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ANAEROBACULUM HYDROGENIFORMANS: INSIGHTS INTO THE NOVEL BIOCHEMICAL AND PHYSIOLOGICAL ABILITY OF A THERMOPHILIC BIOCATALYST THAT CAN UTILIZE BOTH DEFINED AND UNDEFINED SUBSTRATES FOR THE PRODUCTION OF RENEWABLE HYDROGEN ENERGY

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By

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ANAEROBACULUM HYDROGENIFORMANS: INSIGHTS INTO THE NOVEL BIOCHEMICAL AND PHYSIOLOGICAL ABILITY OF A THERMOPHILIC BIOCATALYST THAT CAN UTILIZE BOTH DEFINED AND UNDEFINED SUBSTRATES FOR THE PRODUCTION OF RENEWABLE HYDROGEN ENERGY

A DISSERTATION APPROVED FOR THE

DEPARTMENT OF BOTANY AND MICROBIOLOGY

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Abstract

A novel anaerobic, moderately thermophilic, NaCl-requiring fermentative bacterium, strain OS1^T, was isolated from oil production water collected from Alaska, USA. Cells were Gram-negative, non-motile, non-spore-forming rods (1.7-2.7 x 0.4-0.5 μm). The G+C content of the genomic DNA of strain OS1^T was 46.6 mol%. The optimum temperature, pH and NaCl concentration for growth of strain OS1^T were 55 °C, 7 and 10 g L^{-1}, respectively. The bacterium fermented D-fructose, D-glucose, D-maltose, D-mannose, α-ketoglutarate, L-glutamate, malonate, pyruvate, L-tartrate, L-asparagine, Casamino acids, L-cysteine, L-histidine, L-leucine, L-phenylalanine, L-serine, L-threonine, L-valine, inositol, inulin, tryptone and yeast extract. When grown on D-glucose, 3.86 mol hydrogen and 1.4 mol acetate were produced per mol of substrate. Thiosulfate, sulfur and L-cystine were reduced to sulfide, and crotonate was reduced to butyrate with glucose as the electron donor. 16S rRNA gene sequence analysis indicated that strain OS1^T was related to *Anaerobaculum thermoterrenum* (99.7 % similarity), a member of the phylum *Synergistetes*. DNA-DNA hybridization for strain OS1^T and *A. thermoterrenum* yielded a 68 % relatedness value. Unlike *A. thermoterrenum*, strain OS1^T fermented malonate, D-maltose, tryptone, L-leucine and L-phenylalanine, but not citrate, fumarate, lactate, L-malate, glycerol, pectin or starch. The major cellular fatty acid of strain OS1^T was iso-$C_{15:0}$ (91 % of total). Strain OS1^T also had iso-$C_{13:0}$ 3-OH (3 %), which was absent from *A. thermoterrenum*, and iso-$C_{13:0}$ (2 %), which was absent from *Anaerobaculum mobile*. On the basis of these results, strain

OS1^T represents a novel species of the genus *Anaerobaculum*, for which the name *Anaerobaculum hydrogeniformans* sp. nov. is proposed. The type strain is OS1^T (=DSM 22491^T =ATCC BAA-1850^T).

Chapter 1

Anaerobaculum hydrogeniformans sp. nov., a novel anaerobe that produces hydrogen from glucose

Introduction

The *Synergistetes* phylum of bacteria is an underrepresented phylogenetic The *Synergistetes* phylum of bacteria is an underrepresented phylogenetic cluster of Gram-negative anaerobic rod-shaped bacteria that are related to *Synergistes jonesii* (41). Members of this group have been isolated from a variety of environments including anaerobic digesters (54, 138), the termite hindgut (81), subgingival plaque (74) and petroleum reservoirs (85, 117, 128). The 11 validly described species in this group display diverse physiological properties, but all currently cultivated taxa catabolize amino acids (125). It has been noted that these organisms, although underrepresented by cultivated taxa, are not uncultivable but are present at low cell numbers in microbial communities (27). The genus *Anaerobaculum*, comprised of *Anaerobaculum thermoterrenum* (88) and *Anaerobaculum mobile* (68), is a member of the phylum *Synergistetes*, but their ability to catabolize amino acids has not been studied. However, their capacity to produce hydrogen gas from sugars has been documented (68).

Increasing global demand for fossil fuel reserves and a need to decrease carbon dioxide emissions has driven research towards renewable, carbon-neutral energy sources. Hydrogen has the potential to be a sustainable alternative to some fossil fuels. It is a clean fuel that burns with no carbon dioxide emissions and can be easily converted to electricity by fuel cells (14). Hydrogen has an energy yield of 122 kJ/g, which is 2.75 times greater than that of hydrocarbon derived fuels (45).

Presently about 95 % of the worlds' hydrogen is produced via steam reformation of natural gas, which does not lower net carbon dioxide emissions, and is resource and energy intensive (56). The production of hydrogen from renewable resources such as biomass or waste materials is being explored as a sustainable global fuel source. "Dark-fermentative" bacteria can produce hydrogen from carbohydrates and other waste products (2, 32, 60) without the need for light input (75).

$C_6H_{12}O_6 + 4H_2O \rightarrow 4H_2 + 2CH_3COO^- + 2HCO_3^- + 4H^+$ ($\Delta G^{0'} = -206.3$ kJ/mol)

The Gibbs' free energy of formation was from Thauer *et al*. (113) and is essentially the same as in Kengen *et al*. (48). Four hydrogens can be produced per glucose via this thermodynamically favorable reaction with acetate and carbon dioxide as additional products. However, only a handful of organisms have been isolated which produce close to this amount of hydrogen, such as *Acetomicrobium faecalis* (136), *A. mobile* (68), *Caldicellulosiruptor saccharolyticus* (121), *Thermotoga maritima* (98) and *Thermotoga elfii* (121). An objective of the United States Department of Energy is exploration of novel biological catalysts that can produce four hydrogen per molecule of glucose (17).

The hydrogen producing bacterium strain $OS1^T$ isolated from oil production water is described in this study. On the basis of phenotypic and genotypic evidence,

strain OS1^T is proposed as a new species of the genus *Anaerobaculum*, as *Anaerobaculum hydrogeniformans* sp. nov. The description of the genus is also emended to include the amino acids catabolized by the three species reported to date. Strain OS1^T can produce almost four hydrogen molecules per molecule of glucose, which approaches the theoretical maximum via the aforementioned reaction. It also has the ability to produce hydrogen from a variety of amino acids and other organic acids.

Materials and Methods

Enrichment and isolation

Strain OS1^T was isolated from oil production water collected from Alaska, USA as part of a study of the microbiology of a North Slope oil facility (18). Most probable number (MPN) enumerations of general heterotrophs were conducted at 50 °C using anaerobic half- strength tryptic soy broth (TSB) (Difco) with 1 % NaCl and 100 % nitrogen gas phase (5). The Hungate agar roll tube method was used for isolation (35).

Phenotypic characterization

Exponential phase cells grown on glucose were used for transmission electron microscopy. Cells were fixed with 1 % glutaraldehyde, spread onto carbon-coated Formvar grids and stained with 0.5 % phosphotungstate (pH 7.0). Cells photographed using a JEOL JEM 2000 FX transmission electron microscope. Strain OS1^T was regularly cultivated at 55 °C on anaerobic TSB plus 1 % NaCl. The temperature and NaCl range and optima were determined using TSB as the growth medium. Growth was measured spectrophotometrically at 600 nm using a Spectronic 20D (Thermo Spectronic).

All substrates were added from sterile anaerobic stock solutions to a final concentration of 3 g l^{-1} before inoculation. Glucose grown cells were used as the inoculum for substrate tests. Growth and end product formation were compared to

substrate-unamended controls. Chemicals used in this work were obtained from Sigma unless otherwise noted (Sigma-Aldrich). Growth was measured spectrophotometrically at 600 nm (Spectronic 20D; Thermo Spectronic) (5).

pH range and optima

For determination of the pH range and optimum, TSB was amended with (10 g l^{-1}): HOMOPIPES, pH 4 and 5; MES, pH 6 and 6.5; TES, pH 7 and 7.5; TAPSO, pH 8; TAPS, pH 8.5; CAPSO, pH 9 and 9.5 (111). The final pH was adjusted at 55 °C. Growth was measured spectrophotometrically at 600 nm (Spectronic 20D; Thermo Spectronic). A mineral medium was developed for routine growth (l^{-1}): 10 ml mineral solution (111); 10 ml vitamin solution (111); 10 ml trace metal solution (111); 10 g NaCl; 2 g yeast extract (Difco); 3 g glucose. The medium was buffered with 10 g TES at pH 7.5. The medium was prepared using strict anaerobic technique under a final gas phase of 100 % nitrogen (5).

Alternate electron acceptor analysis

Nitrate reduction and sulfide production was measured using CHEMetrics test kits (CHEMetrics, Inc). Nitrate, cystine, sulfate, thiosulfate and sulfite were added to the medium from sterile stock solutions. Elemental sulfur was added and sterilized as previously described (88). Crotonate reduction was measured by HPLC using a Shimadzu LC-20AT with a SPD-20A UV/vis detector equipped with an Aminex HPX-87H ion exclusion column (Bio-Rad) using 0.002N H_2SO_4 as the mobile phase at a flow rate of 0.9 ml min^{-1}.

Types strains used for comparison

Anaerobaculum thermoterrenum DSM 13490^T and *Anaerobaculum mobile* DSM 13181^T were obtained from the DSMZ and used for controls in the characterization assays. The basal medium used for routine growth of these two organisms was the same as the mineral medium described above. Growth experiments were conducted in triplicate.

Chemotaxonomic analysis

Cellular fatty acid methyl ester (FAME) and polar lipid analysis was conducted on strain OS1^T, *A. thermoterrenum* DSM 13490^T and *A. mobile* DSM 13181^T. Cells were grown at 55 °C on anaerobic TSB supplemented with 1 % NaCl containing a 100 % N_2 gas phase. FAME and polar lipid analysis was carried out by the Identification Service of the DSMZ and Dr. B.J. Tindall, DSMZ, Braunschweig, Germany.

Genomic analysis

Genomic DNA was isolated from strain OS1^T using a modified method of the Marmur procedure (40). The mol% G+C content of the genomic DNA was measured by HPLC as previously described (69), with previously described modifications (1). 16S rRNA gene sequencing was conducted using DNA as a template for PCR amplification using the universal primers 27f, 357f, 704f, 926f, 907r and 1492r corresponding to the *E. coli* numbering system (40). Sequencing of

the PCR products was performed by the Oklahoma Medical Research Foundation (Oklahoma City, OK). Primer sequences were aligned using the Sequencher software suite (Gene Codes Corporation) that resulted in a 1,444-base contiguous DNA sequence. The phylogenetic position of strain OS1^T was assessed via maximum likelihood analysis using the ARB software suite (83). A consensus tree was generated by bootstrapping at values greater than 90 % confidence limit (22) (FIGURE 2). The topology and major branching points of the phylogenetic tree were conserved between neighbor-joining and maximum parsimony analyses in the ARB software suite (Ludwig et al., 2004) (data not shown).

DNA-DNA reassociation analysis was necessary for taxonomic placement of strain OS1^T and was conducted in the laboratory of Dr. Peter Schumann at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) as described by De Ley et al. (15), with the modifications described by Escara & Hutton (20) and Huß et al. (36). Analysis was performed using a model 2600 spectrophotometer equipped with a model 2257-R thermoprogrammer and plotter (Gilford Instrument Laboratories). Rates of renaturation were computed with the program TRANSFER.BAS (38).

Pure and co-culture growth assays

Both *A. thermoterrenum*^{T} and *A. mobile*^{T} have been shown to produce H_2, acetate and carbon dioxide when grown in pure culture on glucose. *A. mobile*^{T} was

shown to grow syntrophically with a methanogen using glucose as the substrate (68). Syntrophic studies were conducted using strain OS1^T and the H_2-oxidizing methanogen *Methanothermobacter thermoautotrophicus* DSM 1053^T. The syntrophic medium was the same mineral medium as described above, but 3 g $NaHCO_3$ was added after the pH adjustment to pH 7.5. All syntrophic and pure culture fermentation studies were carried out in 125 ml crimp sealed serum bottles with 10 ml of medium at 55 °C with glucose (3 g l^{-1}) as the substrate. Acetate was measured by a gas chromatograph (GC) equipped with a flame ionization detector on a Shimadzu GC 8A equipped with a 2 m glass column packed with an 80/120 Carbopak B-DA/4% carbowax resin 20M (Supelco) with helium as the carrier gas at a flow rate of 35 ml min^{-1}. Methane, CO_2 and H_2 were measured with a Shimadzu GC-8A GC equipped with a thermal conductivity detector and a Porapak Q (2 m stainless steel) column (Alltech) using nitrogen as the carrier gas at a flow rate of 30 ml min^{-1}.

Results and Discussion

Phenotypic analysis

Strain OS1^T was the dominant culturable heterotroph present in the production water at 2.3 cells/ml (MPN). The dominant culturable H_2 oxidizer, *Methanothermobacter thermoautotrophicus*, was present at the same cell density (18). Colonies on anaerobic roll tubes were small, circular, smooth and yellowish in color. Cells were Gram-negative, non-flagellated, non-motile, non-spore-forming rods that were 1.7-2.7 x 0.4-0.5 μm in size and occurred singly (FIGURE 1). Strain OS1^T grew optimally at pH 7.5 (range pH 6-9). Strain OS1^T reduced elemental sulfur, thiosulfate and cystine to sulfide when grown in anaerobic TSB + 1 % NaCl. Nitrate, sulfite and sulfate reduction were not observed. Strain OS1^T reduced crotonate to butyrate in the presence of glucose but not with crotonate alone. Sulfur and thiosulfate reduction, as well as crotonate reduction in the presence of glucose, seems to be a universal phenotype of the genus *Anaerobaculum* (68, 88).

Genotypic analysis

16S rRNA gene analysis indicated that strain OS1^T was a member of the genus 16S rRNA gene analysis indicated that strain OS1^T was a member of the genus *Anaerobaculum* (FIGURE 1). *Anaerobaculum thermoterrenum*^{T} and *Anaerobaculum mobile*^{T} are currently the other two described members of this genus (68, 88), which are members of the phylum *Synergistes* (34, 125). The 16S rRNA gene of strain OS1^T was 99.7 % similar to *A. thermoterrenum*^{T} and 97.8 %

similar to *A. mobile*^{T}. It may not be surprising to find a close relative to *A. thermoterrenum*^{T} in oil production water because this type strain was isolated from a similar environment (88). Culture-independent surveys have shown the presence of *Anaerobaculum* strains in petroleum reservoirs (NCBI = GU357467 and EU573105) (25, 46), mining wastewater (NCBI = DQ256300) (26), a solid waste digestor (NCBI = EF559029) (61) and methanogenic reactors (NCBI = AB234001, FN563242, FN563270 and AB274508) (53, 91, 92). *A. mobile* was isolated from an anaerobic wastewater treatment lagoon (68). The above suggests that strains of *Anaerobaculum* may be present in many anaerobic environments, particularly thermophilic ones. The G+C content of strain OS1^T the genomic DNA was 46.6 mol%, compared to *A. thermoterrenum*^{T} (44 %) (88) and *A. mobile*^{T} (51.5 %) (68).

A DNA-DNA reassociation value of 68 ± 4 % (n = 2) was observed when strain OS1^T was hybridized to *A. thermoterrenum*^{T}, which is close but lower than the threshold value of 70 % for the definition of species (105), indicating that strain OS1^T was a distinct species from *A. thermoterrenum*^{T}.

Biochemical characterization

Phenotypic characteristics of strain OS1^T are listed in the species description and in Table 1. Several of these substrates were not tested in the original descriptions of *A. thermoterrenum*^{T} (88) and *A. mobile*^{T} (68), nor was the ability to utilize amino acids as substrates, a characteristic of members of the phylum *Synergistes* (125). The list of

substrates examined for the genus *Anaerobaculum* as well as a comparison of the phenotypes of *A. thermoterrenum*^{T}, *A. mobile*^{T} and strain OS1^T is in Table 1.

As shown in Table 1, strain OS1^T can be readily differentiated from the other species of *Anaerobaculum* by several phenotypic characteristics, such as the ability to utilize malonate, D-maltose and inulin, and the inability to catabolize L-malate, glycerol and starch. Strain OS1^T had an absolute requirement for NaCl for growth, which the other two species do not. In addition, strain OS1^T grew in medium containing up to 7 % NaCl, which is much higher than the maximum of 2 % and 1.5 % observed for cultures of *A. thermoterrenum* (88) or *A. mobile* (68), respectively.

Chemotaxonomic data

The detailed fatty acid profiles for all three species are shown in Table 2. FAME analysis showed that all three species contained iso-$C_{15:0}$ and iso-$C_{11:0}$ fatty acids, with iso-$C_{15:0}$ as the predominant fatty acid. Strain OS1^T contained both iso-$C_{13:0}$ and iso-$C_{13:0\ 3OH}$, however, *A. thermoterrenum* DSM 13490^T contained iso-$C_{13:0}$ and A. mobile DSM 13181^T contained iso-$C_{13:0\ 3OH}$ fatty acids. Polar lipid analysis results are summarized in Table 3. Polar lipid analysis showed that all three species contained diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phospholipids (PL1-PL3) and aminophospolipids (PN1-PN4). Strain OS1^T and *A. thermoterrenum* DSM 13490^T were shown to contain the phospholipids PL4 and PL5 but these were undetected in *A. mobile* DSM 13181^T.

Pure and co-culture growth data

Results from the pure and co-culture work are presented in Table 4. All three species of the genus *Anaerobaculum* produced close to four H_2 per mol of glucose and 1 mol of methane per mol of glucose when grown syntrophically with a H_2-oxidizing methanogen. In pure culture, strain $OS1^T$ produced up to 3.1-4.5 H_2 mmol L^{-1} culture using clarified raw sewage as the substrate (67).

FIGURE 1. Transmission electron micrograph of negatively stained cells of *Anaerobaculum hydrogeniformans* sp. nov. strain OS1^{T}. Bar, 0.5 μm.

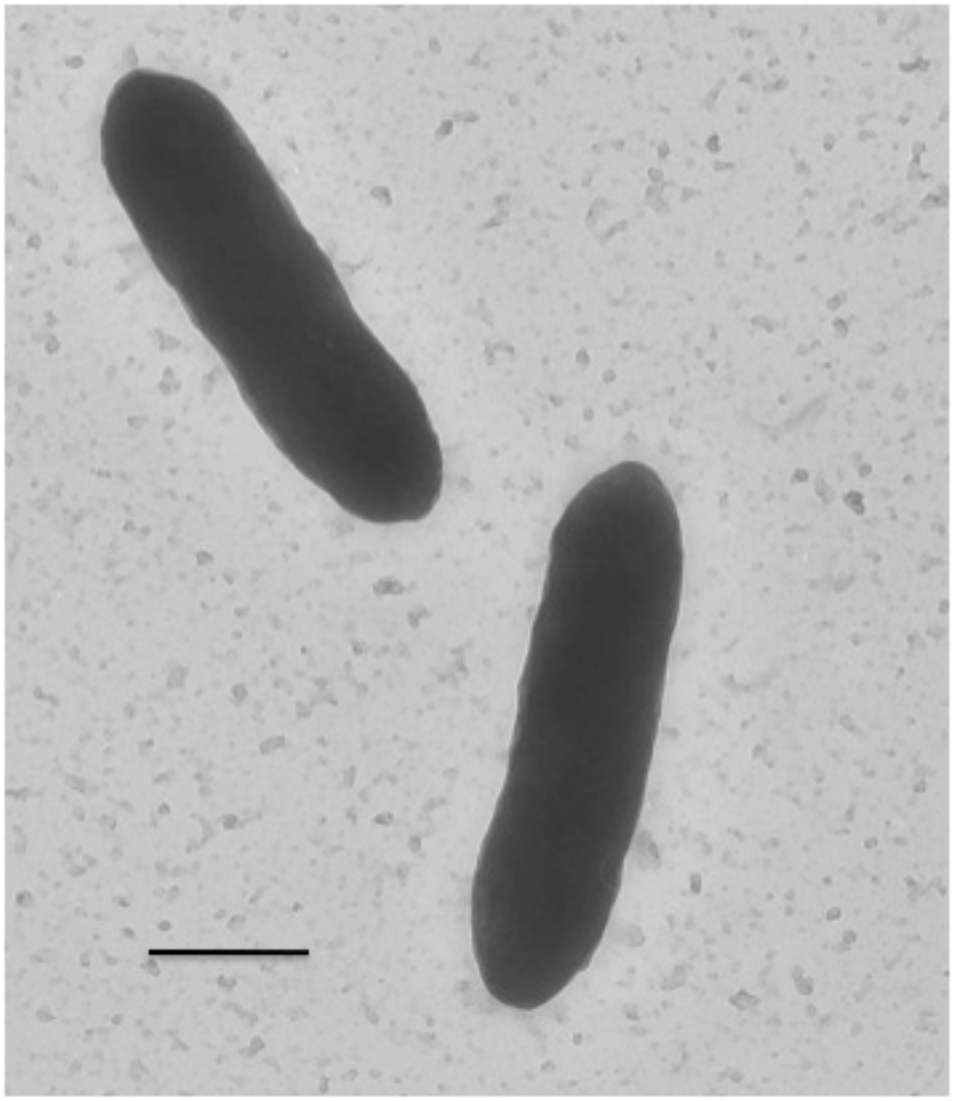


FIGURE 2. Phylogenetic tree based on 16S rRNA gene sequence analysis for *Anaerobaculum hydrogeniformans* sp. nov. and other closely related microorganisms within the phylum *Synergistes*. Identical branches were present in both phylogenetic consensus trees generated by the neighbour-joining, maximum likelihood and the maximum-parsimony algorithms. Bootstrap values are expressed as a percentage of 1000 replications. The bar represents 1 substitution per 10 nucleotide positions.

