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## Introduction

The prediction of oil shortages in the next 10 to 20 years necessitates the development of alternative sources of energy. Popular growing concerns for the environment and the requirement for a renewable source of energy are influencing the future importance of the hydrogen economy. The enzymatic conversion of glucose to H<sub>2</sub> has been demonstrated as a method for H<sub>2</sub> production [1]. However, the maximum stoichiometric yield of H<sub>2</sub> possible from glucose with that pathway is only 1 mole/mole sugar. Increasing the production of hydrogen from biomass-derived glucose and attainment of the maximum molar yield of H<sub>2</sub> can be achieved through the coupling of the enzymes of the pentose phosphate cycle with a hydrogenase. This has been demonstrated to generate 11.6 mol H<sub>2</sub> per mole of glucose-6-phosphate [2].

The focus of this current work is to study optimal requirements and limitations for hydrogen production using the oxidative portion of the pentose phosphate pathway. This process centers on three NADP<sup>+</sup> dependent enzymes, glucose-6 phosphate dehydrogenase (G-6-PDH), 6-phosphogluconate dehydrogenase (6-PGDH) and hydrogenase from *Pyrococcus furiosus*. The dehydrogenases are currently obtained from mesophilic sources, but *Pyrococcus furiosus* is an archaeal hyperthermophile, that grows optimally at 100°C. Therefore, in view of this substantial temperature difference and in order to increase the rates of hydrogen production, additional work is being carried out to isolate the genes for the G-6-PDH, and 6-PGDH from hyperthermophilic Archaeon, *Sulfolobus solfataricus* [3]. The final aim is to eventually express these recombinant enzymes in a mesophilic host such as *Escherichia coli* and incorporate them into an entirely hyperthermophilic *in vitro* enzymatic H<sub>2</sub> production system.

## Materials and Methods

**Materials, enzymes and assays.** Glucose 6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, 6-phosphogluconic dehydrogenase Type V from *Torula* yeast, glucose 6-phosphate, 6-phosphogluconic acid, β-NADP<sup>+</sup> and β-D-glucose were all obtained from Sigma Chemical Company, (St. Louis, M.O.). Hydrogenase from *Pyrococcus furiosus* was purchased from University of Georgia, Athens, G.A., U.S.A. It was purified as described in [4] and assayed by measuring hydrogen evolution from reduced methylviologen in 50 mM Tris (pH 8.0) containing 1 mM sodium dithionite at 80°C and determined to have an activity of 680 units/mL. Spectrophotometric assays were performed on a Unicam (Cambridge, U.K.) UV/vis spectrometer UV4 controlled by Vision v. 3.32 software by Unicam UV/vis

spectrometry (Cambridge, U.K.). Matrex gel Red A affinity media was purchased from Millipore Ltd., Watford, Hertfordshire, U.K.

**Strains and culture conditions.** Cells of *S. solfataricus* strain P1 (DSM 1616) employed for the purification of the 6-PGDH enzyme were kindly provided by Dr. Neil Raven (Centre for Applied Microbiological Research, Porton Down, Wilts., U.K.). All subsequent work employed *S. solfataricus* strain P1 purchased from the American Type Culture Collection (ATCC 35091). Cells were grown in ATCC medium 1304 at 70°C in test tubes. PCR products were cloned into the pCR2.1 vector system (Invitrogen, Carlsbad, California, U.S.A.).

