained from the oxidative portion of the pentose phosphate pathway should be 2:1. However, at the higher temperatures, the cofactor NAD^+(H) is very thermolabile (Zatman *et al*., 1953; Wu *et al*., 1986; Daniel and Danson, 1994) and therefore may result in the observing less H_2 than could actually be produced. Additionally, the greater thermostability of the second enzyme (6PGDH) causes it to keep catalyzing the production of CO_2 using the excess intermediate (6PGa) that is produced due to the greater turnover number of the first enzyme, G6PDH.
Alteration of the pH in the H_2 production system agrees with the results obtained for the optimal pH of the individual PPP enzymes (Fig. 5). However, this is also aided by the fact that the optimal pH for the hydrogenase for oxidizing reactions is also pH 8 (the hydrogenase from *P. furiosus* is a "bifunctional" sulfhydrogenase possessing H_2-oxidizing and S^o-reduction activities that respond differently to pH, temperature and inhibitors (Ma *et al*., 1993)). The level of activity of the hydrogenase at the temperatures used in this study is much reduced (optimal temperature of hydrogenase is over 95°C in H_2-oxidizing activities (Fiala and Stetter, 1986) and 85°C with $NADP^+$ as a cofactor (Ma *et al*., 1994). Despite the inclusion of excess amounts of hydrogenase, the build-up of NADPH may still occur. Therefore, the higher pH values may also be advantageous for the $NADP^+$/NADPH ratio due to the increased stability of NADPH in dilute alkali (Wong and Whitesides, 1981; Chenault and Whitesides, 1987; Woodward and Orr, 1998). The addition of excess NADPH (Fig. 9) does not decrease the rate and yield of H_2 and CO_2 and leads to expected increases in H_2 production, which may also be due to the favorable pH of the reaction buffer.

The effect of including excess amounts of 6-phosphogluconic acid (6PGa) (Fig. 10) indicates that the second enzyme in the pathway (6PGDH) is indeed being overwhelmed by the concentration of the intermediate. An addition of 2.5 mM 6PGa, which is only 3 times the Km for 6PGDH, leads to a 50% reduction in the maximal H_2 and CO_2 that can be obtained. This inhibition is even more evident when 10 mM 6PGa is added, which results in 66% and 19% final yields of H_2 and CO_2, respectively. The maximal theoretical yield also includes the amount of H_2 and CO_2 that can be obtained from the added 6PGa, which is also a substrate. The loss of the 2:1 ratio is caused by the addition of extra 6PGa, which produces one mole of H_2 and CO_2 per mole of 6PGa. The limitation of the second enzyme (6PGDH) is confirmed by the addition of more 6PGDH to the system containing 10 mM initial concentration of glucose-6-phosphate (G6P) (Fig. 11), where the rates and yields of CO_2 production are dramatically increased. The ratio of 1:20 was used to reflect that ratio of the turnover numbers of these enzymes and was expected to prevent the build up of the intermediate, 6PGa. In the case of the 1:1 and the 1:20 (G6PDG:6PGDH) ratios, the G6PDH enzyme appears to be capable of converting all the available G6P to 6PGa and NADPH, and is not inhibited by the high concentration of G6P. Therefore, the majority of the H_2 produced in the 1:1 ratio is due to the NADPH produced by

G6PDH. Increasing the concentration of 6PGDH leads to a 10% increase in the yield of H_2 that can be obtained from 10 mM G6P, but also causes a 25% increase in the theoretical yield of CO_2. The rate of CO_2 production is also increased by 80% on increasing the level of 6PGDH in the reaction system. Despite the observed improvement of H_2 and CO_2 yields, the ratio did not lead to the maximal yields of gases expected from the use of this ratio.

Isolation of a Thermophilic Oxidative Branch of the Pentose Phosphate Pathway

Approach with Sulfolobus solfataricus

The N-terminus MKIGLIGLGIMGYRIAANLAKANKLNLVYDRTQE was obtained for a 48 kDa protein partially purified using affinity chromatography along with the enzyme glucose dehydrogenase (GDH) (41 kDa). Sequence comparison analysis revealed this protein N-terminus to match with a variety of 6-phosphogluconate dehydrogenases. Despite the reported absence of central metabolic pathways employing phosphoryated intermediates in this species (De Rosa *et al*., 1984), very low levels of activity for both the PPP enzymes could be detected in cell cultures grown in enhanced media. The enhanced media contained higher concentrations of carbon sources and this may have influenced the expression of these enzymes. However, the far greater specific activity obtained for the GDH suggests that this is the dominant pathway for the degradation of glucose in *S. solfataricus*.

Despite originating from the same species, the sequences of the 6PGDHs from the two strains of *S. solfataricus* P1 and P2, surprisingly only share 57% sequence identity and 72% sequence similarity. However, the assigning of a function for the P2 gene appears to be based purely on sequence identity and may, in fact, be incorrect. This has since been established by the assigning of a new function of a 3-hydroxybutyrate dehydrogenase.

Approach with Thermotoga maritima

Both genes coding for the enzymes of the oxidative branch of the pentose phosphate pathway have been successfully isolated and cloned from the species *T. maritima* into the cloning vector pCR2.1. However, the failure to obtain fully transformed colonies of JM109 cells i.e. those containing recombinant PPP genes, has required that several trouble-shooting procedures be put in place to determine the specific step that led to the failure of successful ligation. The restriction enzymes are required to be tested to determine their efficiency for excising the DNA at the correct sites. Therefore, the colonies observed growing on ampicillin-containing plates could contain fully-functional non-recombinant pET-15b plasmids. Or it may be possible that the initial restriction digest (and subsequent ligation) were successful and that the resulting plasmids obtained from the *E. coli* strain JM109 colonies do contain the gene inserts, but that the second restriction digest to determine this has not been successful. This work is still in progress.

Conclusions

Characterization of the Mesophilic Oxidative Branch of the Pentose Phosphate Pathway

The system has been optimized in terms of the reaction buffer, pH and temperature. The ratio of G6PDH:6PGDH of 1:20, which reflects the respective turnover numbers of these enzymes, still does not result in the complete conversion of 10 mM G6P to H_2 and CO_2.

Isolation of a Thermophilic Oxidative Branch of the Pentose Phosphate Pathway

Even with the less complex method of growing *S. solfataricus*, the N-terminus that gives 31 – 58% matches with other 6PGDHs, and the observation of low level activity for the PPP enzymes, it appears that it may not be a suitable host for obtaining all the enzymes of the complete pentose phosphate pathway. Both genes coding for the enzymes of the oxidative branch of the pentose phosphate pathway have been successfully isolated and cloned from the species *T. maritima* and expression work is still in progress.

Future Work

- Optimize the expression and purification of recombinant thermophilic oxidative Pentose Phosphate enzymes.
- Purify and characterize the native wild-type enzymes from *T. maritima*.
- Compare hydrogen production by mesophilic and thermophilic G6PDH and 6PGDH.
- Establish that thermophilic enzymes will offer advantage over mesophilic enzymes for H_2 production.

This work will result in new knowledge showing the advantage of using thermophilic enzymes for the generation of molecular hydrogen from renewable energy.

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