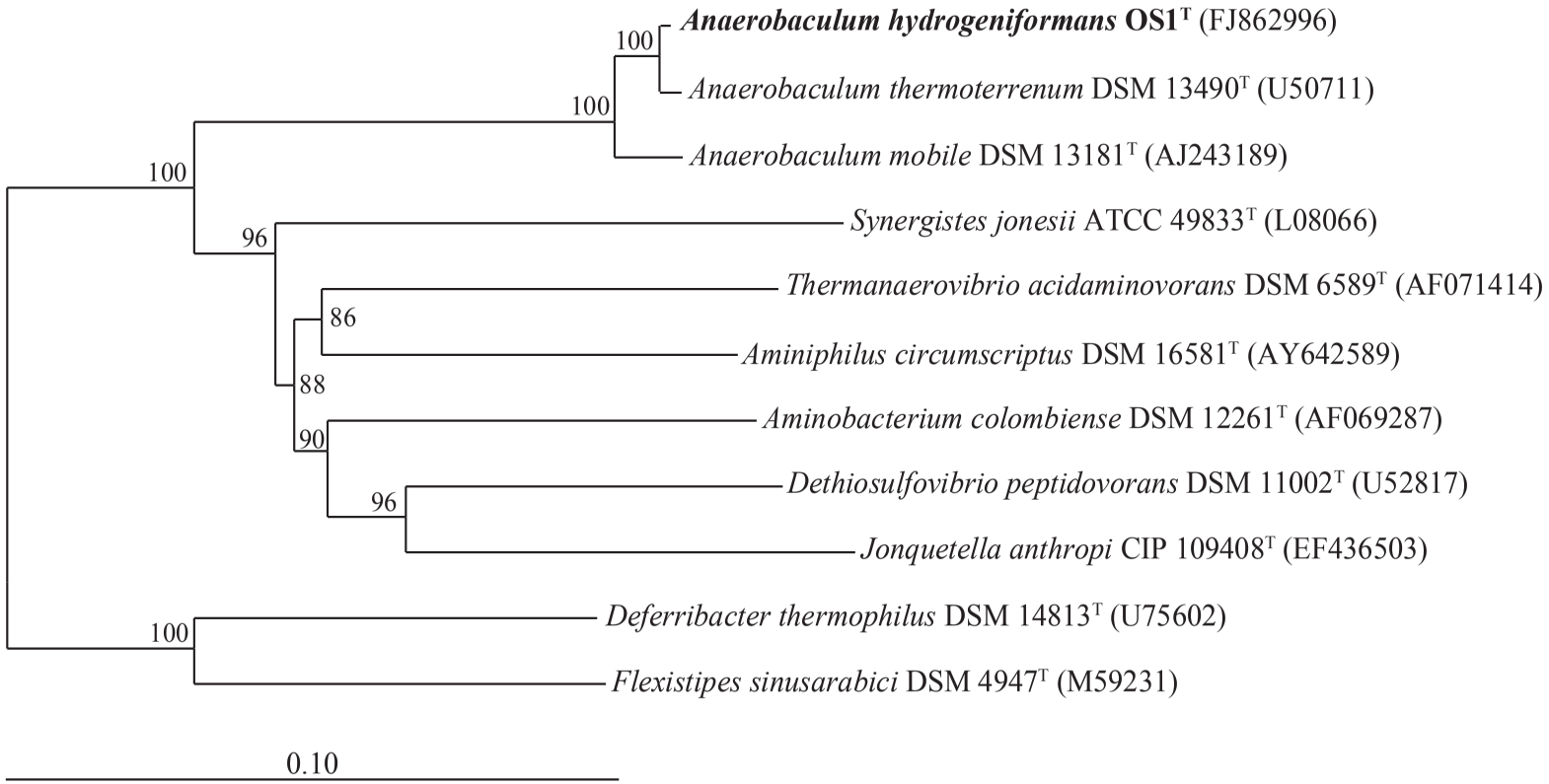

***Anaerobaculum hydrogeniformans* OS1^T** (FJ862996)
Anaerobaculum thermoterrenum DSM 13490^T (U50711)
Anaerobaculum mobile DSM 13181^T (AJ243189)
Synergistes jonesii ATCC 49833^T (L08066)
Thermanaerovibrio acidaminovorans DSM 6589^T (AF071414)
Aminiphilus circumscriptus DSM 16581^T (AY642589)
Aminobacterium colombiense DSM 12261^T (AF069287)
Dethiosulfovibrio peptidovorans DSM 11002^T (U52817)
Jonquetella anthropi CIP 109408^T (EF436503)
Deferribacter thermophilus DSM 14813^T (U75602)
Flexistipes sinusarabici DSM 4947^T (M59231)
100
100
100
96
86
88
90
96
100
0.10

Table 1. Phenotypic comparison of *A. hydrogeniformans* strain OS1^T, *A. thermoterrenum* (DSM 13490^T) and *A. mobile* (DSM 13181^T).

All three species utilized the substrates D-fructose, D-glucose, pyruvate, L-tartrate, Casamino acids, L-asparagine, L-cysteine, L-histidine, L-serine, L-threonine and L-valine. All three species did not utilize D-arabinose, D-cellobiose, cellulose, D-galactose, α-lactose, D-melibiose, D-raffinose, D-rhamnose, sucrose, D-xylose, dextrin, xylan, adonitol, acetate, butyrate, L-aspartate, L-glutamine, glycine, L-lysine, L-methionine, L-proline, L-tryptophan and L-tyrosine.

* Data from (88); † Data from (68)

Characteristic	Strain OS1	*A. thermoterrenum*	*A. mobile*
Growth rate (hr^{-1})	0.011	0.013	0.01
Temperature range (optimum) (°C)	40-65 (55)	28-60 (55)*	35-65 (55-60)†
pH range (optimum)	6.0-9.0 (7.0)	5.5-8.6 (7-7.6)*	5.4-8.7 (6.6-7.3)†
NaCl range (optimum) (g/l)	0.8-70 (10)	0-20 (10)*	0-15 (0.08)†
DNA G+C content (mol%)	46.6	44*	51.5†
Motility	-	-	+
Growth on:			
α-ketoglutarate	+	+	-
Citrate	-	+	-
Fumarate	-	+	-
D-Gluconate	-	-	+
L-Glutamate	+	+	-
Lactate	-	+	-
L-Malate	-	+	+
Malonate	+	-	-
D-Maltose	+	-	-
D-Mannose	+	+	-
Glycerol	-	+	+
Inositol	+	+	-
Tryptone	+	-	+
L-Arginine	-	-	+
L-Leucine	+	-	+
L-Phenylalanine	+	-	+
Inulin	+	-	-
Pectin	-	+	-
Starch	-	+	+

Table 2. Cellular fatty acid composition of *A. hydrogeniformans* strain OS1^T, *A. thermoterrenum* DSM 13490^T and *A. mobile* DSM 13181^T. Values are percentages of total identified fatty acids.

ND, not detected.

Fatty Acid	OS1^T	*A. thermoterrenum*	*A. mobile*
iso-$C_{11:0}$	4.43	12.98	20.74
iso-$C_{13:0}$	1.78	20.96	ND
iso-$C_{13:0\ 3OH}$	3.26	ND	8.53
Iso-$C_{15:0}$	90.53	66.06	70.73

Table 3. Polar lipid composition of *A. hydrogeniformans* OS1^T (1), *A. thermoterrenum* DSM 13490^T (2) and *A. mobile* DSM 13181^T (3).

ND, not detected

Polar lipid	1	2	3
Diphosphatidylglycerol	+	+	+
Phosphatidylglycerol	+	+	+
Phosphatidylethanolamine	+	+	+
Phospholipids			
PL1	+	+	+
PL2	+	+	+
PL3	+	+	+
PL4	+	+	ND
PL5	+	+	ND
Aminophospolipids			
PN1	+	+	+
PN2	+	+	+
PN3	+	+	+
PN4	+	+	+

Table 4. Acetate, H_2 and CH_4 produced by strain OS1^T, *A. thermoterrenum* DSM 13490^T and *A. mobile* DSM 13181^T with glucose in pure and in co-culture with *M. thermoautotrophicus* DSM 1053^T.

Data from this study and (68) (*A. mobile*).

	Glucose consumed	Fermentation products mmol L^{-1}		
	(mmol L^{-1})	H_2	Acetate	CH_4
Pure culture:				
OS1^T	14.1	47.2	19.7	
A. thermoterrenum	7.9	36.4	13.8	
A. mobile	7.5	29.8	15.1	
Co-culture:				
OS1^T	8.4		17.6	8.1
A. thermoterrenum	8.3		16.3	8.7
A. mobile	8.5		16.9	8.5

Conclusions

Strain OS1^T was isolated as the dominant culturable heterotrophic organism from an oil water separation unit in Alaska, USA. 16S rRNA gene analysis indicated that strain OS1^T was a member of the genus *Anaerobaculum* in the phylum *Synergistes*. The 16S rRNA gene of strain OS1^T was too similar to *A. thermoterrenum*^{T} to be classified as a novel species based solely on 16S rRNA gene analysis. DNA-DNA hybridization analysis was very close to the 70 % cut-off value when strain OS1 was hybridized with *A. thermoterrenum*^{T}. However, when chemotaxonomic data from FAME analysis readily differentiated strain OS1^T from both *A. thermoterrenum* and *A. mobile*. Phenotypic characterization further supported the creation of a novel species for strain OS1^T based on the substrate utilization profile strain OS1^T exhibited when compared to *A. thermoterrenum*. The most significant phenotypic characteristic of strain OS1^T is that it requires NaCl for growth and both *A. thermoterrenum* and *A. mobile* do not, and strain OS1^T is capable of growth in medium with up to 7 %, which is much higher than any reported value for the other two members of the genus. On the basis of the genotypic differentiation and phenotypic data presented, both phenotypic and genotypic, strain OS1^T is proposed to be a new species of the genus *Anaerobaculum*, for which the name *Anaerobaculum hydrogeniformans* sp. nov. is proposed.

Emended description of the genus *Anaerobaculum* Menes & Muxi, 2002

Chemoorganotrophic Gram-negative anaerobe. Moderately thermophilic (28-65 °C) and halotolerant to halophilic (0-70 g L^{-1}). Straight to slightly curved rods. Motile by means of a single flagellum or non-motile. In complex media, some strains grow with a sheath-like material extending past the cell poles. Spores not observed. Ferments organic acids and a limited number of carbohydrates to acetate, hydrogen and carbon dioxide. Peptone and yeast extract also fermented. Some amino acids (e.g. L-asparagine, L-cysteine, L-histidine, L-serine, L-threonine or L-valine) can be used as substrates. Utilizes a range of electron acceptors: thiosulfate, sulfur and L-cystine are reduced to sulfide, and crotonate is reduced to butyrate. Cellular fatty acid composition of species of *Anaerobaculum* includes iso-$C_{11:0}$ and iso-$C_{15:0}$, and may or may not contain iso-$C_{13:0}$ and/or iso-$C_{13:0}$ 3-OH. The polar lipid composition of the genus contains diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phospholipids (PL1-PL3), aminophospholipids (PN1-PN3), and may or may not contain phospholipids PL4 and PL5. The DNA G+C content is 44-51.5 mol%. The type species is *Anaerobaculum thermoterrenum*.

Description of Anaerobaculum hydrogeniformans sp. nov.

Anaerobaculum hydrogeniformans (hy.dro.ge.ni.for'mans. N.L. n. *hydrogenum* hydrogen; L. part. adj. *formans* forms; N.L. part. adj. *hydrogeniformans* producing hydrogen).

Cells are 1.7-2.7 x 0.4-0.5 μm. Growth occurs between 40 and 65 °C (optimum 55 °C), from pH 6 to 9 (optimum 7.0), and with 0.8 to 7 % NaCl (optimum 1 %). The DNA G+C content is 46.6 mol%. The cellular fatty acid composition of strain OS1^T is iso-$C_{15:0}$, iso-$C_{11:0}$, iso-$C_{13:0}$ 3-OH and iso-$C_{13:0}$. The polar lipids detected were diphodphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phospholipids (PL1-PL5) and aminophospholipids (PN1-PN4). Cells reduce elemental sulfur, thiosulfate and L-cystine to sulfide using glucose as the electron donor. Sulfate, sulfite or nitrate are not reduced. Crotonate is reduced to butyrate when glucose is present. Growth occurs on D-fructose, D-glucose, D-maltose, D-mannose, α-ketoglutarate, L-glutamate, malonate, pyruvate, L-tartrate, L-asparagine, Casamino acids, L-cysteine, L-histidine, L-leucine, L-phenylalanine, L-serine, L-threonine, L-valine, inositol, inulin, tryptone and yeast extract. D-Arabinose, carboxymethylcellulose, cellulose, D-cellobiose, D-galactose, α-lactose, D-melibiose, D-raffinose, D-rhamnose, sucrose, D-xylose, acetate, butyrate, citrate, fumarate, D-gluconate, lactate, L-malate, oleate, succinate, L-arginine, L-aspartate, L-glutamine, glycine, L-isoleucine, L-lysine, L-methionine, L-proline, L-tryptophan, L-tyrosine, adonitol, glycerol, dextrin, gelatin, pectin, starch or xylan do not support growth. The type strain is OS1^T (=DSM 22491^T =ATCC

BAA-1850^{T}). Strain OS1^{T} was isolated from oil production water collected from Alaska, USA.

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Chapter 2

Biohydrogen from waste feedstocks by *Anaerobaculum hydrogeniformans*

Abstract

The production of hydrogen (H_2) from brewery wastewater (BWW), sweet sorghum juice (SSJ), cheese whey wastewater (WW), raw sewage (RS) and anaerobic digestor sludge (AD) by *Anaerobaculum hydrogeniformans* was evaluated. An optimal ratio of wastewater to mineral medium was obtained for all feedstocks and used for batch fermentations. Clarified wastewaters resulted in higher H_2 yields for BWW, AD, RS and WW. The R_{max} (13 ml H_2 L^{-1} hr^{-1}) for glucose grown cells and when clarified BWW (CBWW) (R_{max} 9 ml H_2 L^{-1} hr^{-1}) was used as the carbon and nutrient source produced 955 ml H_2 L^{-1} and 623 ml H_2 L^{-1}, respectively. When *A. hydrogeniformans* was grown on CBWW or diluted CBWW (1:1), COD was reduced by 99 % and 75 %, respectively. *A. hydrogeniformans* produced 264 ml H_2 L^{-1} and reduced COD by 95% when grown on SSJ diluted 1:4 with mineral medium, which corresponds to 10.3 ml H_2 per g COD reduced. Cells grown on clarified WW produced 174 ml H_2 L^{-1} at a rate of 1.3 ml H_2 hr^{-1} and reduced 87 % of the COD. *A. hydrogeniformans* produced 166 ml H_2 L^{-1} and reduced COD by 84 % when grown on clarified RS. These values are significantly higher than those reported for bacterial fermentations or microbial electrolytic cells. *A. hydrogeniformans* grown on AD produced 22.6 ml H_2 L^{-1} at a rate of 0.8 ml H_2 hr^{-1} and reduced COD by 89 %. This study shows that *A. hydrogeniformans* can be used to produce H_2 from waste feedstocks while significantly reducing COD.

Introduction

Worldwide energy needs have been increasing while fossil fuel reserves are decreasing. This, in combination with concerns about the impact of carbon dioxide emissions on the environment, has spurred interest in the exploration of new sustainable energy sources that could supplement fossil fuels (45). Proposed alternative energy sources include the production of renewable biodiesel, bioethanol and biobutanol. Recently much attention has been paid to the use of H_2 as a fuel for transport and electricity generation.

Hydrogen is considered a clean source of energy, with water as the only end product of combustion. Hydrogen can be produced from renewable raw materials and possesses an energy yield approximately 2.75 times greater than that of hydrocarbon derived fuels (4). Presently, about 90 % of H_2 is generated from fossil fuels by the reaction of natural gas or light oil fractions with steam at high temperatures, methods that are energy-intensive, expensive and unsustainable (72, 126). The production of H_2 from organic wastewaters rather than fossil fuels offers a sustainable alternative H_2 is produced or if the electrons are used in electrical circuits directly (107). Fermentative H_2 production (FHP) is a microbial process that emits less carbon dioxide than conventional thermo-chemical H_2 production (23) and has great potential for H_2 formation from organic wastestreams.

Currently most of the FHP research being conducted on wastewaters is performed with mixed cultures derived from treated anaerobic digester sludge (29).

Most of this research, however, is irreproducible due to variation in seed inoculum and the conditions used for the enrichment of the H_2-producing consortia (37). Here, we present the results from a pure culture, *Anaerobaculum hydrogeniformans*, which can utilize a variety of organic wastewaters for FHP. *A. hydrogeniformans* is a moderate thermophile that produces almost 4 hydrogen molecules per molecule of glucose with carbon dioxide and acetate as the only other end-products (67). This is the first report of a pure culture being used for biological H_2 production and waste stream remediation for many of the wastewaters used in this study.

Materials and methods

Culture and growth conditions:

Anaerobaculum hydrogeniformans was grown in a mineral medium (MM) containing (L^{-1}): 10 mL mineral solution (111), 10 mL vitamin solution (111), 10 mL trace metal solution (111), 2 g yeast extract (Difco), 10 g TES (Research Organics) and 10 g NaCl. The medium (pH 7.5) was prepared using strict anaerobic technique (5). Cultures were grown at 55 °C and fed glucose (3 g L^{-1}) added from a sterile anaerobic stock solution. All chemicals were obtained from Sigma-Aldrich unless stated otherwise.

Feedstocks:

Anaerobic digester sludge (AD) and raw sewage (RS) were obtained from the Norman Wastewater Treatment Plant (Norman, OK). The water treatment plant serves 82,000 citizens and processes an annual monthly average of 12 million G d^{-1}. The pH of both the AD ($pH_{initial}$ 6.9) and RS ($pH_{initial}$ 6.9) was adjusted to 7.5 with NaOH and 1% NaCl was added to each. Sterile anaerobic stock solutions were prepared and kept under 100 % N_2 headspace in stoppered and crimp sealed 18 mm glass tubes (Bellco). Brewer's wastewater (BWW) was collected after primary fermentation and processed immediately upon receipt. The BWW consisted of yeast cells, remaining nutrients and carbohydrates, coagulant and remnant particulates of the brewery process. The BWW was acidified to a pH of 1 with HCl and autoclaved at 121 °C for 20 min to hydrolyze the yeast cells. After autoclaving

the cooled hydrolyzed BWW was then pH adjusted to 7.5 with NaOH and 1% NaCl was added. The resulting BWW was kept frozen at -20 °C until use. Sweet sorghum has been gaining interest as an energy crop due to its ability to grow in subtropical and temperate climates and its high content of fermentable sugars (Antonopoulou *t al.* 2007). The sweet sorghum juice (SSJ) was obtained from Oklahoma State University (Stillwater, OK) and prepared fresh for each assay by first adjusting the pH to 7.5 with NaOH and adding 1 % NaCl. The SSJ was then made anaerobic by boiling and degassing with 100 % N_2. Cheese production wastewater (whey wastewater [WW]) was obtained from Christian Cheese Co. (Kingfisher, OK, USA). WW was prepared fresh for each assay. The unamended WW contained 3 % NaCl (George Christian, personal communication). The pH was adjusted to 7.5 with NaOH and then made anaerobic by boiling and degassing with N_2. If specified, all feedstocks underwent a clarification process by centrifugation (10,000 x g for 45 min at 4 °C) following the adjustment of salinity and pH. Feedstocks were prepared immediately after arrival to the laboratory. Optimization experiments occurred immediately following feedstock preparation. Feedstocks for batch fermentations were stored at -20 °C until needed.

Optimization of growth parameters:

It was reported that *A. hydrogeniformans* produced H_2 from RS alone, but H_2 production could be elevated if RS was supplemented 1:1 with MM containing yeast extract (YE) (67). A range finding experiment was constructed to elucidate the ratio of feedstock to MM for optimal H_2 production. The MM used for this experiment

was the same as described above with the YE removed to ascertain the feasibility of using each waste stream as both the carbon and nutrient source for *A. hydrogeniformans*. In this experiment 100 % feedstock was tested without the addition of MM. Alternatively, a 1:1 mixture of feedstock to MM was analyzed and serially diluted 1:1 to a final ratio of 1:16. The experiment was conducted in triplicate at 55°C in 18 mm stoppered crimp-sealed tubes with 100 % N_2 headspace using strict anaerobic technique. This assay was conducted for both raw and clarified feedstocks.

Batch fermentations:

Batch fermentations were conducted using the optimized parameters from Section 2.3. AD was chosen over CAD to eliminate pretreatment steps. AD, clarified raw sewage (CRS), CBWW and clarified whey water (CWW) were all used undiluted as the substrate and nutrient source based on results discussed in Section 3.1. CBWW was also diluted 1:1 and SSJ was diluted to 1:4 with MM. All assays were conducted in 125 ml serum stoppered and crimp-sealed bottles (Wheaton). Each bottle contained 20 ml of liquid and 100 % N_2 at 0 psig. Bottles were prepared using strict anaerobic technique. Incubations were carried out at 55 °C. Hydrogen gas was measured as discussed in Section 2.5. R_{max} is defined as the maximum amount of H_2 produced per liter culture per hour.

Analytics:

Hydrogen gas was quantified using a Shimadzu GC-8A gas chromatograph equipped with a thermal conductivity detector. Gases were separated with a 50/80

mesh Porapak Q column (2 m x 1/8 in) (Sulpelco). The inlet and detector were both set at 110 °C. Column temperature was held at 100 °C. Data was analyzed using a C-R8A Chromatopac Integrator (Shimadzu). Ultra-high purity N_2 was used as the carrier gas. A Fisher Accumet Basic pH Meter (Fisher Scientific) was used to measure pH. Chemical oxygen demand (COD) was analyzed using Hach test kits for high range COD (Hach). Glucose concentrations were quantified using the glucose-oxidase reaction with the PGO enzyme assay kit (Sigma). Volatile fatty acids (VFAs) were quantified by HPLC using an ICS 3000 pump and AD-25 absorbance detector set at 210 nm (DIONEX). VFAs were separated using an Acclaim Organic Acid HPLC Column (Dionex) at 30 °C using a stepwise gradient consisting of 2 mM methanesulfonic acid (MSA) at a flow rate of 1.0 ml min^{-1} with increasing amounts of 100 % acetonitrile (gradient conducted at 1 ml min^{-1}) to a final concentration of 60 % acetonitrile and 40 % MSA. All chemicals used in this study were obtained from Sigma-Aldrich unless specified otherwise. Initial glucose, COD and VFA profiles for feedstocks are shown in Table 5.

Table 5. Feedstock characterization including initial COD (g L^{-1}), glucose (g L^{-1}) and fatty acid (mM) measurements of the clarified brewer wastewater (CBWW), dilute clarified brewer wastewater diluted (CBWW 1:1), clarified raw sewage (CRS), anaerobic digestor sludge (AD), sweet sorghum juice (SSJ), and clarified whey wastewater (CWW).

	Glucose	CBWW	CBWW (1:1)	CRS	AD	SSJ	CWW
COD (g/L)	10	83	35	2.0	9.0	27	12
Glucose (g/L)	4.44	2.33	1.34	0.00	0.00	1.99	0.05
Fatty acid (mM)							
Formate	10.8	185	113	0.0	3.40	2.70	77.6
Acetate	6.30	10.0	5.80	4.3	2.20	2.80	0.0
Propionate	51.6	29.2	12.2	0.0	1.70	91.8	92.2
Butyrate	0.0	2.90	0.0	0.0	0.0	0.0	0.0
Valerate	40.8	0.0	0.0	39.0	0.0	0.0	0.0

Results and Discussion

Feedstock optimization:

Results from the feedstock optimization assay can be seen in Table 6. Clarified brewer's wastewater (CBWW) produced more H_2 than brewer's wastewater (BWW) alone. When CBWW was diluted 1:1 with MM, H_2 production showed a slight increase (564 ml H_2 L^{-1} and 647 ml H_2 L^{-1}, respectively). BWW and CBWW were the only feedstocks that allowed for the production of H_2 at significant levels when diluted up to 1:16 and produced more H_2 than any other feedstock tested. *A. hydrogeniformans* could produce a substantial amount of H_2 from the BWW alone with no dilution or clarification.