**Purification of 6-phosphogluconate dehydrogenase.** *S. solfataricus* cell paste was resuspended in 50 mM MES buffer, pH 5.5 with 20 mM MgCl<sub>2</sub>, containing 1 mM PMSF, at approx. 0.2 g of cells/ml. Cells were broken by four 30-s bursts of sonication on ice with an MSE 150-W Ultrasonic Disintegrator. Soluble cell extract was obtained by centrifugation at 25000 g for 1 h at 4 °C. The insoluble pellet was discarded. The resuspension buffer was also used throughout the purification process. Gel filtration was carried out on a HiLoad 16/60 Superdex 200 prep grade 30pg column (0.32 cm×60 cm) (Pharmacia Biotech, Upsalla, Sweden). The gel-filtration fractions containing 6-PGDH activity, were then loaded on to a HiTrap Q anion-exchange column (Pharmacia Biotech). The column was washed with the same buffer and the bound protein was then eluted using a gradient of 0–1.5 M NaCl. The fractions containing 6-PGDH activity were then pooled and dialysed in a centricon (Millipore, U.K.) to remove the salt. A column containing Matrex gel Red A media (Millipore, U.K.) (2cm x 30cm) was equilibrated in resuspension buffer. The column was then washed with the same buffer and the bound protein was eluted using a gradient of 0–1.5 M NaCl. Following preparative gel electrophoresis, the band corresponding to 6-PGDH activity was electroblotted on to a hydrophobic PVDF membrane (Millipore Ltd., Watford, Hertfordshire, U.K.). The N-terminal sequence was then determined on an Applied Biosystems 470 gas-phase sequencer, coupled to an Applied Biosystems 120 phenylthiohydantoin analyzer.

**Experimental apparatus for the measurement of hydrogen.** A continuous flow system was constructed to measure hydrogen and carbon dioxide production based on the system as shown and described in [1,5]. A modification was made with the inclusion of a CO<sub>2</sub> analyzer in line prior to the H<sub>2</sub> sensor.

**Gene cloning and sequencing.** Degenerate PCR primers were designed based on each end of the 34-residue N-terminal amino acid sequence obtained from the purified 6-PGDH. PCR, using *S. solfataricus* genomic DNA as template, yielded a product of approximately 100-bp. This is currently being cloned into the pCR2.1 vector system.

## Results and Discussion

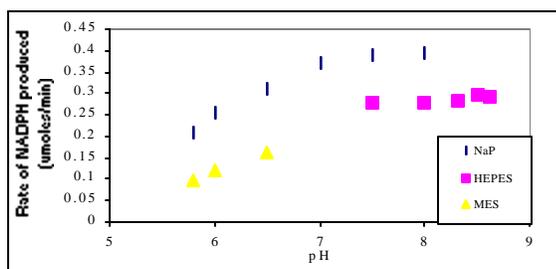
**Characterization of mesophilic pentose phosphate enzymes.** Kinetic characterization of the two individual enzymes revealed the following respective data :

**Table 1. Kinetic data for mesophilic pentose phosphate enzyme**

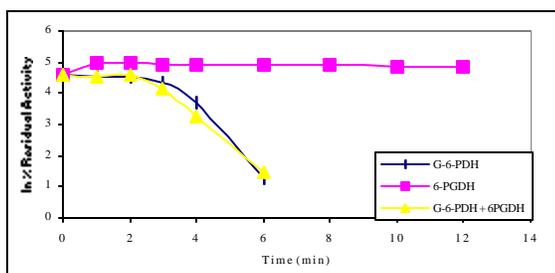
Glucose-6-phosphate dehydrogenase		
Substrate	K <sub>m</sub> (mM)	V <sub>max</sub> (μmoles/min)
glucose 6-phosphate	0.53	0.415

NADP <sup>+</sup>	0.068	0.25
6-Phosphogluconate dehydrogenase		
Substrate	Km (mM)	Vmax (μmoles/min)
6-phosphogluconic acid	1.65	0.22
NADP <sup>+</sup>	0.12	0.23

The optimal temperature and pH for the G-6-PDH from *Leuconostoc mesenteroides* are 30°C and pH 7.8, respectively and for 6-PGDH from *Torula yeast* are pH 7.6 and 50°C respectively. The optimal pH of the two enzyme pathway was also determined to be approximately pH 8 (Fig. 1). 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<sub>2</sub> and CO<sub>2</sub> from 2.5 mM glucose-6-phosphate. Further studies on the alteration of pH and temperature are currently in progress. Thermal inactivation studies of the mesophilic enzymes (Fig. 2) has revealed that G-6-PDH and 6-PGDH possess the following t<sub>50</sub> 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 6-PGDH enzyme has not influenced its thermal stability in comparison to the G-6-PDH enzyme.