Anaerobic digestor sludge (AD) grown cells produced 151 ml H_2 L^{-1}. However, clarified anaerobic digestor (CAD) sludge produced more H_2 (199 ml H_2 L^{-1}). CRS grown cells produced more H_2 than RS (199 ml H_2 L^{-1} and 120 ml H_2 L^{-1}, respectively). CRS grown cells produced H_2 at a dilution up to 1:8. The amount of H_2 produced in the RS and dilute RS was contradictory to previous data that reported H_2 production at higher amounts when the RS was diluted (67).

When sweet sorghum juice (SSJ) was used as a feedstock, H_2 production was not detected until the SSJ was diluted 1:4 with MM. This could be due to a compound present in raw SSJ that inhibited *A. hydrogeniformans* at higher concentrations. Also, the need for additional MM could be explained by the relatively low amounts of phosphorus and nitrogen found in sweet sorghum extracts (3). Cells grown on clarified sweet sorghum juice (CSSJ) did not yield H_2 during

this assay. *A. hydrogeniformans* did not produce significant H_2 when grown on any dilution of whey waste water (WW). However, clarified WW (CWW) could be used as a carbon and nutrient source for H_2 production. It should reiterated that Maune and Tanner used YE in the diluent for their study (67) and YE was left out of the MM in this study in order to ascertain if each feedstock could be used for both a carbon and nutrient source.

Results from this assay were used to determine ratios, if any, of feedstock to MM for batch fermentation studies. AD was used for further analysis even though the CAD produced more H2. AD was used to eliminate the pretreatment of CAD prior to use. Undiluted CRS and CWW and the 1:4 dilution of SSJ with MM were used for subsequent experiments. CBWW and CBWW 1:1 were assayed as potential feedstocks because A. hydrogeniformnans produced similar amount of H2 from each. A. hydrogeniformans was also grown on a MM with glucose as a positive control. Abiotic controls were used to ensure the sterility of the feedstocks during testing. H2 production was not observed in any of the abiotic controls in this study.

Table 6. Amount of H_2 gas produced (ml H_2 L^{-1}) as feedstocks were diluted with mineral medium. Feedstocks were brewer wastewater (BWW), clarified brewer wastewater (CBWW), anaerobic digestor sludge (AD), clarified anaerobic digestor sludge (CAD), raw sewage (RS), clarified raw sewage (CRS), whey water (WW), clarified whey wastewater (CWW), sweet sorghum juice (SSJ) and clarified sweet sorghum juice (CSSJ)**.**

	Dilution of feedstock in mineral medium				
Feedstock	0	1:1	1:4	1:8	1:16
BWW	447	388	145	139	51.0
CBWW	564	647	436	318	108
AD	151	39.0	0.00	0.00	0.00
CAD	199	2.00	38.0	0.00	0.00
RS	120	73.0	60.0	97.0	0.00
CRS	199	156	169	107	0.00
WW	0.00	14.0	26.0	0.00	0.00
CWW	104	55.0	105	0.00	0.00
SSJ	0.00	0.00	200	176	115
CSSJ	0.00	0.00	0.00	0.00	0.00

Batch fermentations:

Glucose vs. brewery wastewater:

Results from this experimental setup are summarized in Table 7. Glucose was used as a benchmark pure substrate for comparison to H_2 production from wastewaters. *A. hydrogeniformans* produced the most H_2 when grown on glucose (FIGURE 1). Glucose, CBWW and CBWW 1:1 grown cultures produced 955, 623 and 625 ml H_2 L^{-1} respectively (FIGURE 4). When *A. hydrogeniformans* was grown on CBWW and CBWW 1:1, COD was reduced by 99 % and 75 %, respectively. This is very promising because remediation of brewery wastewater is of particular concern (39). If brewery wastewater is not treated and reacts with chlorine in a typical water treatment facility, carcinogenic substances can be created by halogenated disinfection by-products formed during the treatment process (89). This is the first attempt to treat brewery wastewater with a pure culture. Brewery wastewater (101) and rice wine wastewater (139) have been remediated using seed sludge as the inoculum. However, little has been reported about COD reduction in brewery wastewater. From a remediation standpoint it can be concluded that *A. hydrogeniformans* can be used to significantly reduce COD in brewery wastewater.

Stoichiometric conversion of substrates to H_2 is also a valuable measurement in assessing fermentation performance. CBWW and CBWW 1:1 contained 2.5 and 1.3 g L^{-1} glucose, respectively (Table 7), and *A. hydrogeniformans* produced 4.2 and 9.5 H_2 per glucose when grown on CBWW 1:1 and CBWW, respectively. While glucose grown cells approached the theoretical maximum of 4 H_2 per mol of glucose consumed, *A. hydrogeniformans* produced or superseded this amount when

grown on CBWW 1:1 or CBWW. This finding is likely due to the complex nature of the brewery wastewater and shows the efficacy of brewery wastewater as a potential feedstock for H_2 production by *A. hydrogeniformans*, which is also confirmed by the removal of 99 % of the COD in the CBWW.

Another valuable measurement in assessing fermentation performance is the production of H_2 per reduction of COD. Due to the complex nature of all feedstocks tested and the ability of *A. hydrogeniformans* to use substrates other than glucose, H_2 production per gram COD reduced is reported in this study. Glucose grown cultures of *A. hydrogeniformans* produced 116 ml H_2 g^{-1} COD reduced and produced the most H_2 g^{-1} COD reduced than any other feedstock tested. When grown in the CBWW and the CBWW diluted 1:1 6.68 and 26.4 ml H_2 g^{-1} COD was produced respectively, suggesting that the addition of the MM will increase the amount of product per g COD from brewery wastewater.

Sweet sorghum juice:

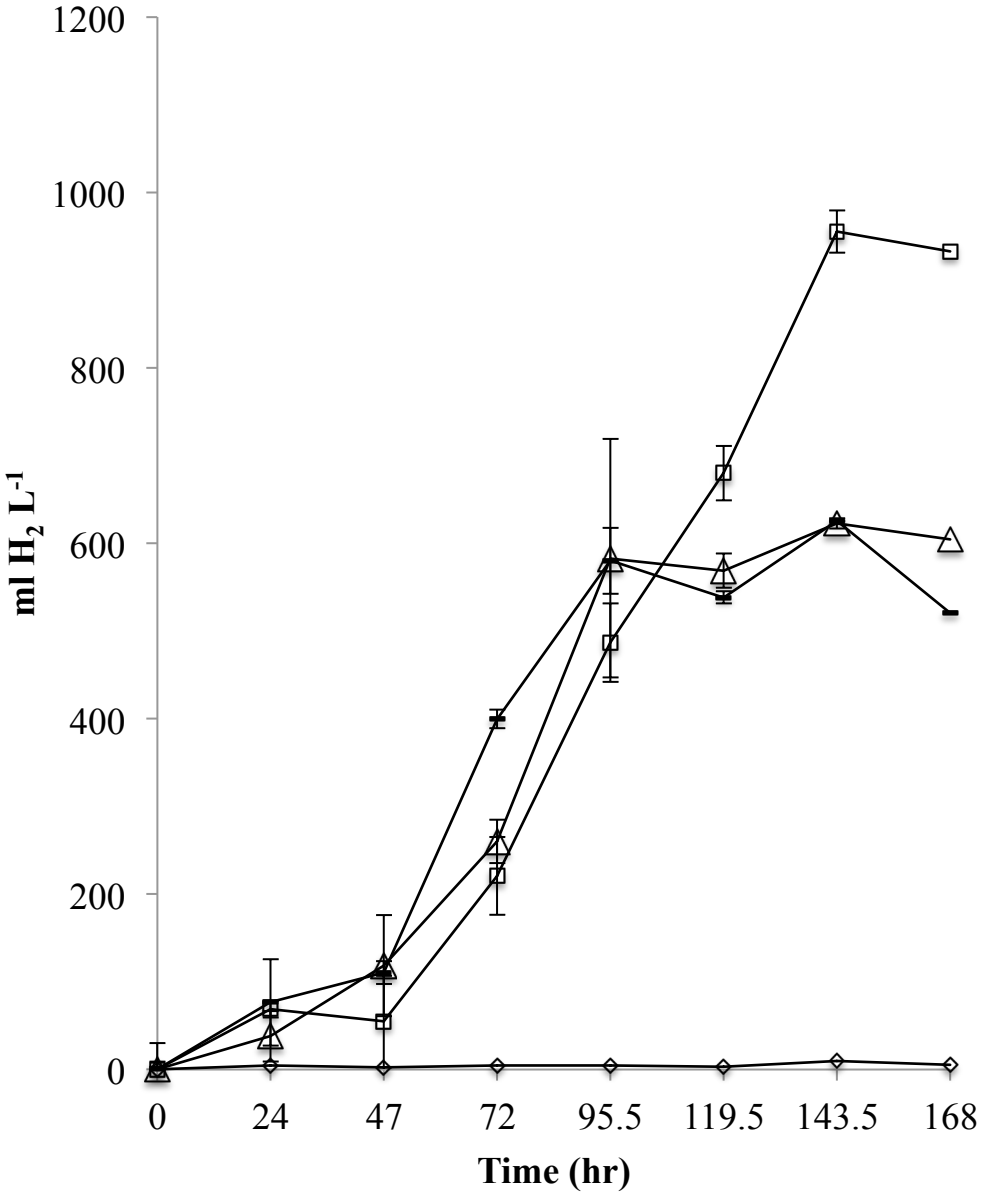
Sweet sorghum is an "energy crop" that is gaining attention as a potential feedstock for renewable energy because of its high yield of biomass and fermentable sugars (12). The constitutive composition of the plant is about 50 % sugars (glucose, xylose, cellulose and xylan) (3, 12), and therefore is a potential feedstock for H_2 production. When a mixed microbial community was used as inoculum with SSJ only 126 ml of H_2 was produced (100). Additionally, previous research showed that *Caldicellulosiruptor saccharolyticus* and *Ruminococcus albus* produced H_2

Table 7. A summary of the performance of *Anaerobaculum hydrogeniformans* when grown on glucose, clarified brewer wastewater (CBWW), clarified brewer wastewater diluted 1:1 (CBWW (1:1)), sweet sorghum juice (SSJ). clarified whey water (CWW), clarified raw sewage (CRS) and anaerobic digestor sludge (AD).

	ml H_2 L^{-1}	ml H_2 g^{-1} COD	R_{max} (ml H_2 L^{-1} hr^{-1})	COD_{Δ} (%)
Glucose	955	116	13	85
CBWW	623	6.68	9	99
CBWW (1:1)	625	26.4	9	75
SSJ	264	10.3	1.6	95
CWW	174	17.1	1.4	87
CRS	166	91.2	1.3	84
AD	22.6	2.81	-0.1	89

FIGURE 3. Amount of H_2 produced (ml H_2 L^{-1}) from cultures grown with mineral medium alone (◇), mineral medium and glucose (□), clarified brewer's wastewater alone (△) and clarified brewer's wastewater diluted 1:1 with minimal medium (-).

Error bars represent standard deviations of replicate samples (n=4)



from SSJ at a rate of approximately 10 mmol L^{-1} h^{-1} (12) and 0.7 mmol L^{-1} h^{-1} (80), respectively.

In this study, *A. hydrogeniformans* produced 0.22 mmol L^{-1} h^{-1}. This large discrepancy might be explained by the substrate utilization capacity of the different organisms. *C. saccharolyticus* can grow on glucose, xylose, cellulose and sucrose (12) and *R. albus* is a cellulolytic species that can also grow on glucose, xylose and sucrose (Ntaikou et al., 2008). *A. hydrogeniformans*, however, can only grow on glucose, but not xylose or sucrose.

As noted above, a 1:4 dilution of sweet sorghum juice (SSJ) in MM was used for this experiment, which had an initial glucose concentration of 2.0 g L^{-1}. *A. hydrogeniformans* produced 264 ml H_2 L^{-1} (FIGURE 4), corresponding to 10.3 ml H_2 per g COD reduced (Table7). There was 1.99 g L^{-1} of glucose present initially and *A. hydrogeniformans* consumed about 1 g L^{-1} using SSJ as a feedstock, translating to 14.6 H_2 $glucose^{-1}$. The amount of H_2 produced per glucose consumed surpasses the theoretical yield of 12 H_2 per glucose if glucose is totally oxidized to H_2 and CO_2 (113) and far surpasses the theoretical maximum of 4 H_2 per mol of glucose if H_2, CO_2 and acetate are produced (113). This suggests *A. hydrogeniformans* utilizes a carbon source other than glucose during the fermentation process as was observed during the CBWW fermentation. The amount of fatty acids in the SSJ did not increase or decrease after fermentation with the exception of the accumulation of acetate. Therefore *A. hydrogeniformans* did not use fatty acids as the source of carbon. The substrate for H_2 production has not been

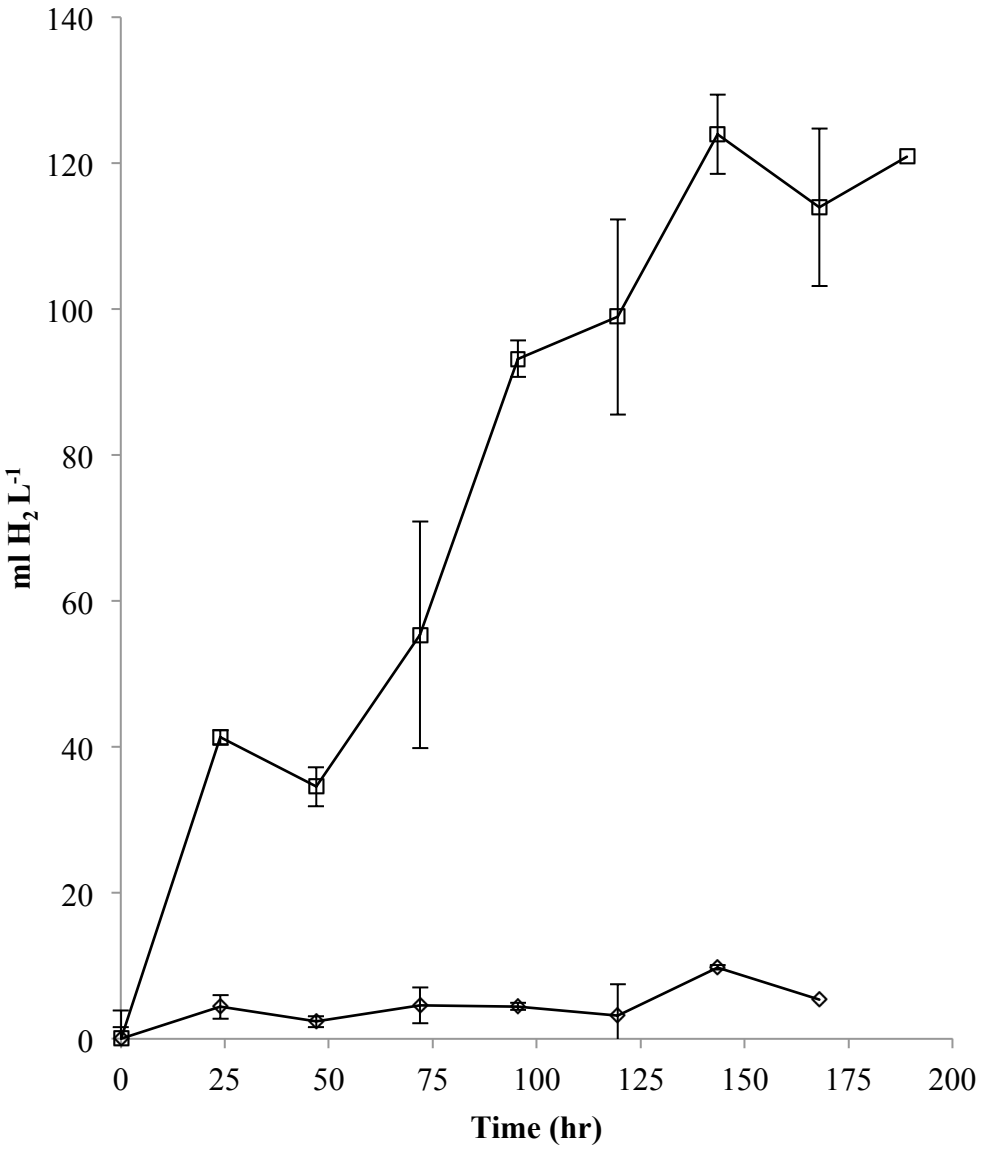
determined. The benefit of using *A. hydrogeniformans* is the reduction in COD of up to 95 %, which is much higher than the 20 % COD reduction previously reported (3), however, the H_2 produced g^{-1} COD reduced was only 10.3.

Cheese whey wastewater:

The cheese manufacturing industry generates large amounts of concentrated wastewater characterized by high biological and chemical oxygen demand that can cause organic loading problems in local sewage treatment systems (16). Carbohydrates (e.g. lactose), proteins and fats are the main contributors to the organic load of these wastewaters (102). The WW used in this study had a significantly lower COD (12 g L^{-1}) than that of previously reported wastewater (69 g L^{-1}) (16). When grown on crude cheese whey, *Clostridium saccharoperbutylacetonicum* yielded up to 7.89 mmol H_2 g^{-1} lactose and removed 97 % of the sugars (23). There was a negligible amount of glucose present in the WW (0.06 g L^{-1}) and *A. hydrogeniformans* does not grow on lactose (unpublished data), which suggests that the organism grew on proteins, amino acids and/or lipid components in the WW. Most of the literature available on cheese wastewater remediation focuses on H_2 production from lactose and not the other constitutive portions of the wastewater, such as using cultures of *Clostridium saccharoperbutylacetonicum*, which produced up to 7.89 mmol H_2 g^{-1} (24). *A. hydrogeniformans* produced H_2 at a maximum rate of 174 ml h^{-1} (FIGURE 5) and produced 17 ml H_2 g^{-1} COD reduced (Table 7). Future studies could be conducted using H_2-producing cocultures for total remediation of cheese wastewater.

FIGURE 4. Amount of H_2 produced (ml H_2 L^{-1}) from a time course study conducted with cultures of *A. hydrogeniformans* grown with minimal medium alone (◇) and sweet sorghum juice diluted 1:4 with minimal medium (SSJ) (□).

Error bars represent standard deviations of replicate samples (n=4)



Domestic wastewater/raw sewage:

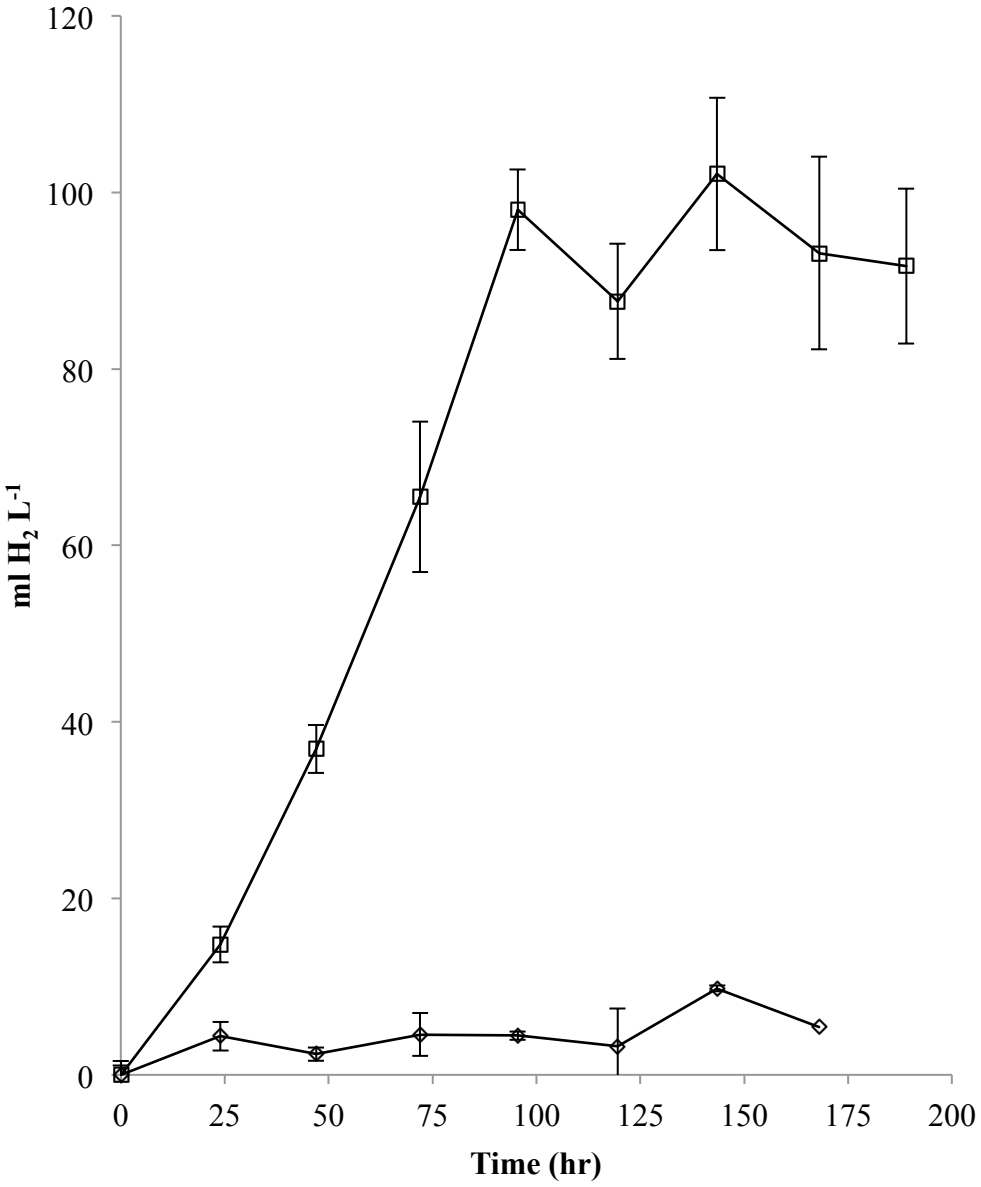
Domestic wastewater or raw sewage is an abundant, ubiquitous renewable waste stream. Surprisingly, limited research has been conducted on the fermentation of raw sewage. Previously conducted studies used concentrated raw sewage (RS) as a feed stock (119). The RS used in this study was first subjected to a clarification process (CRS). *A. hydrogeniformans* produced 166 ml H_2 L^{-1} (FIGURE 6) from RS and reduced COD by 84 %, which corresponded to 91.2 ml H_2 g^{-1} COD reduced. Aside from glucose, RS was the best overall H_2 producing feedstock per gram COD reduced. These values are significantly higher that that reported for bacterial fermentations or microbial electrolytic cells in which mixed cultures were used as the inoculum (63, 119). The pH of the CRS incubations was only reduced by 0.04 during fermentation, which is similar to previous findings (119). *A. hydrogeniformans* produced H_2 from compound(s) other than glucose because glucose was not detected in the RS. The VFA profile obtained for the RS grown cultures of *A. hydrogeniformans* was unchanged after growth with the exception of the accumulation of acetate. Therefore, *A. hydrogeniformans* was not growing on glucose or VFAs, but some other compound(s) that has yet to be determined. This is the first report of raw sewage being used for biological H_2 production via fermentation by a pure culture.

Anaerobic digestor sludge:

Waste-activated sludge, or anaerobic digestor sludge, from a wastewater treatment plant contains high levels of organic matter making it a potential substrate

FIGURE 5. Amount of H_2 produced (ml H_2 L^{-1}) from a time course study conducted with cultures of *A. hydrogeniformans* grown with minimal medium alone (◇) and clarified cheese whey wastewater (CWW) (□).

Error bars represent standard deviations of replicate samples (n=4)



for renewable H_2 (129). Anaerobic digestors act as the second stage of wastewater treatment in which microorganisms produce H_2 and CO_2 and, if properly maintained, the methanogenic population converts H_2 and CO_2 to CH_4 (119). There is limited data showing biohydrogen production from anaerobic digestor sludge (AD) using pure cultures. *A. hydrogeniformans* produced 22.6 ml H_2 L^{-1} (FIGURE 7) at a rate of 0.8 ml H_2 hr^{-1} and reduced COD by 89 % (Table 7). Cultures grown on AD produced the lowest amount of H_2 when compared to other waste feedstocks in this study (FIGURE 7). The same can be said for the amount of H_2 g^{-1} COD reduced (Table 7). Batch fermentations yielded approximately half the H_2 produced during the initial feedstock optimization studies. Freezing and thawing AD prior to the batch fermentation studies could have destroyed bioavailable compounds. The pH of unbuffered AD increased from 7.5 to 8.4 during fermentation, which indicates that *A. hydrogeniformans* may have been growing on proteins and/or amino acids and producing ammonia. A study conducted by Wang *et al*. showed H_2 production rates as high as 0.6 mmol H_2 g^{-1} COD for anaerobic digestor sludge using *Clostridium bifermentans* (129). However, it was not reported if the pH of the reactor was controlled by addition of a buffer. *A. hydrogeniformans* produced 0.12 mmol H_2 g^{-1} COD reduced. It should be noted that 8.4 is the upper limit of the pH range of growth for *A. hydrogeniformans* (Chapter 1).

FIGURE 6. Amount of H_2 produced (ml H_2 L^{-1}) from a time course study conducted with cultures of *A. hydrogeniformans* grown with minimal medium alone (◇) and clarified raw sewage (CRS) (□).

Error bars represent standard deviations of replicate samples (n=4)

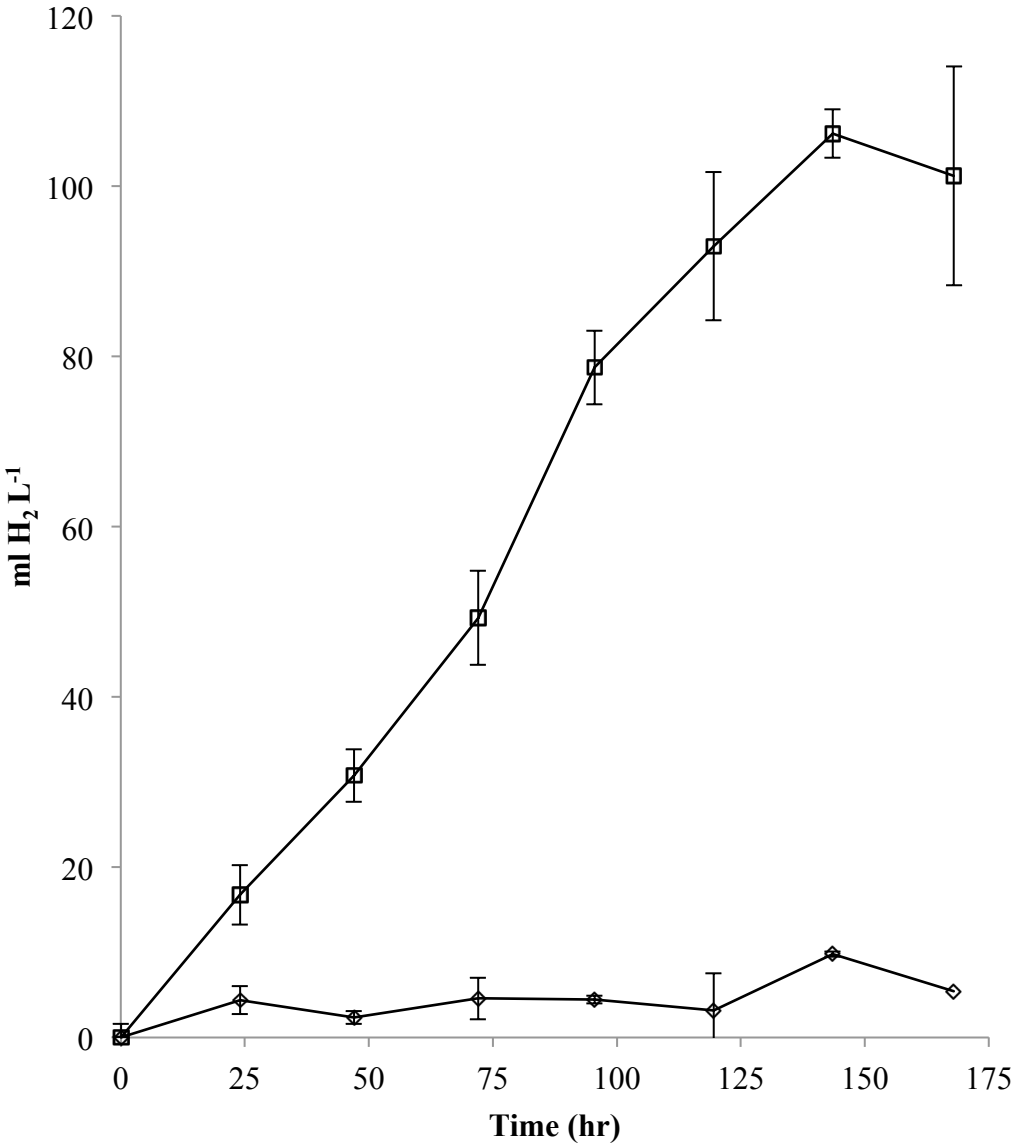
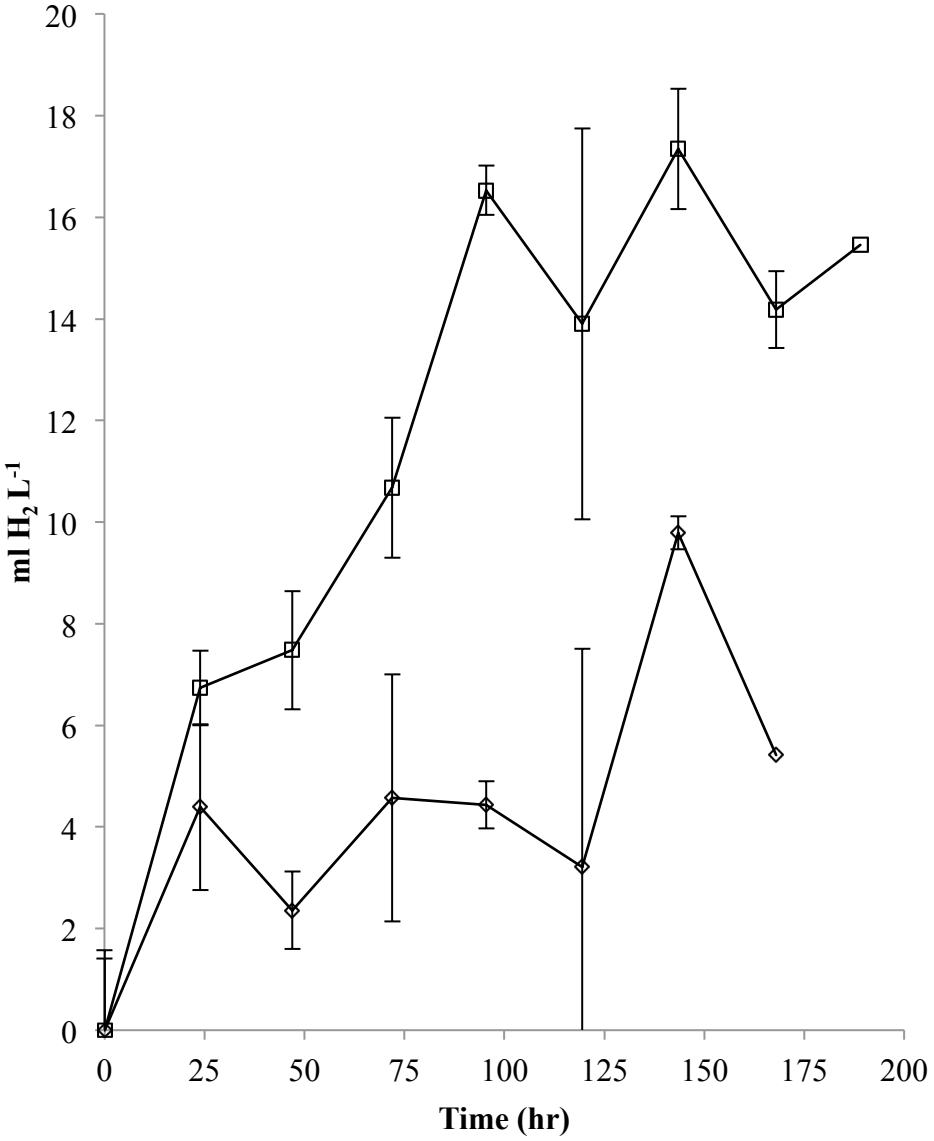


FIGURE 7. Amount of H_2 produced (ml H_2 L^{-1}) from a time course study conducted with cultures of *A. hydrogeniformans* grown with minimal medium alone (◇) and undiluted anaerobic digestor sludge (AD) (□).

Error bars represent standard deviations of replicate samples (n=4)



Conclusions

Biological H_2 production by pure cultures of *A. hydrogeniformans* can be achieved from various renewable wastewaters as well as pure glucose. *A. hydrogeniformans* produced close to the theoretical maximum of 4 H_2 per molecule of glucose when grown in a mineral medium containing glucose. Brewery wastewater seems to be the most promising waste feedstock because it can be used as both a carbon and nutrient source for cultures of *A. hydrogeniformans* and allowed for the production of H_2 at the same rate as glucose grown cultures. Sweet sorghum juice, cheese whey wastewater, raw sewage and anaerobic digestor sludge could all be used for the generation of H_2 as well. Anaerobic digestor sludge grown cultures of *A. hydrogeniformans* produced the least amount of H_2. As seen with the sweet sorghum juice, it is important to evaluate the proper ratio of nutrients and wastewater load when conducting studies with raw waste feedstocks. This study, using pure cultures of *A. hydrogeniformans*, offers a waste-remediation, energy-generating strategy for all feedstocks tested.

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Chapter 3

Influence of culture parameters and product inhibition on H_2 production by *Anaerobaculum hydrogeniformans*

Abstract

Anaerobaculum hydrogeniformans is a thermophilic bacterium that has potential for biological hydrogen (H_2) production. Results from a 5 L fermentor trial showed there were limiting factors in the fermentation broth. Only 50 % of the glucose was utilized before the culture of *A. hydrogeniformans* started to lyse. The aim of this study was to determine the optimal fermentation conditions for *A. hydrogeniformans* and elucidate limiting factors during fermentation. *A. hydrogeniformans* grew optimally and produced H_2 from 0.2 M to 0.6 M, and tolerated up to 1.2 M NaCl, making it the most NaCl tolerant organism producing approximately 4 H_2 per mole of glucose. *A. hydrogeniformans* was able to grow in the presence of initial sodium/potassium acetate concentrations of 240 mM. However, growth was limited compared to the control. Growth and H_2 production was observed in the presence of a much higher concentration of the inorganic salts than that of the Na-acetate. Therefore ionic strength was not responsible for the inhibition, but the presence of acetate. Inhibition of total glucose oxidation was also affected by a decrease in the culture pH, where by pH adjustment with additional buffer or bicarbonate during growth resulted in total glucose oxidation. Hydrogen removal from the headspace marginally increased glucose utilization and growth rates, but the total H_2 production rate was raised from 17.4 mmol H_2 L^{-1} min^{-1} to 39 mmol H_2 L^{-1} min^{-1} after the H_2 was removed. Glucose was not oxidized totally, in cultures with lowered H_2 partial pressures, supporting the hypothesis that increasing H_2 partial pressure is not the only limiting factor during fermentation. Growth rate

and glucose utilization increased when the concentration of yeast extract was increased in the medium. The increase in growth rate did not change the fermentation stoichiometry, but did increase the overall growth performance thus raising the volumetric H_2 production rate by 50 %.

Introduction

A hydrogen-based economy fueled by renewable sources including biomass, is getting increased attention. Currently, approaches to large-scale hydrogen (H_2) production include steam reforming of methane and coal gasification (115). These methods use fossil fuels and contribute to an increase in global CO_2 emissions. There have been many bacterial species capable of producing H_2 from sugar substrates of biomass wastes (30, 33). The current measure for the description of an optimal H_2 producer is the amount of H_2 produced per mole of substrate. The theoretical maximum ratio of H_2 produced per mole of glucose is 4, and is obtained when acetate is the sole end-product of fermentation (113). A relatively small number of microorganisms approach this limit due to thermodynamic limitations and metabolic requirements (31, 73).

The microorganisms that produce close to 4 H_2 per mol of glucose are generally thermophilic species, such as members of the *Thermotoga*, *Caldicellulosiruptor*, *Thermococcales* and *Pyrococcus* (135) (Table 8). Mesophilic bacteria, such as enterics and clostridia, can have relatively high H_2 productivities under certain conditions, but usually only produce about 1-2 H_2 per mol of glucose (11), unless genetic modifications are made, in which yields increased from about 0.09 to 0.8 mol H_2 per mol substrate (64). Thermophilic microorganisms have been hypothesized to be potential prolific H_2 producers, but they have not been studied to any extent from the perspective of genetic/metabolic engineering (11). Research into the optimization of fermentation parameters such as nutrient availability, pH,

Table 8. Various cultures grown on glucose under optimum temperatures, and the total H_2 yield (mmol L^{-1}) and H_2 produced per mole of glucose.

NR= not reported

Organism	Temp (°C)	Mode of operation	H_2 (mmol L^{-1})	Yield (H_2 glucose^{-1})	Reference
Clostridium butyricum	30	Continuous	78-111	1.4-2.0	(47)
Enterobacter aerogenes	37	Batch	50	0.56	(86)
Anaerobaculum hydrogeniformans	55	Batch	90	4.0	This study
Clostridium thermocellum	60	Continuous	NR	1.6	(58)
Thermotoga elfii	65	Batch	32	1.4	(42)
Caldicellulosiruptor saccharolyticus	70	Batch	60	4.0	(43)
Thermotoga neapolitana	77	Batch	45	3.9	(73)
Thermotoga maritima	80	Batch	70	4.0	(98)
Pyrococcus furiosus	100	Batch	NR	3.5	(49)

substrate concentration and organic/inorganic end product inhibition have been conducted on some cultures and each can effect the overall performance of H_2 producing microorganisms (30, 77).

Nutrient availability and substrate concentration have proven to be crucial factors in fermentation performance of thermophilic H_2 producers. Ferchichi *et al*. (24) showed that an increase in both H_2 production rate and yield of H_2 per mol of glucose could be raised dramatically in cultures of *Clostridium saccharoperbutylacetonicum* with yeast extract supplementation. An increase in rate of H_2 production was also seen in cultures of *C. saccharolyticum* and *Thermotoga elfii* when yeast extract or casamino acid concentrations were increased (121). Initial substrate concentration can influence fermentation performance. Cultures of *C. saccharoperbutylacetonicum* responded with a 33 % increase in yield of H_2 per glucose and a 130 % increase in H_2 production rate when the initial glucose concentration was doubled (24).

Controlling the pH of the fermentation medium contributes to the rate of H_2 production and substrate utilization. Addition of a extra buffer or bicarbonate allowed cultures of *Thermotoga neapolitana* to utilize all of the glucose in the medium and subsequently produce more H_2. However, the yield of H_2 per mol of glucose was unchanged (73). Acetate inhibits the growth of many microorganisms (55), and it may act as an physiological uncoupler of growth when in both the undissociated or dissociated form (7, 130). van Neil *et al.* (122) showed that *C.*

saccharolyticus exhibited Na-acetate inhibition to the same extent as inorganic salts such as NaCl and concluded that the ionic strength of the Na-acetate was the inhibitory agent and not necessarily the concentration of acetic acid in the medium

Sensitivity to H_2 partial pressures (P_{H2}) seems to be one of the most common traits among H_2 evolving microorganisms. An increase in P_{H2} can result in the production of alternate reduced end products, changing the fermentation stoichiometry (11). In order to overcome this obstacle, researchers have developed methods such as negative headspace pressure (65), gas sparging (70, 78) and gas stripping (52, 134) methods to increase overall H_2 production. The use of total pressure reduction in a bioreactor by vacuum has proven to be a useful (65), but this increased the risk of contamination (50) and energy required to maintain an underpressurized system. Nitrogen sparging has been used in many studies and has shown to be beneficial in lowering the P_{H2} enough to increase the yield of H_2 per mol of substrate (77). The use of CO_2 stripping has also been used to significantly increase H_2 yields by lowering P_{H2} in mixed cultures (52), but a major drawback in using CO_2 as a stripping gas is the formation of bicarbonate, which is accompanied by acidification of the medium and increased osmotic pressures (134). Wilquist *et al*. (134) used this method on cultures *C. saccharolyticus* that tolerated NaCl up to 440-425 mM but with negative effects due to inhibitory solute concentrations that rose to around 0.25 osm/kg H_2O. It has been hypotesized that cultures of *T. neapolitana* or *P. furiosus* would be better suited for CO_2 sparging because they can tolerate solute concentrations of 0.46 M (0.92 osm/kg H_2O) (11) and 0.5 M (1

osm/kg H_2O) (90) respectively (135), however this hypothesis has yet to be tested. If a culture is capable of withstanding high concentrations of solutes, then CO_2 stripping may be a viable technique for lowering the P_{H2}.

A novel thermophilic organism under consideration for biological hydrogen production is *Anaerobaculum hydrogeniformans* (our paper). *A. hydrogeniformans* is a rod-shaped, gram-negative bacterium that grows optimally at 50-55 °C, and can oxidize glucose to H_2, acetate and CO_2 in a ratio close to the theoretical maximum (our paper) and produce hydrogen from renewable waste streams such as raw sewage (67). The goal of this work was to determine various limiting factors, such as nutrients, solute concentration, acetate tolerance, and hydrogen inhibition during the fermentation of glucose. By elucidating the limiting factors in fermentation conditions when using *A. hydrogeniformans* it is possible to develop a targeted approach to fermentor/process design and medium optimization for future large-scale studies.

Materials and methods

Culture and medium

Anaerobaculum hydrogeniformans strain OS1 (DSM 22491^T) was grown in a mineral medium containing (L^{-1}): 10 mL mineral solution (111), 10 mL vitamin solution (111), 10 mL trace metal solution (111), 1 g yeast extract (Difco), 10 g TES (Research Organics) and 10 g NaCl. The medium (pH 7.5) was prepared using strict anaerobic technique (5) and contained an initial headspace of 100 % N_2. Cultures were grown at 55 °C and fed glucose added from a sterile anaerobic stock solution to a final concentration of about 22 mM unless otherwise stated.

5 L batch fermentation

A. hydrogeniformans was grown in a 5 L BioFlo 110 fermentor (New Brunswick Scientific) with a working volume of 3 L. The medium used for the fermentation was the same as described above. 6 g L^{-1} of glucose was used as the carbon and energy source and added from a sterile anaerobic stock to the fermentation vessel after autoclaving. The pH of the fermentation broth was not adjusted during growth. pH measurements were taken using a gel filled pH probe (New Brunswick Scientific), and pH data was collected using the AFS-Biocommand bioprocessing software suite version 2.6 (New Brunswick Scientific). A heating blanket and cooling finger connected to an external water supply controlled the vessel temperature. 100 % N_2 was sparged in the fermentor using the sparging tubing supplied with the fermentor (New Brunswick Scientific) to ensure

positive pressure was maintained. Headspace gas was released through an exhaust port that was connected to a 5 mL glass luer lock syringe filled with glass wool and retrofitted with a tubing connector. Gas samples were collected by flushing the headspace out gas into 10 ml stoppered and crimp sealed serum bottles (Wheaton). A vent needle was placed in the stopper and the gas was allowed to flush for approximately 5 min to ensure sample uniformity. Liquid samples were taken out of the sample port, which was fitted with a one-way luer lock stopcock. The collection tubing was flushed with 5 ml of fermentation broth before samples were collected to ensure sample uniformity. Optical density, H_2 and acetic acid concentrations were quantified for each time point. The amount of H_2 produced was calculated by correlating the % H_2 measured in the 10 ml stoppered bottle to the amount of headspace in the fermentation vessel.

Growth conditions

Unless otherwise stated, cultures were grown in 125 mL stoppered and crimp-sealed bottles, using 20 mL of medium was the working volume for all conditions tested. Control cultures consisted of the medium described in section 2.1. Oxygen tolerance was tested by the addition of O_2 into the headspace from a sterile stock to final gas phase concentrations of 10, 20, 50 and 70 μmol L^{-1}. The effect of NaCl or KCl was examined a minimum inhibitory concentration (MIC) assay by the addition of 0, 0.09, 0.17, 0.32, 0.51, 0.68, 0.86, 1.2 and 1.54 M NaCl or KCl in the medium described in section 2.1. 10 ml of medium was anoxically

prepared and placed in 18 mm stoppered and crimp-sealed tubes containing a 100 % N_2 gas phase for the MIC assay. Cultures were allowed to incubate for 14 days at 55 °C after which time cultures growth and H_2 production was quantified. A time course experiment was conducted in which the minimum, mid-point and maximum concentration of NaCl was supplemented in the medium (0.2, 0.6 and 1.2 M, respectively). Inhibition by sodium acetate was first quantified by means of an MIC assay in which initial concentrations of 0, 8, 17, 50, 83, 167, 250 and 333 mM Na-acetate was added prior to inoculation. The MIC assay was conducted in the same medium, tubes, gas phase, temperature and time used in the NaCl MIC assay. After 14 days of incubation H_2 and growth was quantified. The MIC results led a time course assay in which either 0 or 240 mM Na or K-acetate was added from a sterile anoxic stock prior to inoculation. During the time course experiment H_2, growth and glucose utilization was quantified. The inhibition of glucose oxidation due to lowering of the pH was tested by the addition of 1 ml 1.5 × TES buffer solution (pH 7.5) or the addition of 1 ml 1 M $NaHCO_3$ from sterile stock solutions at 74 hrs. To test the effect of low H_2 partial pressures H_2 was evacuated and exchanged with 100 % N_2 after 74 hr of growth. Yeast extract additions were conducted prior to inoculation to a final concentration of 0, 2 or 8 g L^{-1}.