**Figure 1.** The effect of pH on the activity of the mesophilic oxidative branch of the pentose phosphate pathway.



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

**In vitro hydrogen production system.** 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. Additional monitoring of CO<sub>2</sub> evolution confirmed the stoichiometry of H<sub>2</sub> to CO<sub>2</sub> to be 2:1 (data not shown). Variation of the initial glucose 6-phosphate concentrations has revealed that the optimal requirements for the complete *in vitro* pathway appear to be dependent on the

concentration of the second enzyme in the pathway, 6-PGDH. i.e. it is the rate limiting step in the pathway. Further studies are in progress on the variation of all the other conditions in order to achieve the optimal system in terms of rate and overall yield of hydrogen production.

**Isolation of thermophilic 6-PGDH.** The hyperthermophilic counterpart of this system is being established using enzymes isolated from the Archaeon *Sulfolobus solfataricus*. Purification of the 6PGDH from *S. solfataricus* cell pastes revealed a protein with approximate molecular weight of 48 kDa. The N-terminal sequence - MKIGLIGLIGIMGYRIAANLAKANKLNLVYDRTQE - has been found to align with a number of 6PGDHs from other species as shown in Table 2 [8], which also includes a putative 6PGDH from the alternative strain P2, of the same species. Surprisingly, despite originating from the same species, the sequences of the 6-PGDHs from these two strains P1 and P2 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 and may in fact be incorrect. Degenerate primers designed from the N-terminal sequence have produced a PCR fragment of approximately 100bp from genomic DNA for use in hybridization experiments. In view of the tandem positioning of the 6-PGDH and the G-6-PDH genes in the genome of the extremophilic and ancient eubacteria *Aquifex aeolicus* [9], it is expected that sequencing of the hybridized clone may reveal a glucose 6-phosphate dehydrogenase in its flanking regions. Sequencing is in progress.

**Table 2. N-terminal Alignment of 6-Phosphogluconate Dehydrogenases From All Three Domains of Life**

E. coli	MS-KQQIGVVGMVAVMGRNLALNIESRGYTVSVFNRSREKTEE	40
S. typhimurium	MS-KQQIGVVGMVAVMGRNLALNIESRGYTVSVFNRSREKTEE	40
Synechococcus	MA-LQQFGLIGLAVMGENLALNIERNFSLTVYNRTAEKTEA	40
T. maritima	-M-KSHIGLIGLAVMGQNLALNIARKGYKVSIVYNRTAQRTEE	39
D. melanogaster	MSGQADIALIGLAVMGQNLILNMDKGFVVCAYNRTVAVKVE	42
Homo sapiens	MA-QADIALIGLAVMGQNLILNMDHGFVVCAYNRTVSKVDD	41
S. cerevisiae	MS--ADFGLIGLAVMGQNLILNADHGFTVCAVYNRVTSKVDH	40
S. solf. P2	---MKVGFIFLIGIMGFPMASNLLKAGYDLTVYNRVTEKAEK	34
S. solf. P1	---MKIGLIGLIGIMGYRIAANLAKANKLNLVYDRTQEKIER	33
A. aeolicus	--MKTFLFLIGLRGMSALAYRLKNRGWEIYGYSTRVQTRER	38

## Conclusions

The present mesophilic system appears to offer a satisfactory system for the complete metabolism of glucose. However, the limitation of the second enzyme, 6-PGDH for higher concentrations of substrate and the degree of thermal instability of G-6-PDH at 60°C necessitates the establishment of an entirely thermophilic system. **Acknowledgement.** Mrs. J. Young (Zeneca Pharmaceuticals, Macclesfield, U.K.) for the amino acid sequencing and Frank Larimer (ORNL, Oak Ridge, T.N., U.S.A.) for DNA sequencing. This work is supported by the US Department of Energy.

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