Analytical methods

Growth was monitored using a Beckman DU series 600 Spectrophotometer (Beckman) set to a wavelength of 600 nm. Glucose concentrations were quantified

using the glucose-oxidase reaction with the PGO Enzyme assay kit (Sigma). Acetate was quantified using a Shimadzu GC-8A equipped with a flame ionization detector (Shimadzu). Compounds were separated with a glass column (2 m x 5 m) packed with 80/120 mesh carbopack with 4 % carbowax (Supelco). The inlet and detector were both set to 200 °C. The column temperature was held at 155 °C. Ultra high purity He was used as the carrier gas. Headspace pressure was quantified by the use of a digital pressure gauge (Cole-Parmer). Hydrogen was quantified with a Shimadzu 8A gas chromatograph equipped with a thermal conductivity detector. Gasses were separated with a stainless steel column (2 m x 1/8 in) packed with a 50/80 mesh Porapak Q (Supelco). The inlet and detector were bet at 110 °C and the column temperature was held at 100 °C. Data was analyzed using a C-R8A Chromatopac Integrator (Shimadzu). All chemicals used in this study were obtained from Sigma-Aldrich unless otherwise specified. All gas measurements were made at STP.

Data analysis

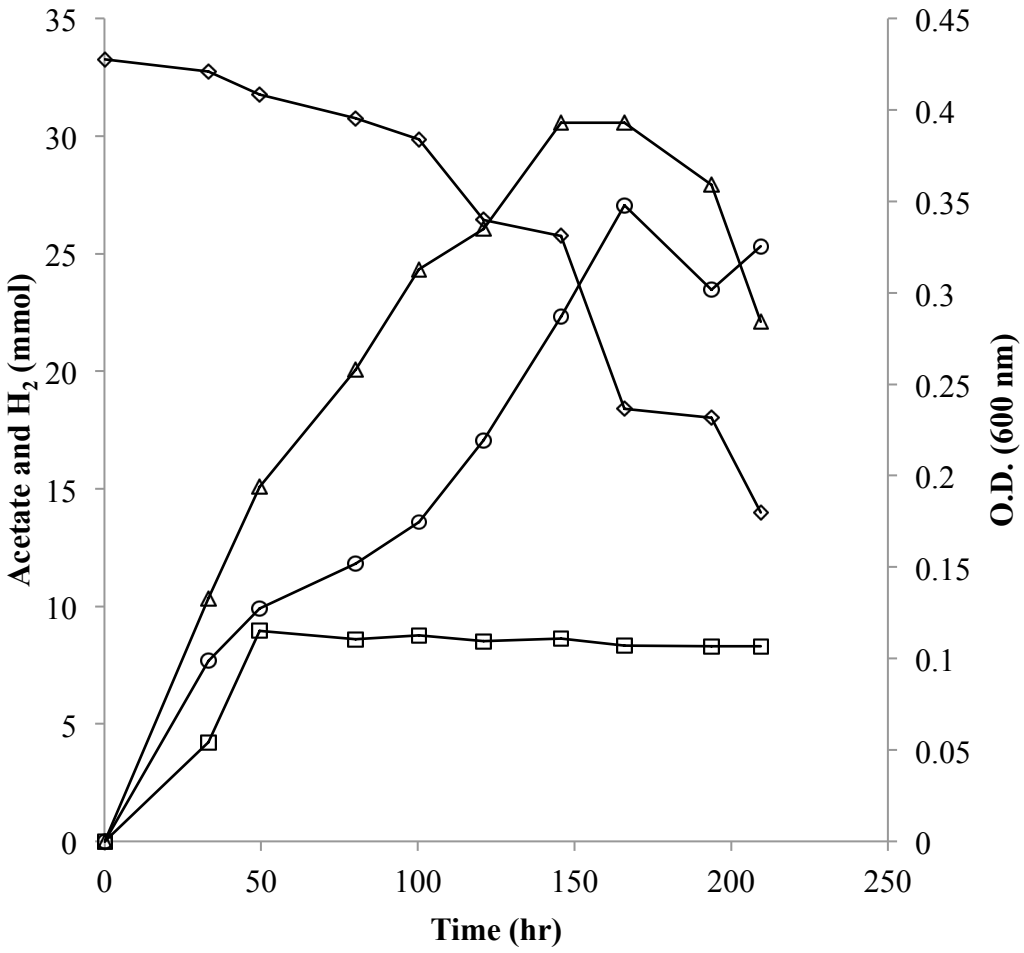
Maximum H_2 production, glucose utilization and growth rates were calculated during the exponential growth phase of *A. hydrogeniformans* from 50 to 96 hrs. The overall volumetric productivity of H_2 production was calculated as the total H_2 produced (mmol L^{-1}) over the time span of the entire experimental set as previously described (73). Total glucose oxidized, H_2 produced and final yield of mol H_2 mol glucose^{-1} were calculated after experimental sets were completed.

Results and Discussion

5 L batch fermentation

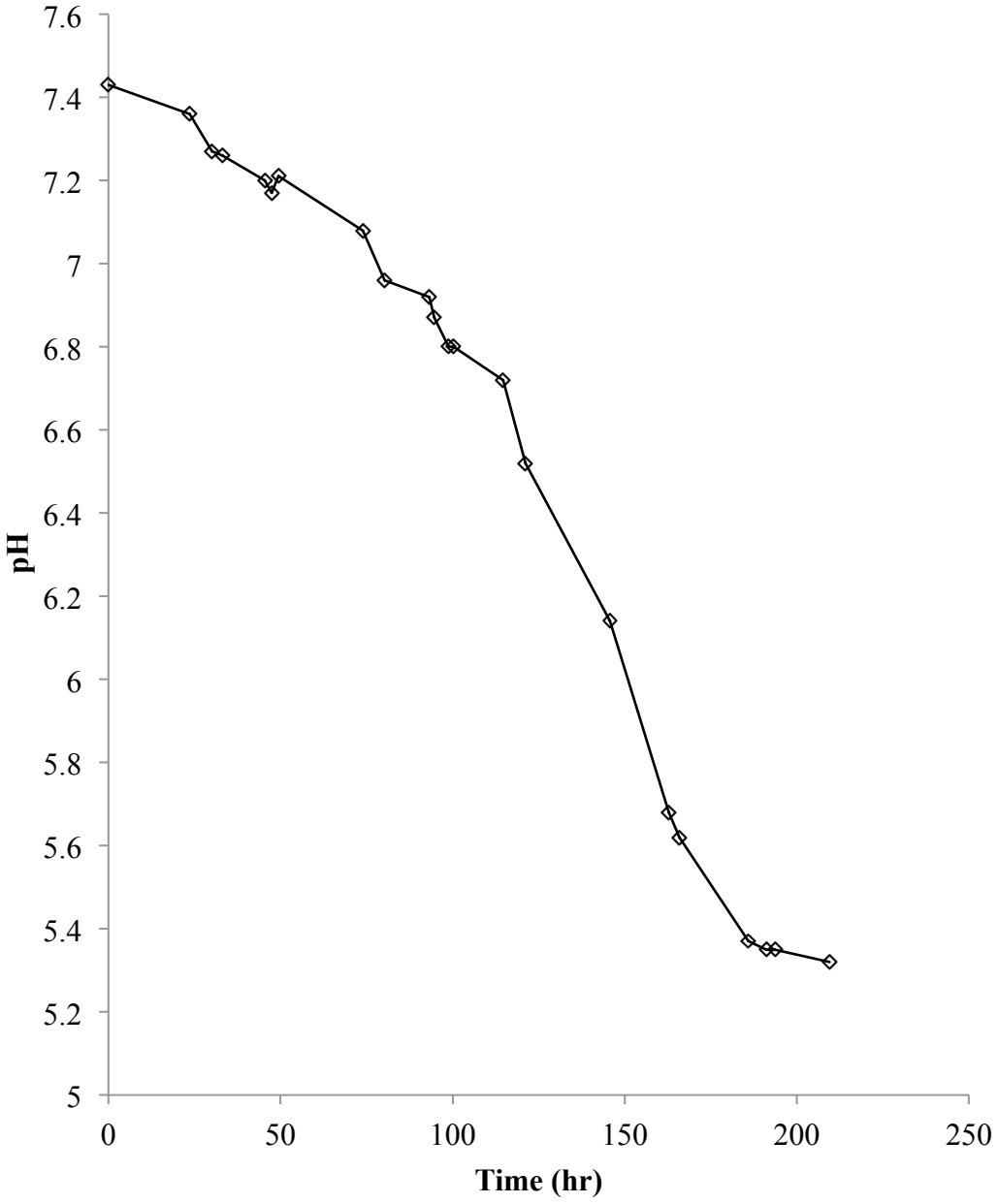
The results from the 5 L fermentor run are summarized in FIGURE 8. Growth, glucose oxidation and product formation started by the first time point (24 hr). Hydrogen production remained constant at 8 mmol L^{-1} by 48 hrs until the completion of the experiment. Actual total H_2 produced could not be quantified because the flow rate of the N_2 into the reactor was not measured before starting the reactor. However, it is possible to qualitatively see that H_2 was being produced at about the same rate N_2 was being added to the reactor, as evident by the steady H_2 measured in the exhaust. There was a steady decrease in glucose concentration throughout the experiment, which was accompanied by an increase in acetate concentration in the fermentation medium. Throughout the experiment about 2 mol acetate was produced per mol of glucose, which is indicative of the fermentation stoichiometry previously described for *A. hydrogeniformans* (Chapter 1). Although glucose was steadily oxidized throughout the experiment, only half of the glucose was consumed by *A. hydrogeniformans* and a decrease in cell density was observed prior to total glucose oxidation. This could have been due to a drop in pH as a result of acetic acid accumulation, which dropped below the known pH range for growth of *A. hydrogeniformans* (FIGURE 9). Another possible explanation for the decrease in cell numbers could have been due to nutrient limitation. Whatever the cause, the observation that there are limitations that need to be determined to ensure optimal cell growth and product recovery needs to be addressed.

FIGURE 8. Acetate (mmol) (○), H_2 (mmol) (□), glucose (mmol) (◇) and optical density (600 nm) (△) quantified from the 5 L batch fermentation experiment using glucose as the substrate for the H_2 producing anaerobe *A. hydrogeniformans*.



*H_2 measurements were made from the exhaust flow from the fermentor, however the flow rate of the exhaust was not quantified, thus after 48 hr $H_2$was being produced at the same rate as the exhaust.

FIGURE 9. pH measurements taken during the batch fermentation of glucose in a 5 L reactor.



Fermentation parameter optimization

Overall productivity and H_2 yield per mol glucose

The overall productivity of H_2 production (Q_{H2}) remained steady in most conditions tested (0.489 ± 0.038 mmol L^{-1} hr^{-1}). There was an increase in the overall productivity when the conditions for growth proved to be more favorable, such as increasing the yeast extract in the culture medium from 2 g L^{-1} to 8 g L^{-1} (0.950 ± .001 mmol L^{-1} hr^{-1}). Increasing the initial glucose from 3 g L^{-1} to 6 g L^{-1} (0.747 ± 0.009 mmol L^{-1} hr^{-1}) or 9 g L^{-1} (0.785 ± 0.006 mmol L^{-1} hr^{-1}) also increased the Q_{H2}. In conditions that exhibited cell inhibition, such as growth in the presence of 7 % NaCl (0.168 ± .001 mmol L^{-1} hr^{-1}), initial concentrations of 240 mM Na-acetate (0.326 ± 0.002 mmol L^{-1} hr^{-1}) or 240 mM K-acetate (0.325 ± 0.023 mmol L^{-1} hr^{-1}) the Q_{H2} was decreased. The Q_{H2} for calculated for *A. hydrogeniformans*, under the control conditions established in the Material and Methods section, was about half of that reported for *T. neapolitana* (73). However when conditions were more favorable the overall Q_{H2} was higher than that reported for *T. neapolitana* (73). In all conditions tested in this study *A. hydrogeniformans* produced close to the theoretical maximum of 4 mol H_2 per mol glucose. Table 9 shows the yields observed under the various conditions tested. When cultures did not have high growth, glucose utilization or H_2 production, *A. hydrogeniformans* still produced 3.17 ± 0.12 mol H_2 per mol glucose and a maximum of 3.99 ± 0.05 mol H_2 per mol glucose. These data are indicative of the range of conditions that *A. hydrogeniformans* can be cultured in without significantly altering the fermentation stoichiometry.

Effect of controlled pH, H_2 partial pressure, initial glucose and nutrients

Glucose remaining after growth ceased under most of the conditions tested led to the assumption that glucose oxidation was inhibited in cultures of *A. hydrogeniformans*. Experimental sets were created in which pH, H_2 partial pressure and nutrient limitations were tested to elucidate the limiting factors. Inhibition from lowering of the pH was tested by pH adjustment experiments, of which growth and H_2 results are shown in FIGURE 10. These assays were conducted by an injection of 1 ml anoxic 1.5× TES buffer (pH 7.5) or 1 ml of 1 M $NaHCO_3$ at 74 hrs of growth. Both methods of adjusting the pH influenced the amount of glucose consumed and subsequent H_2 production. When the pH was adjusted all available glucose was consumed in roughly in 44 hrs (FIGURE 10) and there was an increase in the amount of total H_2 produced (86.25 ± 0.06 mmol H_2 L^{-1} for the buffer addition; 85.52 ± 0.03 mmol H_2 L^{-1} for the $NaHCO_3$ addition) when compared to the unamended control (73.69 ± 3.9 mmol H_2 L^{-1}). There was also an increase in glucose oxidation and growth rate (Table 9). After the assay was terminated pH measurements were made to verify that the pH of the medium was altered. The pH for the control cultures was 6.25 ± 0.02 where the pH for the 1.5× buffer was 6.98 ± 0.01 and 7.12 ± 0.01 for the $NaHCO_3$ injected cultures. This allowed for the generation of more cell mass as well as an increase in H_2 production (Table 9). Munro et al. (73) made a similar observation in which cultures of *T. neapolitana* were tested in the same fashion and described increased glucose oxidation and H_2 production. However, the yield of H_2 per glucose was unchanged. Similar results are reported here in that controlling of the pH did not result in a higher yield

Table 9. Maximum H_2 production rate ($MaxH_2$) during exponential growth phase (mmol L^{-1} hr^{-1}), overall volumetric H_2 productivity spanning the time frame of entire experiment (Q_{H2}) (mmol L^{-1} hr^{-1}), glucose utilization rate (mmol L^{-1} hr^{-1}), growth rate (min^{-1}), final yield of H_2 (mmol L^{-1}), initial concentration of glucose in the culture medium (mmol L^{-1}), total glucose utilized (mmol L^{-1}) and yield of H_2 per glucose (mol mol^{-1}) under all conditions tested for *Anaerobaculum hydrogeniformans.*

Control cultures were unamended and grown in the conditions and medium described in the materials and methods with 3 g L^{-1} glucose, 2 g L^{-1} yeast extract and 1 % NaCl. Hydrogen was allowed to accumulate in the headspace and the pH was not adjusted.

ND = not determined

	Max H_2	Q_{H2}	Glucose utilization rate	Growth rate	Final H_2	Initial glucose	Glucose consumed	Yield
Control	0.89 ± 0.01	0.452 ± 0.003	0.149 ± 0.01	1.26 ± 0.42	73.69 ± 3.9	17.71 ± 0.32	16.65 ± 0.36	3.76 ± 0.20
3.5 % NaCl	1.04 ± 0.05	0.443 ± 0.002	0.159 ± 0.06	1.68 ± 0.60	71.08 ± 6.2	22.80 ± 0.26	16.33 ± 0.25	3.36 ± 0.52
7. 0 % NaCl	0.087 ± 0.02	0.168 ± 0.001	0.029 ± 0.01	0.54 ± 0.06	27.95 ± 2.4	22.78 ± 0.30	8.22 ± 0.46	3.44 ± 0.06
8 g L^{-1} yeast extract	1.20 ± 0.01	0.950 ± 0.001	0.280 ± 0.06	2.31 ± 0.54	87.20 ± 1.2	22.80 ± 0.01	22.80 ± 0.01	3.99 ± 0.05
H_2 removed	ND	ND	1.79 ± 0.02	1.88 ± 0.12	ND	23.56 ± 0.29	18.08 ± 1.22	ND
Plus 1.5 × buffer at 74 min	0.987 ± 0.01	0.520 ± 0.010	0.308 ± 0.02	1.67 ± 0.02	86.25 ± 0.06	22.74 ± 1.70	22.74 ± 1.70	3.75 ± 0.12
Plus 1 ml 1 M $NaHCO_3$ at 74 min	0.994 ± 0.01	0.509 ± 0.010	0.285 ± 0.01	1.32 ± 0.18	85.52 ± 0.03	22.68 ± 1.01	22.68 ± 1.01	3.77 ± 0.09
6 g L^{-1} glucose	1.101 ± 0.01	0.747 ± 0.009	0.216 ± 0.02	1.40 ± 0.12	71.67 ± 1.23	34.48 ± 0.84	18.13 ± 0.08	3.85 ± 0.12
9 g L^{-1} glucose	1.30 ± 0.02	0.785 ± 0.006	0.366 ± 0.03	1.43 ± 0.05	75.37 ± 0.95	65.36 ± 0.22	18.53 ± 0.10	3.89 ± 0.09
240 mM initial Na-acetate	0.357 ± 0.07	0.326 ± 0.002	0.191 ± 0.03	0.99 ± 0.30	38.58 ± 2.53	22.73 ± 0.36	11.73 ± 0.46	3.17 ± 0.12
240 mM initial K-acetate	0.503 ± 0.02	0.325 ± 0.023	0.077 ± 0.02	0.88 ± 0.30	38.04 ± 2.77	22.56 ± 0.25	10.64 ± 0.86	3.58 ± 0.03

of H_2 per glucose (3.75 ± 0.12 in buffer added, 3.77 ± 0.09 in $NaHCO_3$ added; 3.76 ± 0.20 in unamended), however, it did show that the controlling of pH in fermentations using *A. hydrogeniformans* is a critical component, as observed previously in studies with *T. neapolitana* (73).

Hydrogen partial pressure (P_{H2}) plays a significant role in the generation of H_2 by hydrogenic microorganisms (59, 77, 122). Cultures of *A. hydrogeniformans* were assayed to see if P_{H2} effected growth and glucose oxidation. When H_2 was removed from the headspace glucose utilization and growth increased in pre H_2 removal vs. post H_2 removal (FIGURE 11). Not all of the glucose was utilized in the H_2 evacuated cultures, supporting the hypothesis that there are other limiting factors in fermentations. The H_2 production rate prior to H_2 removal was 1.19 mmol H_2 L^{-1} min^{-1}, after which the production rate increased to 2.70 mmol H_2 L^{-1} min^{-1}. This data is promising in that the rate in which H_2 is produced can be significantly increased by the removal of H_2 during fermentation.

Nitrogen, namely from yeast extract, in the medium has been noted to have an effect on the production of H_2 (24, 98, 121). Increasing the concentration of yeast extract did not alter the fermentation performance of *C. saccharolyticus* or *Thermotoga elfii* (121), even though yeast extract is required for the growth of *T. elfii* (87, 121). *A. hydrogeniformans* also requires yeast extract for growth. Increasing the concentration from 2 g L^{-1} to 8 g L^{-1} increased all of the factors tested (Table 9). However, the increase in growth rate did not change the H_2 yield, which agrees with the study on *T. elfii* and *C. saccharolyticus*, but did increase the growth rate, increasing the volumetric H_2 production rate by 50 %.

FIGURE 10. Glucose concentration in pH unamended control (◇) and pH adjusted cultures of *A. hydrogeniformans* with either 1.5 × buffer (pH 7.5) (□) or 1 ml addition of anoxic 1 M $NaHCO_3$ (△) after 74 hr of growth.

Error bars represent standard deviation of replicate cultures (n=3).

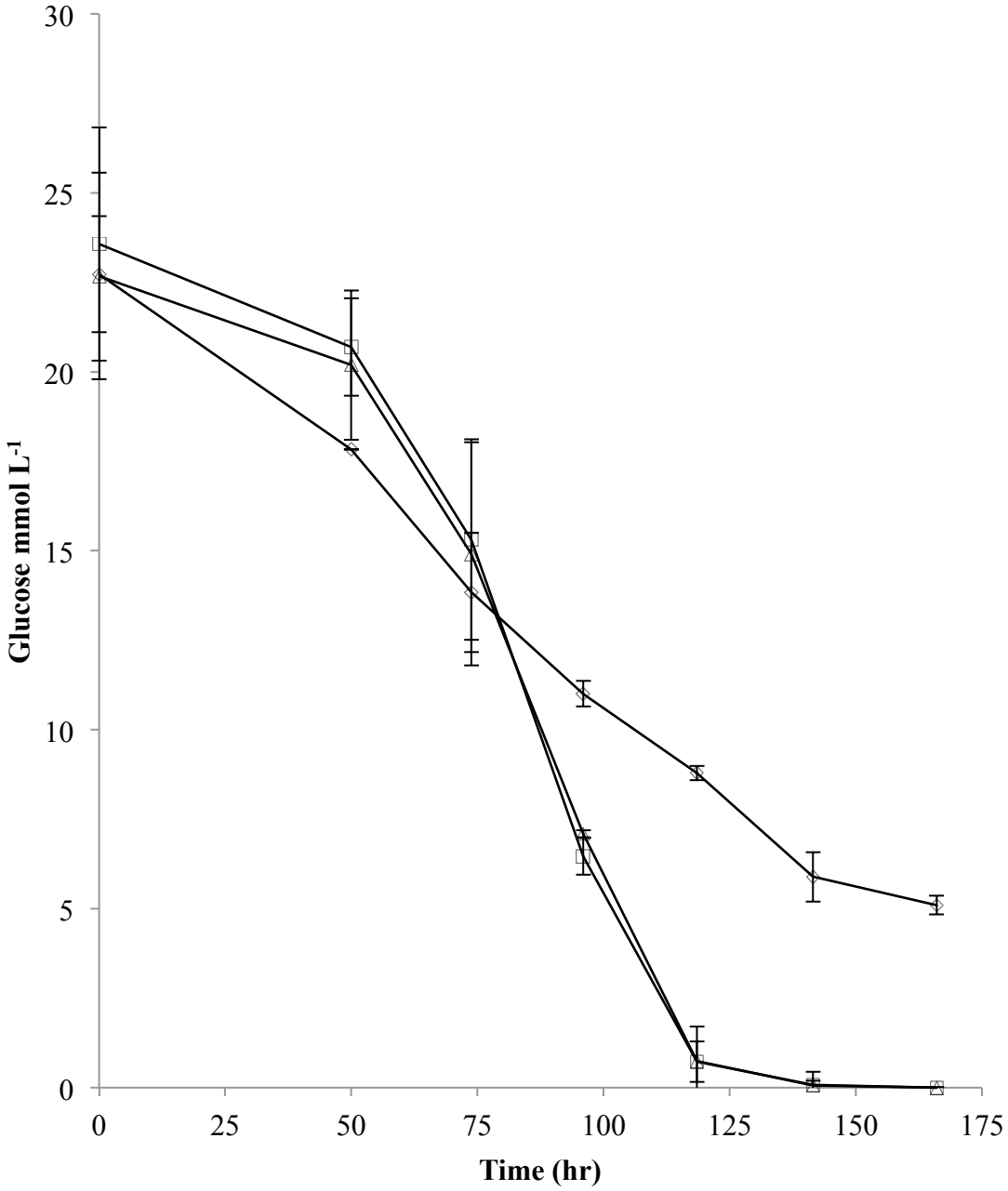
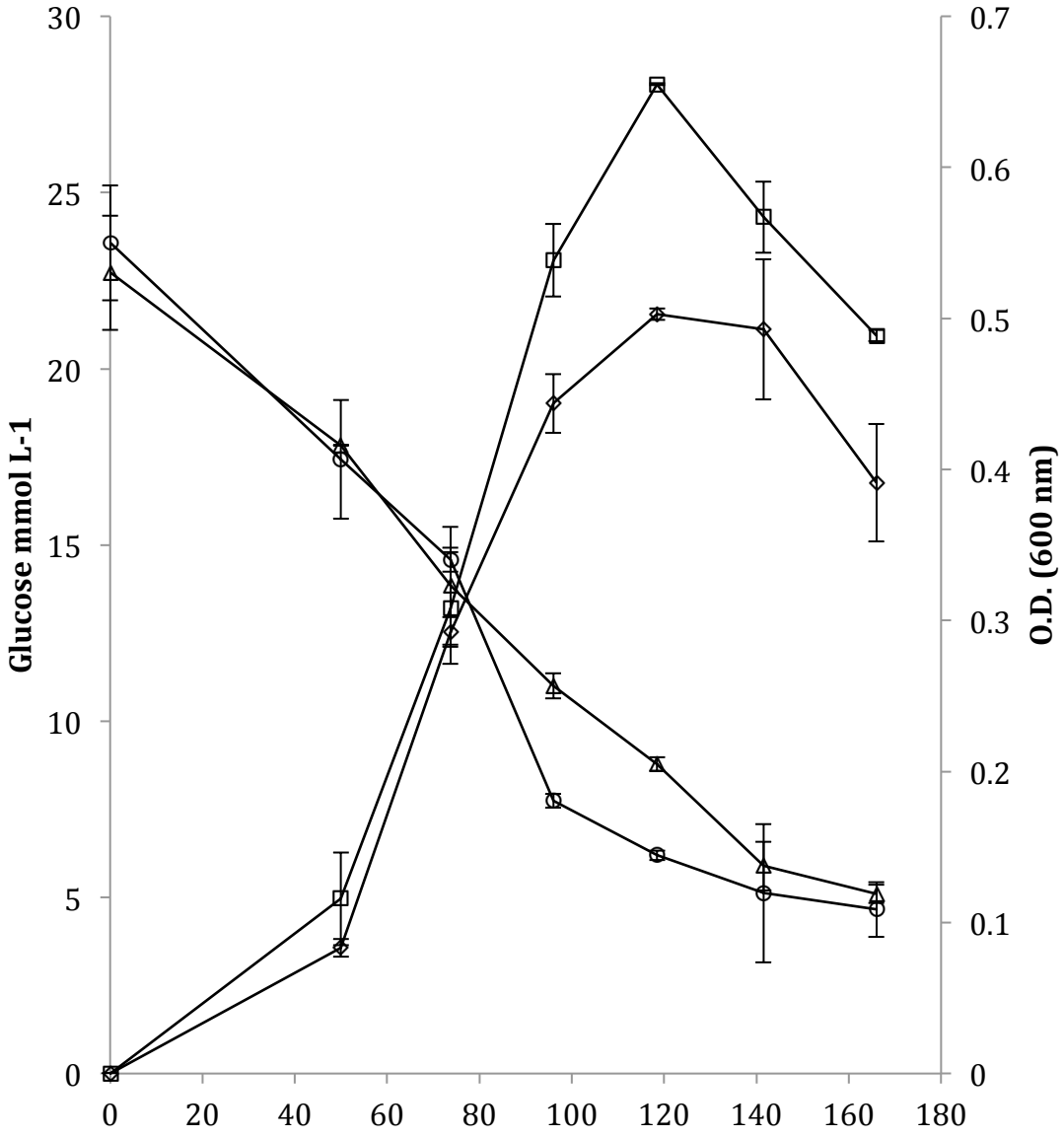


FIGURE 11. Time course of growth and glucose utilization by A. hydrogeniformans when H_2 was evacuated at 74 hrs and the unamended control; (△) glucose concentration in control cultures; (○) glucose concentration in H_2 evacuated cultures; (◇) growth in control cultures; (□) growth in H_2 evacuated cultures.

Error bars represent standard deviation of replicate cultures (n=3).



Initial glucose concentration has been noted to play a large role in the fermentation performance of various microorganisms. Ferchichi et al. (24) showed that cultures of *Clostridium saccharoperbutylacetonicum* responded with a 33 % increase in yield of H_2 per glucose and a 130 % increase in H_2 production rate when initial glucose concentrations were raised from 20 g L^{-1} to 40 g L^{-1}. A similar trend was observed in cultures of *T. maritima* and *T. neapolitana* (79). *A. hydrogeniformans* was grown with an initial concentrations of either 17, 34 and 65 mM glucose. There was an increase of 37 % and 28 % in glucose utilization rates when the 34 and 65 mM glucose grown cultures compared to that of the 17 mM control respectively. Interestingly, the 34 and 65 mM glucose grown cultures oxidized roughly the same amount of glucose (18.13 ± 0.08 in the 34 mM; 18.53 ± 0.50 in the 65 mM); where as the 17 mM glucose grown cultures only oxidized 16.65 ± 0.36 mM. The reason for this is not yet known, but the increase in growth rate might suggest that glucose concentration could play a role in the uptake of glucose by *A. hydrogeniformans*. The total amount of H_2 produced in the 17, 34 and 65 mM glucose grown cultures was about the same in all three conditions (Table 9). This could have been due to nutrient availability, culture pH or H_2 accumulation during fermentation.

Inhibition by NaCl or KCl

The ability to withstand high solute concentrations within a fermenting system increases the options for H_2 removal from the system by CO_2 stripping (70, 134). Another benefit of this technology after the H_2 is separated is the production of a 100 % CO_2 gas phase, allowing for an inexpensive treatment process that will sequester any CO_2

emission from the fermentation process (120). CO_2 stripping is not without it's disadvantages. With the addition of CO_2 as the stripping gas, bicarbonates are produced in the culture medium, which increases the solute concentration, which can lead to culture inhibition and even cell lysis (134). The thermophile *Caldicellulosiruptor saccharolyticus* can only tolerate salt concentrations up to 0.4-0.425 M (122) which was not enough to withstand the solute concentration CO_2 stripping introduced (134). The hyperthermophiles *T. neapolitana* and *Pyrococcus furiosus* can grow in the presence of 0.46 M NaCl and 0.5 M NaCl respectively (11, 90), which lead to the hypothesis that both *T. neapolitana* and/or *P. furiosus* could be tolerant to high enough solute concentrations that CO_2 stripping may be a viable technique for controlling P_{H2} during fermentations (135). The minimum inhibitory concentration (MIC) assay of NaCl conducted with *A. hydrogeniformans* showed growth and H_2 production from a range of 0.09 to 1.2 M (FIGURE 14), making it the highest known NaCl tolerant organism that still produces approximately 4 H_2 per mole of glucose (135). The optimum NaCl concentration for *A. hydrogeniformans* ranges from 0.2 to 0.6 M NaCl (FIGURE 14). The H_2 production rate, final amount of H_2 produced, glucose consumed and yield of H_2 $glucose^{-1}$ were all very similar when *A. hydrogeniformans* was grown in 0.2 or 0.6 M NaCl (FIGURE 15). Potassium chloride was also tested in the same concentrations, however, neither growth nor H_2 were detected, meaning that NaCl is a required mineral for growing *A. hydrogeniformans*. These data are significant in that, by withstanding high solute concentrations, gas stripping with CO_2 may become an optional technique employed for controlling H_2 partial pressures during large-scale fermentations.

FIGURE 12. Growth of *A. hydrogeniformans* with 0 g L^{-1} (◇), 2 g L^{-1} (□) and 8 g L^{-1} (△) yeast extract using glucose as the substrate.

Error bars represent standard deviation of replicate cultures (n=3).

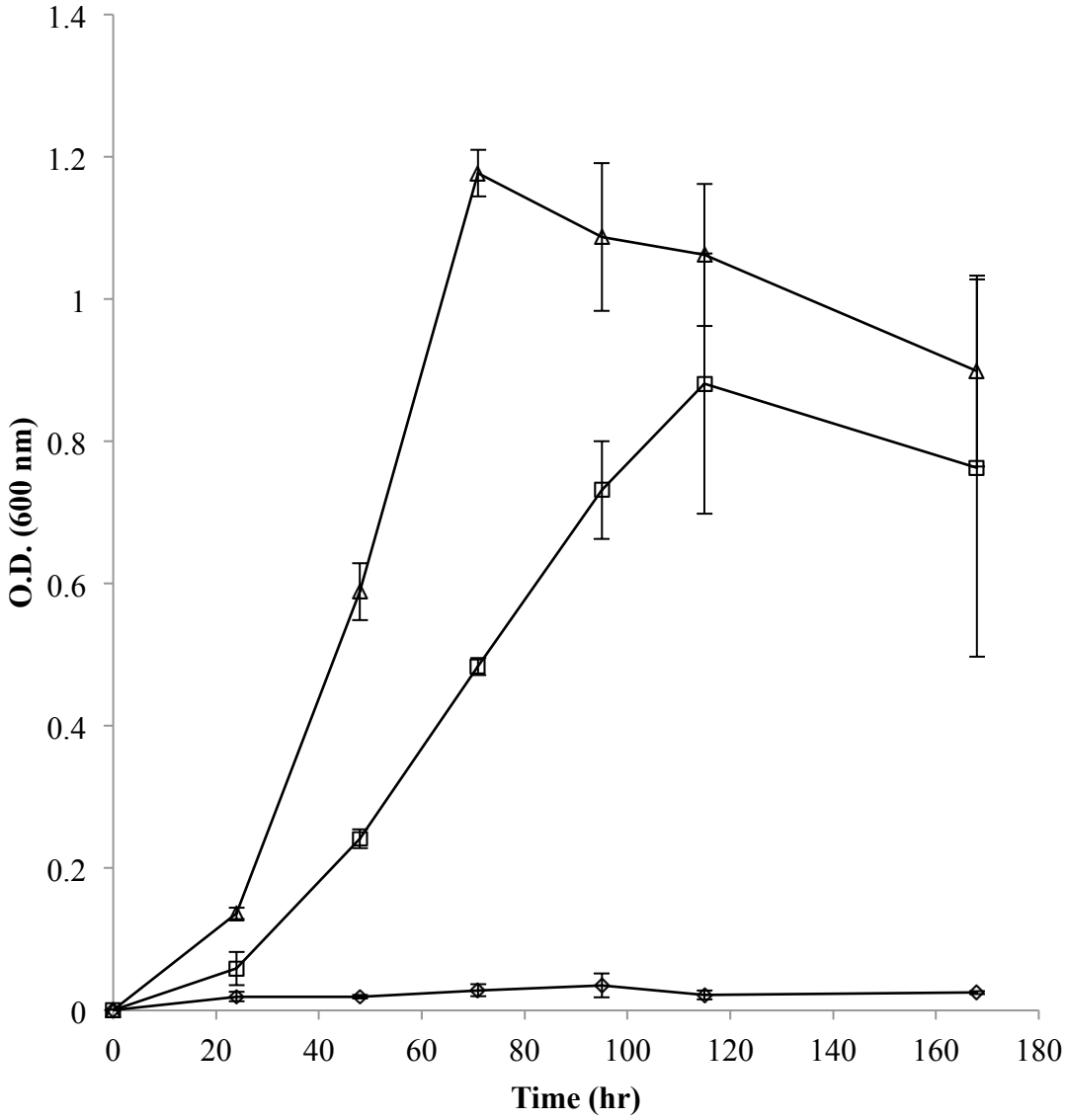
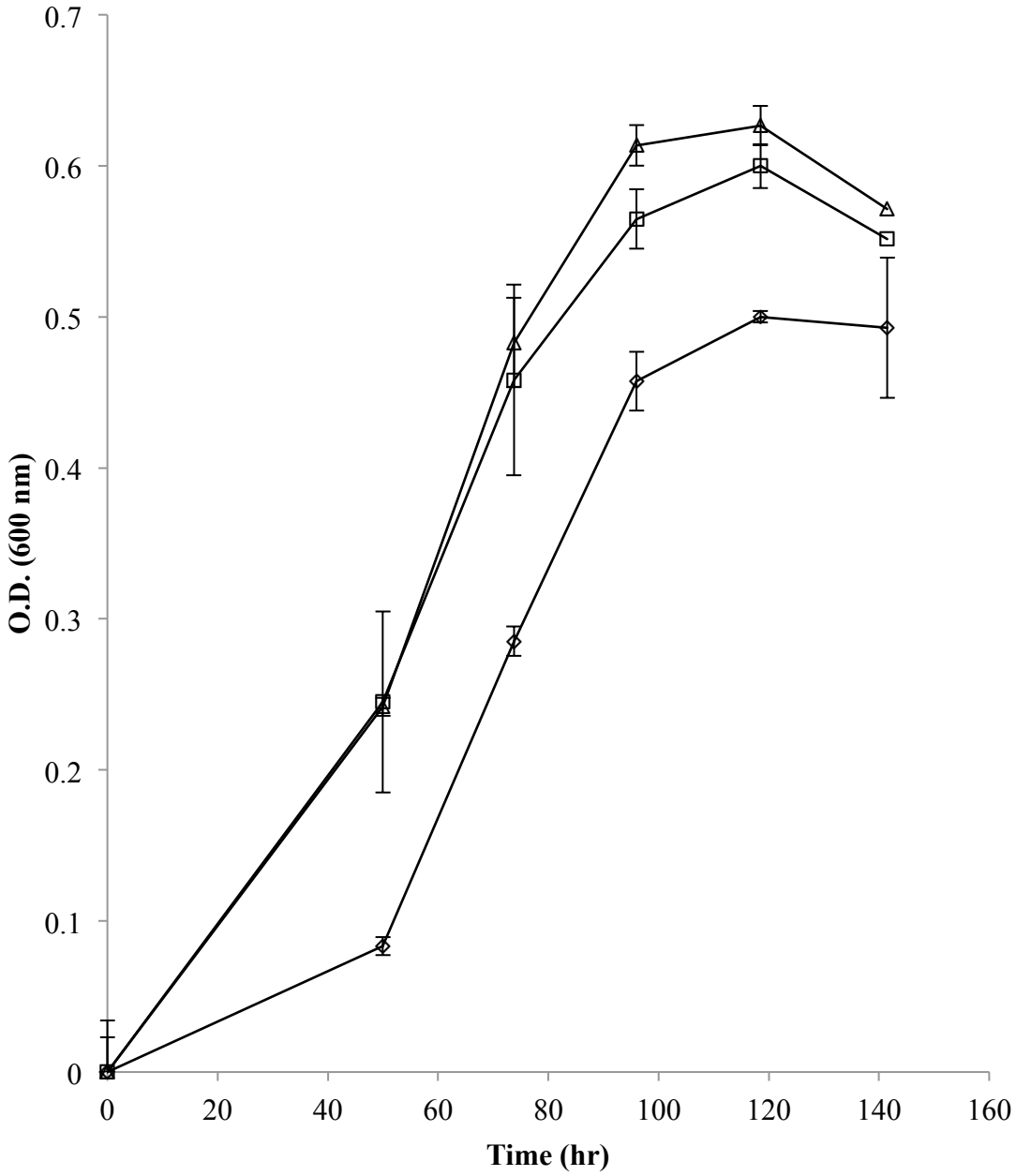


FIGURE 13. Growth measured by optical density at 600 nm when cultures of *A. hydrogeniformans* were grown with initial glucose concentrations of 3 g L^{-1} (◇), 6 g L^{-1} (□) and 9 g L^{-1} (△).



Inhibition by sodium or potassium acetate

The inhibition of thermophilic growth by acidic end products has tested previously and shown to be a valid concern (122, 133). Sensitivity of organic fermentation products investigated in *C. saccharolyticus* (122), described a critical inhibitor concentration of sodium acetate (192 mM) and potassium acetate (206 mM). van Neil et al. (122) concluded that the inhibition was due to ionic inhibition by sodium and not because of critical acetic acid concentrations (122), which has also been described in other cultures (130). *A. hydrogeniformans* was able to grow in the presence of initial sodium/potassium acetate concentrations up to 240 mM (FIGURE 17). However, H_2 production, glucose utilization and growth rates as well as total H_2 produced and glucose oxidation were all significantly lower than culture grown without an acetate addition (Table 9). However, as stated above, *A. hydrogeniformans* can tolerate 600 mM NaCl without inhibiting growth. Thus, the concentration of acetate is the key component not the sodium or potassium ion. This result could pose a problem in large-scale fermentations. However, to reach acetate concentrations of up to 240 mM or higher, *A. hydrogeniformans* would have to consume at least 120 mM of glucose. By using a continuous fermentation system critical acetate concentrations should be easily regulated.

FIGURE 14. Result of the initial NaCl minimum inhibitory concentration assay using cultures of *Anaerobaculum hydrogeniformans* taken after 14 days of incubation in which % H_2 detected in the headspace (bars) and growth quantified by optical density at 600 nm (■) are shown.

Error bars represent standard deviation of replicate cultures (n=3)

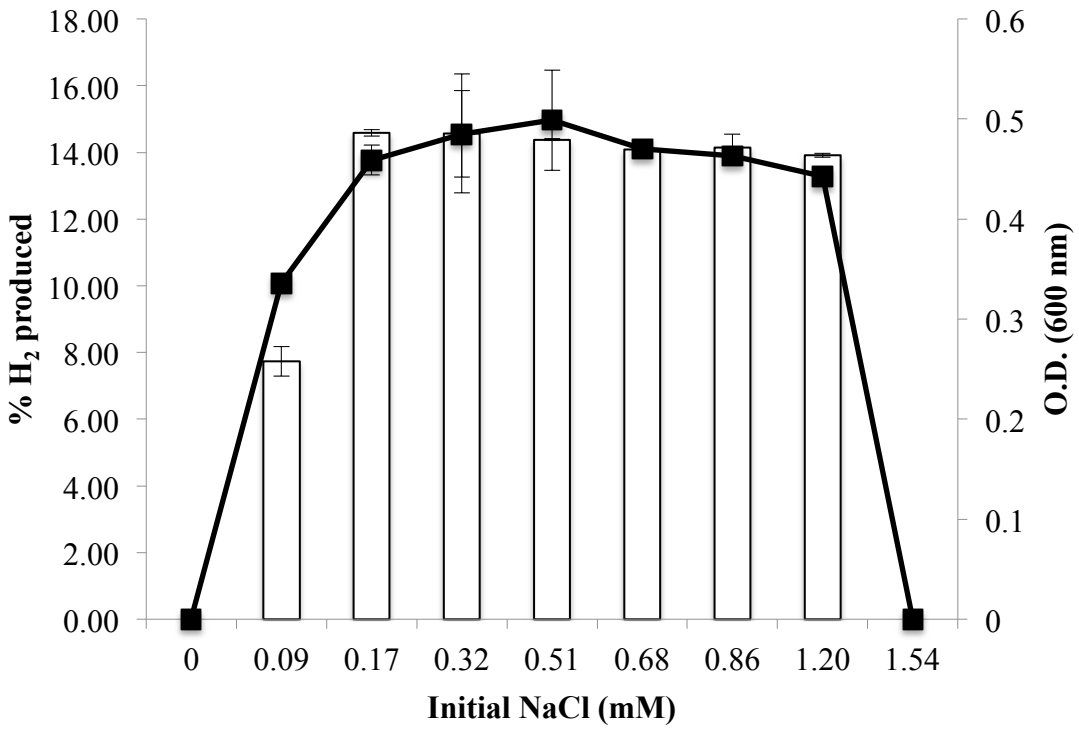
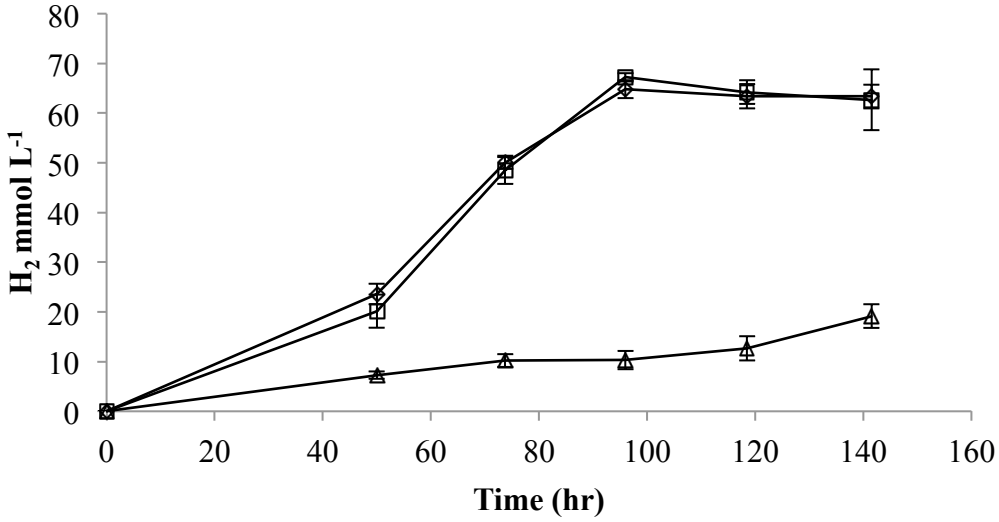


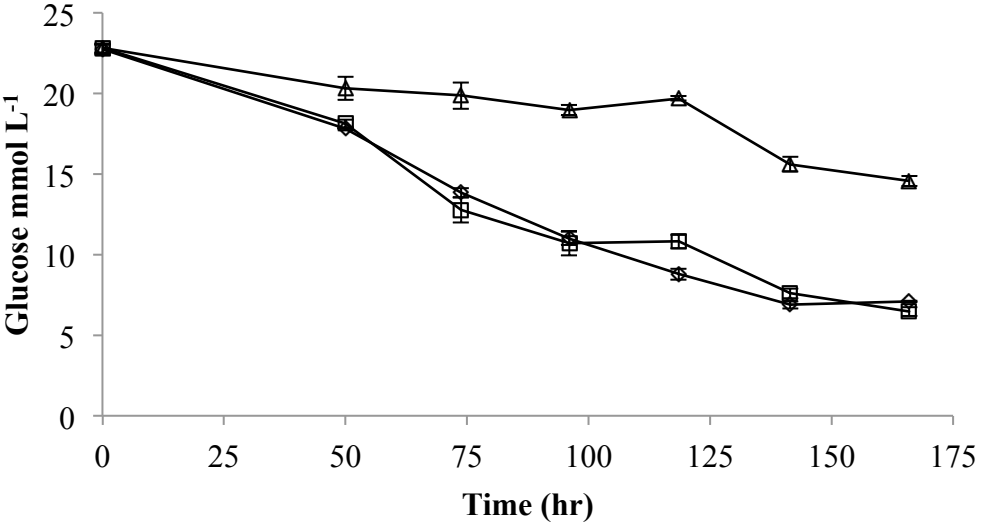
FIGURE 15. H_2 (a) and glucose (b) concentration produced by cultures of *A. hydrogeniformans* with an initial salt concentration of 0.2 (◇), 0.6 (□) and 1.2 (△) M NaCl.

Error bars represent standard deviation of replicate cultures (n=3).

(a)



(b)



Aerotolerance

Microaerobic metabolism in *T. neapolitana*, another 4 H_2 producing microorganism, has been both observed (124) and contradicted in the literature. The study conducted by van Ooteghem et al. (124) showed an increase in H_2 production and yield in microaerobic conditions by means of an alternate physiological pathway. This finding was not reproducible in subsequent studies where the use of a microaerobic pathway was questioned (19, 73). *A. hydrogeniformans* is routinely grown in anaerobic medium without the addition of a reducing agent. The effect of O_2 on cultures of *A. hydrogeniformans* was conducted to test the hypothesis of microaerobic metabolism. *A. hydrogeniformans* was able to tolerate O_2 concentrations in the gas phase up to 50 μmol L^{-1}. The addition of O_2 did not alter the fermentation stoichiometry (Table 10), but it did lower the total amount of glucose utilized until inhibiting O_2 concentrations were reached and growth ceased. These results agree with the latter studies conducted on *T. neapolitana* (19, 73), which showed the addition of O_2 did not increase the H_2 to glucose ratio in cultures of *T. neapolitana*, which is evidence against an alternate microaerophilic physiological pathway of glucose oxidation.

FIGURE 16. Result of the initial Na-acetate minimum inhibitory concentration assay using cultures of *Anaerobaculum hydrogeniformans* taken after 14 days of incubation in which % H_2 detected in the headspace (bars) and growth quantified by optical density at 600 nm (■) are shown.

Error bars represent standard deviation of replicate cultures (n=3)

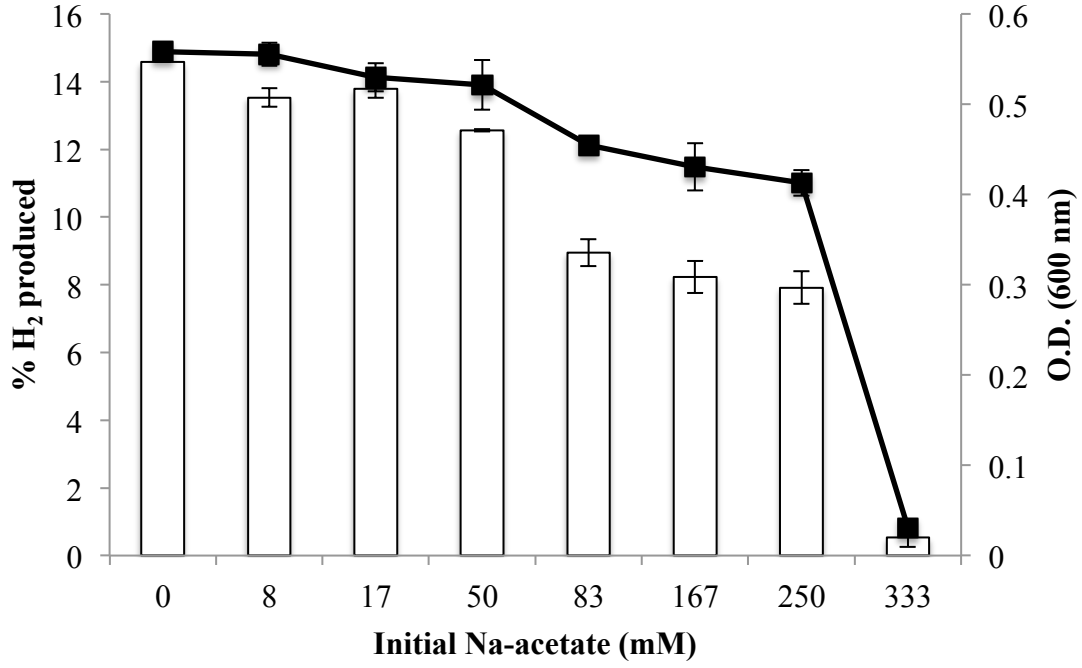


Table 10. Effect of initial O_2 additions on glucose consumption, hydrogen and acetate production for cultures by *A. hydrogeniformans*.

O_2 ($\mu mol\ L^{-1}$)	Glucose consumed ($mmol\ L^{-1}$)	H_2 ($mmol\ L^{-1}$)	Acetate ($mmol\ L^{-1}$)
0	9.1	34.6	18.2
10	8.5	31.7	15.7
20	6.0	22.2	12.0
50	6.0	22.5	10.3
70	0.0	0.33	0.00

Conclusions

A. hydrogeniformans is a potential biological catalyst for H_2 production because it not only produced 4 mol H_2 per mol of glucose, but it does so at the lowest temperature reported to date (Table 1), which aids in the technical feasibility of biological H_2. Under various conditions in this work the yield of H_2 per glucose remained above 3 and was often the thermodynamic limit of 4. The preliminary 5 L batch fermentation run showed that glucose was not totally oxidized by the time *A. hydrogeniformans* reached the stationary and death phase of the culture cycle. The oxidation of glucose by *A. hydrogeniformans* was shown to be dependent on various factors during fermentation, first being P_{H2}, as seen with many other H_2 producing microorganisms. If the H_2 partial pressure is kept low then growth and substrate utilization rates were increase when compared to unamended cultures. Removal of H_2 can be achieved by multiple techniques such as sparging with N_2 or gas stripping with CO_2, which may have detrimental effects. From this research it can be hypothesized that ionic solute concentrations have little effect on the growth of *A. hydrogeniformans* until molar amounts of NaCl are reached, which allows alternate methods of controlling P_{H2}. This work suggests that a continuous fermentation system would be the most optimal for H_2 evolution using *A. hydrogeniformans* as the biological catalyst. This hypothesis was reached by the observation that acetate concentrations in the fermentation broth can inhibit both growth and H_2 production, which could be an issue because acetate is the major organic acid end product. Nutrient limitation and/or pH play a role in the growth of *A. hydrogeniformans*. These data provide key insights into fermentor and medium design for large-scale fermentations using *A. hydrogeniformans* for the production of H_2. Future work

concentrating on H_2 production with A. hydrogeniformans as a biological catalyst should focus on continuous control of pH and product removal with the goal of maximizing substrate utilization and H_2 production.

Acknowledgements

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Chapter 4

The use of a novel eubacterial glyceraldehyde-3-phosphate oxidoreductase during the fermentation of glucose to acetate, CO_2 and H_2 by the anaerobic eubacterium *Anaerobaculum hydrogeniformans*

Abstract

Anaerobaculum hydrogeniformans is an anaerobic organism that grows optimally at 55 °C and is capable of producing up to the thermodynamic maximum of 4 H_2 per glucose. The aim of this study was to elucidate the pathway *A. hydrogeniformans* utilizes for the oxidation of glucose to acetate, H_2 and CO_2. Enzyme activities for phosphoglucose isomerase (1548 nmol min^{-1} mg $protein^{-1}$), phosphofructokinase (87 nmol min^{-1} mg $protein^{-1}$), fructose-1,6-bisphosphate aldolase (296 nmol min^{-1} mg $protein^{-1}$), 2-phosphoglycerate enolase (314 nmol min^{-1} mg $protein^{-1}$) and pyruvate kinase (79 nmol min^{-1} mg $protein^{-1}$) were all detected when assaying for enzyme activity using cell free extracts of *A. hydrogeniformans,* suggesting the presence of the Embden-Meyerhof-Parnas pathway of glycolysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was not be detected in *A. hydrogeniformans* using NAD^+ or $NADP^+$ as electron acceptors. However enzyme activity for a glyceraldehyde-3-phosphate oxidoreductase was detected (41 nmol min^{-1} mg $protein^{-1}$). The oxidation of pyruvate to acetate is accomplished by a pyruvate:oxidoreductase (2150 mmol min^{-1} mg $protein^{-1}$), phosphotransacetylase (785 nmol min^{-1} mg $protein^{-1}$) and acetate kinase (1270 nmol min^{-1} mg $protein^{-1}$). There was no detectable activity of pyruvate dehydrogenase under the assay condition examined in this work. Labeling of the C-1 and C-3 of glucose showed the production of CO_2 from the 3-C of glucose and the incorporation of the C-1 of glucose into the methyl position of acetate, which is indicative of the Embden Meyerhof pathway and further confirmed by the detection of key metabolites from glucose grown cultures of *A. hydrogeniformans*. This is the first report of an eubacterial species that oxidizes glucose to acetate, H_2 and CO_2 via a ferredoxin dependent pathway, such as that

previously thought to only be present in hyperthermophilic archaeal species. This is also the first report of any microorganism that possesses the activity of both NADH/NADPH and ferredoxin oxidizing soluble hydrogenases. *A. hydrogeniformans* also possessed a membrane bound ferredoxin dependent hydrogenase (61 nmol min^{-1} mg $protein^{-1}$).

Introduction

Anaerobaculum hydrogeniformans is an obligate anaerobic thermophilic eubacterium with an optimum growth temperature of around 55 °C (Chapter 1). The organism, isolated from oil production water from Alaska, USA, is a member of the division *Synergistetes* (41). Previous studies have shown that *A. hydrogeniformans* can withstand high H_2 partial pressures and produces close to the theoretical maximum of 4 H_2 per molecule of glucose (67) at 55 °C via the following reaction, which was described by Thauer *et al*. (113):

$$C_6H_{12}O_6 + 4H_2O \rightarrow 4H_2 + 2CH_3COO^- + 2HCO_3^- + 4H^+ \quad (\Delta G^{0'} = -206.3 \text{ kJ/mol})$$

To date, *A. hydrogeniformans* is the only organism described that can produce 4 mol H_2 per mol glucose below the temperature of 65 °C (48). Physiological characterization of the central metabolic pathway of *A. hydrogeniformans* should elucidate how this anaerobe is capable of producing H_2 up to 0.2 atm (Chapter 3).

Since the classification of living organisms into the three domains of life (137), there have been numerous comparative studies that have shown that many of the features observed in archaeal species are in fact variations of previously established themes found in eubacterial or eukaryotic species (118). Central metabolic processes have been one of the major comparatively studied phenotypes between member of the Archaea and the Bacteria. To date there have been three major glycolytic pathways for the oxidation of glucose to pyruvate, albeit with variations on a theme. These are the Embden Meyerhof

Parnas pathway (EM), the Entner-Doudoroff pathway (ED) and the Pentose Phosphate Pathway (PPP). All three of these pathways can be elucidated with the detection of enzyme activities and by using stable isotope labeling at the 1 and 3 carbon positions of glucose (99). FIGURES 17, 18 and 19 show variations of how ^{13}C-glucose can be used to elucidate which pathway of glycolysis is being used. If the EM pathway is used for central carbon metabolism the C-1 of glucose will be incorportated into the methyl position of acetate and the C-3 of glucose will be released as CO_2. If the ED pathway is being used the C-1 of glucose will be released as CO_2 and the C-3 of glucose will be incorporated into the methyl position of acetate. Finally, if the PPP is being used the C-1 of glucose will be released as CO_2 and the C-3 will be incorporated into the carboxyl position of acetate.

The fermentation of glucose can lead to the evolution of H_2 gas. There are a number of isolates, both mesophilic and thermophilic, which have been shown to produce H_2 from the oxidation of glucose via the EM pathway with a very distinct set of enzymes that carry out the pathway. There are two distinct enzymatic steps, the oxidation of glyceraldehyde-3-phosphate (GAP) and pyruvate, which dictate the thermodynamic constraints on the amount of H_2 that can be produced. Enteric bacteria, such as *Escherichia coli,* use a NAD^+ dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and a pyruvate:formate lyase (11). The described thermophilic eubacterial species use the same GAPDH as the enterics. However they use a pyruvate ferredoxin oxidoreductase (POR) for the oxidation of pyruvate (11). The final variation on the EM pathway is the substitution of a glyceraldehyde-3-phosphate oxidoreductase (GAPOR) for the GAPDH and the use of the POR pyruvate decarboxylation. No matter what

central metabolic pathway is being utilized, there is a finite amount of H_2 that can be produced until the production of H_2 becomes thermodynamically unfavorable (48).

The need to maintain low H_2 partial pressures can best be explained when looking at the H_2 forming reactions. During glycolysis, reducing equivalents are produced in the form of NADH (from the oxidation of glycaraldehyde-3-phosphate [GAP] by GAP dehydrogenase) and also in the form of reduced ferredoxin (ferredoxin dependent oxidation of pyruvate by pyruvate:ferredoxin oxidoreductase). At the midpoint the redox potential of the couples NAD^+/NADH and $ferredoxin_{ox}$/$ferredoxin_{red}$ are -320 mV and -398 mV, respectively (113). Thermodynamically, the H_2 evolving reactions using NADH or ferredoxin dependent hydrogenases are distinct from one and other and described by the following reactions calculated by Kengen *et al*. (48):

$$NADH + H^+ \rightarrow NAD^+ + H_2 \qquad (\Delta G^{0'} = 18.1 \text{ kJ/reaction})$$

$$2\ Ferredoxin_{red} + 2\ H^+ \rightarrow 2\ Ferredoxin_{ox} + H_2 \qquad (\Delta G^{0'} = 3.1 \text{ kJ/reaction})$$

As noted in the above reaction the use of ferredoxin for the production of H_2 is a more favorable reaction.

Within a H_2 evolving system, electron carriers need to be recycled on a continual basis for catabolism to proceed. In *Thermotoga maritima* (98) and *Caldicellulosiruptor saccharolyticus* (135), this is accomplished by the production of alternative reduced end products such as lactate and/or ethanol which recycle the intercellular NAD^+ and NADH. The thermodynamics of the NAD^+/NADH ratio and the oxidized ferredoxin/reduced ferredoxin have been shown to be more favorable as temperature increases ($\Delta G^0 = \Delta H - T\Delta S^0$) (108). Kengen *et al*. (48) showed theoretically at 25 °C a system comprised of

ferredoxin and not NADH was favorable at H_2 partial pressures of 0.1 atm and favorable at 1 atm at 50-70 °C, while the use of NADH only becomes favorable at H_2 partial pressures of 0.0005 atm at 25 °C and 0.001 atm for 50-70 °C. To date the only microorganisms that possess a system comprised of ferredoxin mediated reactions, such as glyceraldehyde-3-phosphate:oxidoreductase (GAPOR) and POR, are hyperthermophilic archaeal species (44, 48, 118).

Here, we report the characterization of a glycolytic pathway and enzyme system previously thought to be only used by species in the archaeal domain (118). We also report the use of a dual hydrogenase system consisting of cytosolic hydrogenases dependent on both NADH and ferredoxin. In the present study, *A. hydrogeniformans* was subjected to enzyme activity, ^{13}C-glucose, ^{14}C-glucose and metabolite detection studies. The derived pathway of glucose oxidation by *A. hydrogeniformans* only uses ferredoxin, similar to that described for the hyperthermophilic archaeon *P. furiosus* (118), and not the NAD(P)H/ferredoxin system used by other hydrogenic eubacteria, such as *T. maritima* (98) and *C. saccharolyticus* (135).

FIGURE 17. The enzyme and labeling pattern resulting from the use of the Embden-Meyerhof-Parnas pathway of glycolysis when cells are grown on 1-^{13}C-glucose and 3-^{13}C-glucose.

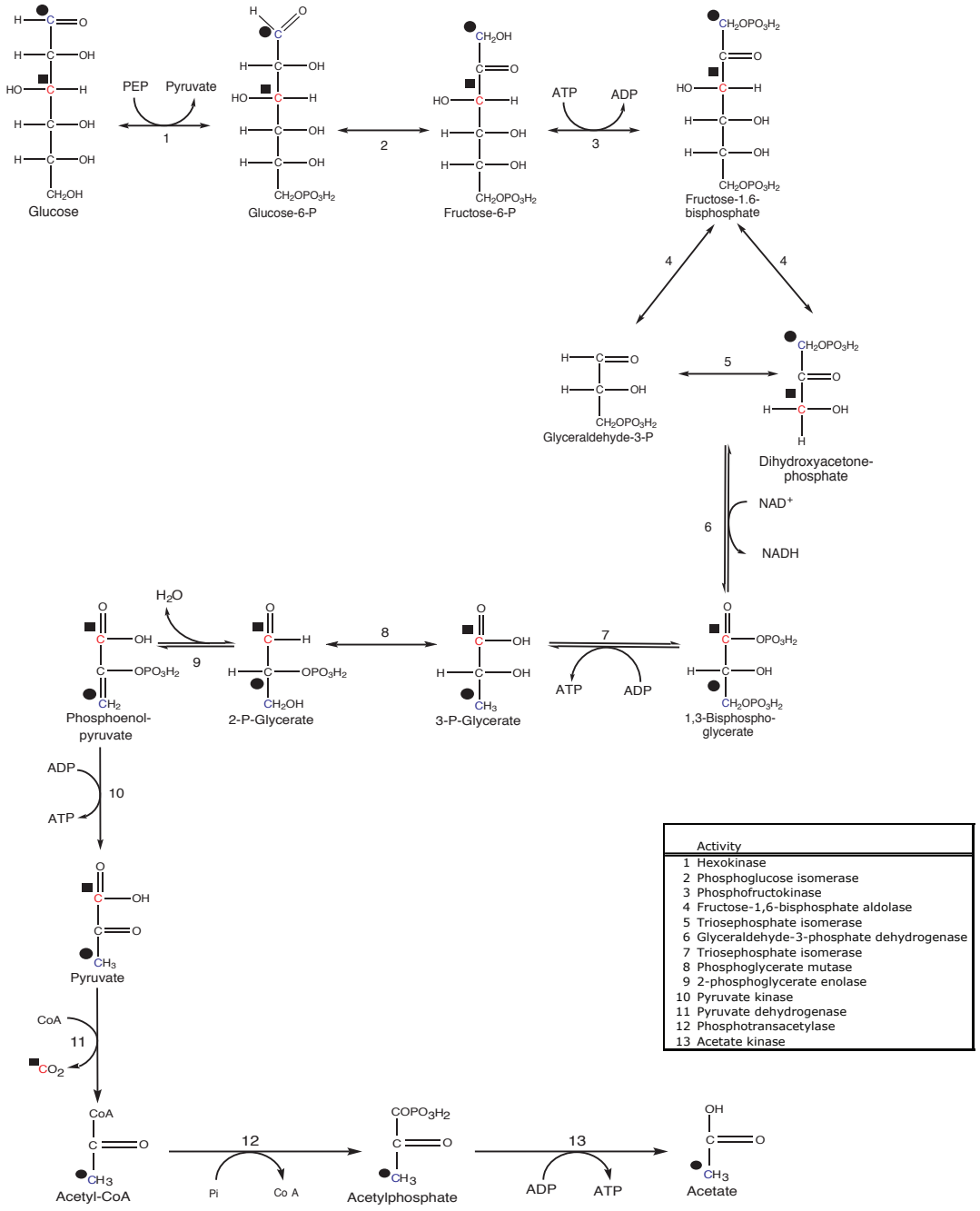


FIGURE 18. The enzyme and labeling pattern resulting from the use of the Entner-Doudoroff pathway of glucose oxidation when cells are grown on 1-^{13}C-glucose and 3-^{13}C-glucose.

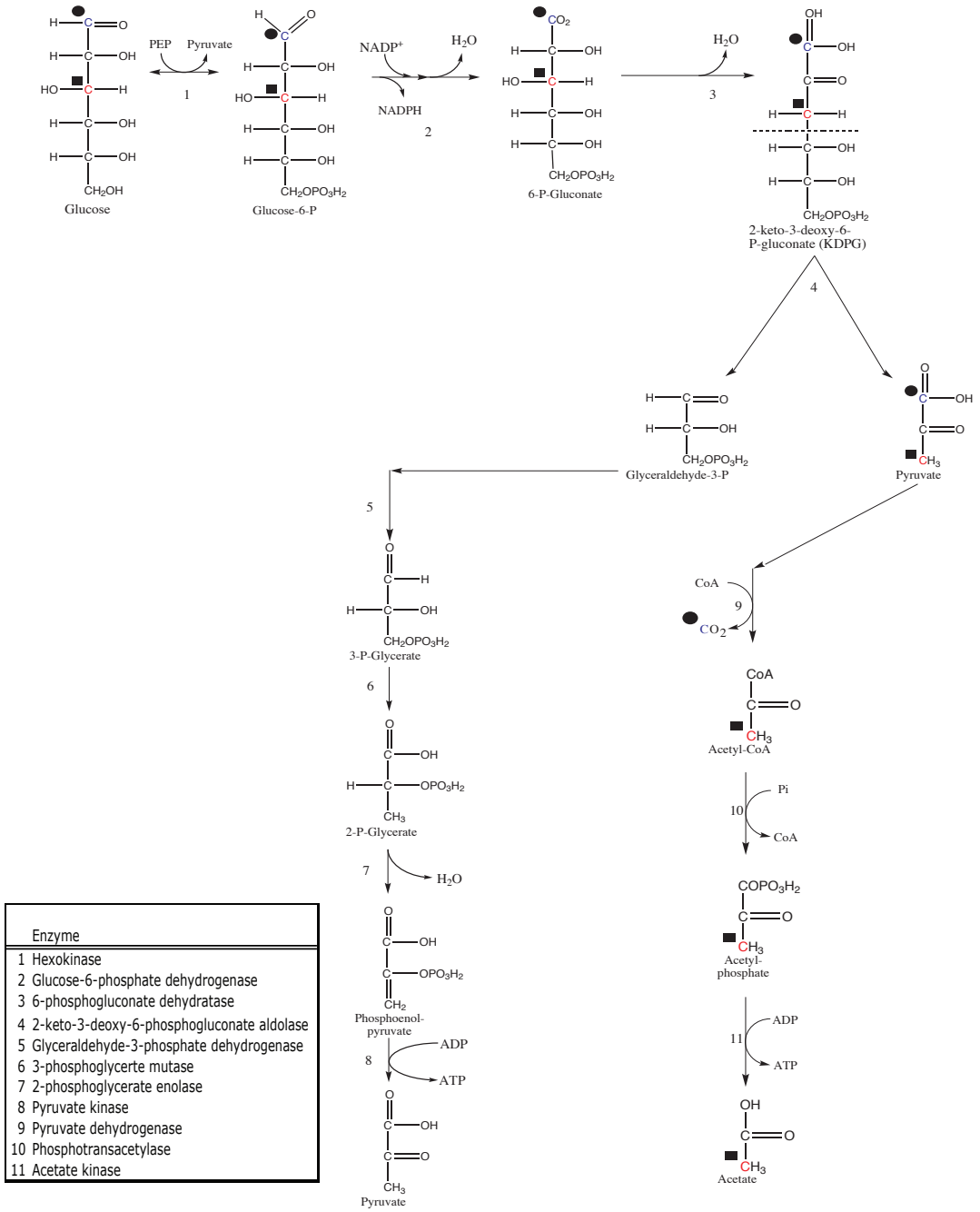
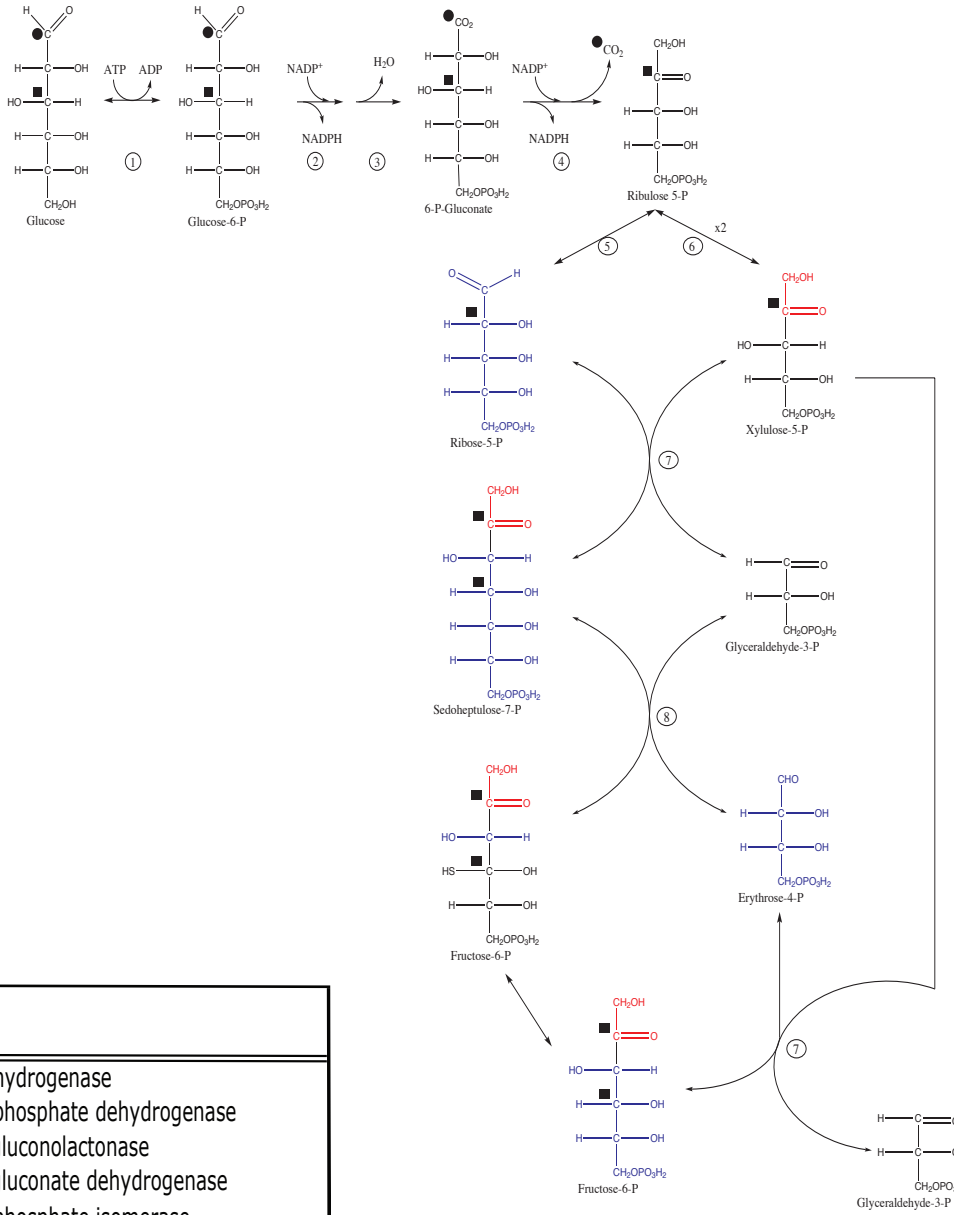


FIGURE 19. The enzyme and labeling pattern resulting from growth on 1-^{13}C-glucose and 3-^{13}C-glucose using the Pentose Phosphate Pathway of glucose oxidation.



Enzyme
1 Glucose dehydrogenase
2 Glucose-6-phosphate dehydrogenase
3 6-phosphogluconolactonase
4 6-phosphogluconate dehydrogenase
5 Pentose-5-phosphate isomerase
6 Pentose-5-phosphate epimerase
7 Transketolase
8 Transaldolase

Materials and methods

Bacteria and growth conditions

A. hydrogeniformans strain OS1^T was routinely cultivated on the medium previously described (Chapter 1) using glucose as the carbon and energy source and grown at 55 °C. *Lactococcus lactis* DSM 20481and *Leuconostoc mesenteroides* DSM 20343^T were obtained from the DSMZ. *L. lactis* was grown on TSB (Difco) and grown at 38 °C. *L. mesenteroides* was grown on TSB at 30 °C.

Cell free extract preparation

Cells in late log phase were harvested by centrifugation (6,000 x g, 30 min and 4 °C) and washed once with 100 mM 3-(N-morpholino)propanesulfonic acid (MOPS) pH 7.2 with 1 mM dithiothreitol (DTT). Cell pellets were frozen in liquid nitrogen or used immediately to prepare cell-free extracts. Cell pellets were resuspended in 100 mM MOPS pH 7.2 with 1 mM DTT and disrupted using a French press at 83 MPa. The lysate was centrifuged (20,700 x g, 10 min, 10°C) and dispensed to cryovials for storage in liquid nitrogen or used immediately for enzyme assays. Small, interfering molecules were removed by passing the extract over a Sephadex G-25 column.

Membrane and cytosolic preparation were prepared by ultracentrifugation of cell-free extracts (100,000 x g, 60 min, 10°C). The pellet was washed once with 100 mM MOPS pH 7.2 with 1 mM DTT and resuspended in the same buffer. Membrane preparations were assayed for fructose-1,6-bisphosphate aldolase to check for contamination with cytosolic proteins.

Air was excluded during all steps and only anoxically-prepared, reduced solutions were used.

Enzyme assays

Unless otherwise indicated, activities were determined at 50 °C in N_2-flushed septum-sealed quartz cuvettes. Solutions were prepared anoxically by boiling and cooling under 100 % N_2. Additions were made using N_2-flushed syringes. Specific activities were determined over the range where activity was linear with protein concentration. Minus substrate and boiled cell-free extract controls were conducted for each assay. Assays following the loss or production of NAD or NADP were measured at 340 nm ($E= 6.22 \times 10^3\ M^{-1}\ cm^{-1}$). Assays containing methyl viologen or benzyl viologen were measured at 578 nm using extinction coefficients of 9780 and 8650 $M^{-1}\ cm^{-1}$, respectively. Buffer pH was set at 50 °C or using the appropriate ΔpK_a/°C to compensate for the difference between ambient temperature and assay temperature pH.

Hexokinase (HK)

Hexokinase activity was assayed by following ATP- or ADP-dependent formation of glucose-6-phophate coupled to the glucose-6-phosphate dehydrogenase-catalyzed reduction of NADP. Activity was measured in a 1 ml mixture containing 100 mM Tris-HCl (pH 8), 1mM DTT, 10 mM glucose, 0.3 mM NADP, 2 U glucose-6-phosphate dehydrogenase (G6P-DH), and 2 mM ATP or ADP (98).

Glucose: PEP phosphotransferase

Glucose: PEP phosphotransferase activity was assayed in a 100 mM Tris buffer (pH 7.4) containing 1 mM DTT, 10 mM $MgCl_2$, 1 mM NADP, 15 mM glucose, 10 mM phosphoenolpyruvate (PEP), and 3 U G6P-DH in 1 ml. Cell-free extracts and membrane preparations were assayed (57).

Phosphofructokinase

Phosphofructokinase activity was measured by enzymatically coupling the fructose-1,6-bisphosphate produced to NADH oxidation using fructose-1,6-bisphosphate (FBP) aldolase, triosephosphate isomerase (TIM), and glycerophosphate dehydrogenase. The 2 ml assays contained 100 mM MOPS (pH 7.2), 1 mM DTT, 10 mM $MgCl_2$, 5 mM fructose-6-phosphate, 0.3 mM NADH, 6 U aldolase, 8 U TIM, and 2 U glycerophosphate dehydrogenase. This assay was conducted as previously described by Selig *et al*. (99). However it was modified by the use of MOPS instead of Tris buffer.

Fructose-6-phosphate (F6P) phosphokinase

F6P phosphokinase activity was assayed in a modification of (110) that contained 100 mM MOPS pH 7.2, 2 mM F6P, 25 mM potassium phosphate, 80 μM CoA, 2 mM NAD, 6 mM malate (disodium salt), 2 mM DTT, 2.8 U citrate synthase, and 55 U malate dehydrogenase.

Fructose-1,6-bisphosphate (FBP) aldolase

FBP aldolase activity was measured by enzymatically coupling the glyceraldehyde-3-phosphate and dihydroxyacetone phosphate produced to NADH oxidation using TIM and glycerophosphate dehydrogenase. Two ml assays contained 100 mM MOPS (pH 7.2), 1 mM DTT, 10 mM $MgCl_2$, 5 mM fructose-1,6-bisphosphate, 300 μM NADH, 8 U TIM, and 2 U glycerophosphate dehydrogenase.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

GAPDH activity was measured by following the glyceraldehyde-3-phosphate -dependent reduction of NAD or NADP. The 2 ml reaction mixture contained 100 mM MOPS pH 7.2, 1 mM DTT, 20 mM fructose-1,6-bisphosphate, 20 mM potassium phosphate, 300 μM NAD or NADP, and 1.8 U aldolase.

Glyceraldehyde-3-phosphate oxidoreductase (GAPOR)

GAPOR activity was determined by measuring the glyceraldehyde -3-phosphate -dependent reduction of methyl viologen. The assays contained, 2 mM methyl viologen, in a 100 mM HEPES (N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) pH 8.0. Assays were also performed in 20 mM potassium phosphate pH 8.0.

Glyceraldehyde dehydrogenase (GADH)

GADH activity was measured by following the glyceraldehyde-dependent reduction of NAD or NADP. The 2 ml reaction mixture contained 100 mM MOPS pH 7.2, 1 mM DTT, 25 mM glyceraldehyde, and 300 μM NAD or NADP.

Glyceraldehyde oxidoreductase (GAOR)

GAOR activity was determined by measuring the glyceraldehyde-dependent reduction of methyl viologen. The assays contained 25 mM glyceraldehyde and 2 mM methyl viologen.

Pyruvate kinase (PK)

PK activity was determined by coupling the pyruvate produced from PEP to NADH oxidation using lactate dehydrogenase (LDH). The 2 ml assay contained 100 mM MOPS, 1 mM DTT, 5 mM $MgCl_2$, 2 mM ADP, 400 μM NADH, 2 mM PEP, and 6 U LDH. This assay was modified from the original publication by Schäfer and Schönheit (95) by changing the amount of ADP used from 5 mM to 2 mM.

Pyruvate dehydrogenase (PDH)

PDH activity was measured by following the pyruvate-dependent reduction of NAD or NADP. The reaction mixture contained 100 mM MOPS pH 7.2, 1 mM DTT, 1 mM sodium pyruvate, 100 μM coenzyme A (CoA), and 300 μM NAD or NADP (96).

Pyruvate:ferredoxin oxidoreductase (POR)

POR activity was assayed in a mixture containing 100 mM MOPS pH 7.2, 1 mM DTT, 1 mM sodium pyruvate, 100 μM CoA, and 2 mM methyl viologen (96).

Glucose oxidoreductase (GOR)

GOR activity was determined by measuring the glucose-dependent reduction of methyl viologen. The assays contained 25 mM glucose, 1 mM DTT, and 2 mM methyl viologen in 100 mM MOPS pH 7.2.

Glucose dehydrogenase

Glucose dehydrogenase activity was examined using three methods. The glucose-dependent reduction of NAD(P) was followed in a reaction mixture containing 100 mM MOPS pH 7.2, 1 mM DTT, 50 mM glucose, and 300 μM NAD or NADP. The second method followed the reduction of DCPIP (2,6-dichlorophenolindophenol) at 600 nm. This reaction mixture contained 100 mM MOPS pH 7.2, 50 mM glucose, 50 μM DCPIP, and 1 mM PMS (phenazine methosulfate). The third method followed the reaction in the reverse direction from gluconate to glucose using a fixed time assay. Ten mM gluconate and 0.8 mM NADH or NADPH were added to 100 mM MOPS pH 7.2 and incubated at 55 °C for 50 min. The amount of glucose formed was measured using the PGO enzymes (glucose oxidase/peroxidase) to quantitate glucose (Sigma Technical Bulletin, product number P7119).

NAD(P)H: methyl viologen oxidoreductase

NAD(P)H: methyl viologen oxidoreductase activity was determined by following the reduction of methyl viologen (2 mM) at 589 nm in the presence of 2 mM NADH or NADPH in a 100 mM MOPS pH 7.2 buffer containing 1 mM DTT.

Gluconate dehydratase

Gluconate dehydratase assays were created, in which the activity was coupled to the endogenous 2-keto-3-deoxygluconate (KDG) aldolase (if present) and the methyl viologen- reducing glyceraldehyde oxidoreductase to the dehydration of gluconate. The assay contained 100 mM MOPS pH 7.2, 1 mM DTT, 25 mM potassium gluconate, and 2 mM methyl viologen. Gluconate dehydratase activity was also assessed in a 2-ml volume containing 100 mM MOPS pH 7.2, 1 mM DTT, 5 mM gluconate 300 μM NADH, and 4 U of lactate dehydrogenase.

2-phosphoglycerate enolase

The activity for 2-phosphoglycerate enolase was determined by coupling the PEP formed to the oxidation of NADH by pyruvate kinase and lactate dehydrogenase. The 2 ml assay contained 100 mM MOPS pH 7.2, 1 mM DTT, 10 mM MgCl2, 2 mM ADP, 300 μM NADH, 2 mM 2-phosphoglycerate, 6 U pyruvate kinase, and 9 U lactate dehydrogenase.

Glycerate kinase

Glycerate kinase activity was determined by coupling the PEP formed to the oxidation of NADH by 2-phosphoglycerate enolase, pyruvate kinase and lactate dehydrogenase.. The 2 ml assay contained 100 mM MOPS pH 7.2, 1 mM DTT, 10 mM $MgCl_2$, 2 mM ADP, 300 μM NADH, 2 mM D,L-glycerate, 10 U enolase, 6 U pyruvate kinase, and 9 U lactate dehydrogenase.

6-Phosphogluconate dehydrogenase

The activity of 6-phosphogluconate dehydrogenase was measured by following the 6-phosphogluconate -dependent reduction of NAD or NADP. The 2 ml reaction mixture contained 100 mM MOPS pH 7.2, 1 mM DTT, 5 mM 6-phosphogluconate, and 300 μM NAD or NADP.

Transaldolase and transketolase

Transaldolase and transketolase were assayed in an 800 μl reaction mix containing 100 mM MOPS pH 7.2, 0.2 mM NADH, 2 U glycerophosphate dehydrogenase, 20 U TIM, and 0.6 mM erythrose-4-phosphate. The transaldolase assay also contained 0.6 mM fructose-6-phosphate and the transketolase assay also contained D-xylulose-5-phosphate and 0.25 mM thiamine pyrophosphate. This method was altered from the previous description (66) by the use of MOPS buffer instead of a TEA buffer.

Glucose-6-phosphate dehydrogenase /oxidoreductase

The assay for glucose-6-phosphate dehydrogenase contained 100 mM MOPS pH 7.2, 0.3 mM NAD or NADP, and 5 mM glucose-6-phosphate. The assay for glucose-6-phosphate oxidoreductase contained 100 mM MOPS pH 7.2, 1 mM DTT, 2 mM methyl viologen, and 5 mM glucose-6-phosphate.

Pyruvate:formate lyase

Pyruvate:formate lyase was assayed in a modification of (110) containing 100 mM MOPS pH 7.2, 20 mM sodium pyruvate, 80 μM CoA, 2 mM NAD, 6 mM malate (disodium salt), 2 mM DTT, 2.8 U citrate synthase, and 55 U malate dehydrogenase.

Phosphoglucose isomerase

Phosphoglucose isomerase was determined in a 1-ml assay containing 100 mM MOPS pH 7.2, 5 mM F6P, 0.3 mM NADP, and 1 U G6P-DH by the modification of the hexokinase assay previously described.

Other oxidoreductases

Oxidoreductase activity was determined using formate, 6-phosphogluconate, gluconate, glucose, and glucose-6-phosphate as substrates. Assays were performed in a 100 mM HEPES buffer pH 8.0 containing 1 mM DTT and 5 mM substrate. Assays were conducted using both methyl viologen and benzyl viologen in 3 mM concentrations.

Gluconate and 2-ketogluconate kinases

Gluconate and 2-ketogluconate kinases were determined in reaction mixtures containing 50 mM TAPS (N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid) pH 8, 10 mM $MgCl_2$, 2.5 mM PEP, 0.3 mM NADH, 2 mM ATP or ADP, 5 U lactate dehydrogenase, and 4 U pyruvate kinase in 1 ml. Assays contained 10 mM gluconate or 2- ketogluconate.

2-keto-3-deoxygluconate (KDPG) aldolase

KDPG aldolase was measured using the assay of Skoza and Mohos (103) and Gottschalk and Bender (28) as modified by Buchanan (10). Cell-free extracts were added to 50 mM MOPS pH7.2 containing 5 mM gluconate or 5 mM D,L-glyceraldehyde and 5 mM sodium pyruvate (reverse direction) and incubated at 55 °C for 10 min. One hundred µl samples were removed and stopped by adding 10 µl of a 12% TCA (w/v) solution followed by centrifugation (16,000 x g, 5 min) to remove precipitated proteins. Fifty µl of the supernatant was oxidized with 125 µl of 25 mM periodic acid in 0.25 M H_2SO_4. After 10 min at room temperature 250 µl of 2 % sodium arsenite in 0.5 M HCl was added to terminate oxidation. One ml of 0.3 % (w/v) thiobarbituic acid was added and developed by heating for 10 min at 100 °C. A sample was removed, added to an equal volume of DMSO (dimethyl sulfoxide), and read at 549 nm. The extinction coefficient for the chromophore was 6.78 x 10^4 M^{-1} cm-^{1}.

Acetyl-CoA synthases (ACS) and adenylate kinase

Activities for ACS (AMP-forming), ACS (ADP-forming), and adenylate kinase were determined aerobically in a 100 mM TEA buffer (pH 7.5) containing 10 mM $MgCl_2$, 375 µM NADH, 1 mM PEP, 2.2 U pyruvate kinase, and 2.8 U lactate dehydrogenase. The assay for ACS (AMP-forming) also contained 480 µM CoA, 5 mM ATP and 2.8 U myokinase. The activity for ACS (ADP-forming) was determined using the same components but with the omission of myokinase. The adenylate kinase assay also contained 2 mM ATP and 2 mM AMP.

Phosphotransacetylase (PTA)

PTA activity was determined by the arsenolysis method of Stadtman (106) combined with the hydroxymate assay. The reaction mix (333 µl) contained the following: 10 mM Tris pH 8, 6 mM acetyl phosphate, 100 mM cysteine, 1 mM CoA, and cell-free extract. After a 10 min incubation at 37 °C sodium arsenate was added to 50 mM to start the reaction. Tubes were incubated for an additional 45 min at 37 °C and then diluted 1:1 with 2 M hydroxylamine neutralized with potassium hydroxide. The reaction was stopped by the addition of trichloroacetic acid (5 % final concentration) after 5 min incubation at 37 °C. The volume was brought to 1.5 ml by the addition of 2.5 % $FeCl_3$ in 2 M HCl, and centrifuged at 16,000 x g for 5 min. Tubes were read at 540 nm against a water blank. A standard curve was constructed for acetyl phosphate using the same assay conditions. Assays lacking CoA and acetyl phosphate, or containing boiled cell-free extracts were performed as controls.

Acetate kinase (AK)

Acetate kinase was determined by a modification of the assay of Bowmann *et al*. (8) based on that of Lipmann and Tuttle (62). Potassium acetate was substituted for sodium acetate and succinate was omitted. The assay contained in a 300 µl volume: 50 mM Tris-HCl pH 8.3, 10 mM ATP, 10 mM $MgCl_2$, 0.5 M hydroxylamine (neutralized to pH 7 with potassium hydroxide), and 20 mM potassium acetate. Assays were performed using 5 different protein concentrations (0 to 30 µl of cell-free extract). For each protein concentration, minus ATP, minus acetate, and boiled cell-free extract controls were done.

The reaction mixture was incubated at 37 °C for 5 min and stopped by the addition of 450 µl of ferric reagent (10 % $FeCl_3$, 3 % trichloroacetic acid, in 0.7 N HCl). The tubes were centrifuged for 5 min at 13,000 x g to remove precipitation before reading at 535 nm. The molar extinction coefficient for acetyl phosphate under these conditions is 594 M^{-1} cm^{-1}. Activities were calculated by subtracting the background rates of the minus ATP, and minus acetate controls.

Hexulose-6-phosphate synthase (HPS) and 6-phosphohexuloisomerase (PHI)

The combined activity of HPS and PHI were assayed by the production of formaldehyde from fructose-6-phosphate. The assay was conducted as previously described by Orita *et al*. (84). Briefly the assay was carried out in 50 mM MOPS buffer pH 8.0, with 10 mM dithiothreitol, 25 mM $MgCl_2$ and 200 mM fructose-6-phosphate. The reaction was carried out in 10 ml anoxic, stoppered and crimp-sealed vials, incubated at 55 °C and the reaction was initiated by the addition of cell-free extracts of *A. hydrogeniformans*. Formaldehyde was quantified using the Nash reagent (76) and also by the HPLC method described by Summers (109).

Formate dehydrogenase (FDH)

Formate dehydrogenase was assayed in 50 mM TAPS pH 8, 10 mM $MgCl_2$, 10 mM sodium formate, and 5 mM NAD or NADP.

Hydrogenase

Hydrogenase and the ability to reduce NAD^+ or $NADP^+$ with H_2 were measured using cell-free extracts, membrane preparations, and the cytosolic fraction. The hydrogenase assay contained 3 mM methyl viologen in 100 mM HEPES pH 8. NAD or NADP reduction was followed in a reaction mixture containing 100 mM HEPES pH 8 and 5 mM NAD or NADP. The reactions were started by the addition of 70 kPa H_2.

Labeling experiments

^{14}C-glucose experiments

To examine the fate of the first position of glucose during glucose oxidation by *A. hydrogeniformans* ^{14}C-1-glucose was added to 20 ml cultures in 125 ml serum bottles. The medium used in this experiment was the same as described above. 16 mmol glucose was added as the substrate and supplemented with 22.2 x 10^6 dpm of ^{14}C-1-glucose once glucose oxidation was observed by the PGO enzyme assay (Sigma). Samples were taken once there was no more detectable glucose in the medium. Before samples were collected 1 ml of 6 N HCl was added to the bottles to ensure all of the carbonates were removed from solution. Radioactivity was monitored using a liquid scintillation counter (LS 1701; Beckman) using the Ultima-Flo AT (Packard) scintillation cocktail. Total radioactivity in the gas phase was conducted as described by Tanner *et al*. (112). Briefly, the radioactivity was determined by the absorption of 1 ml of headspace gas (collected with a syringe equipped with a mininert valve) into ~~a~~ 100 µl of 0.2 N NaOH solution that was then subject to liquid scintillation counting. Liquid samples were also collected and subjected to scintillation counting. One ml of culture medium was added to 9 ml of scintillation cocktail and read by liquid scintillation counting. Radioactivity of the CO_2

was calculated as the difference between the radioactivity in the gas sample and that absorbed into the base. A flow chart of the radiolabeled protocol can be seen in FIGURE 20.

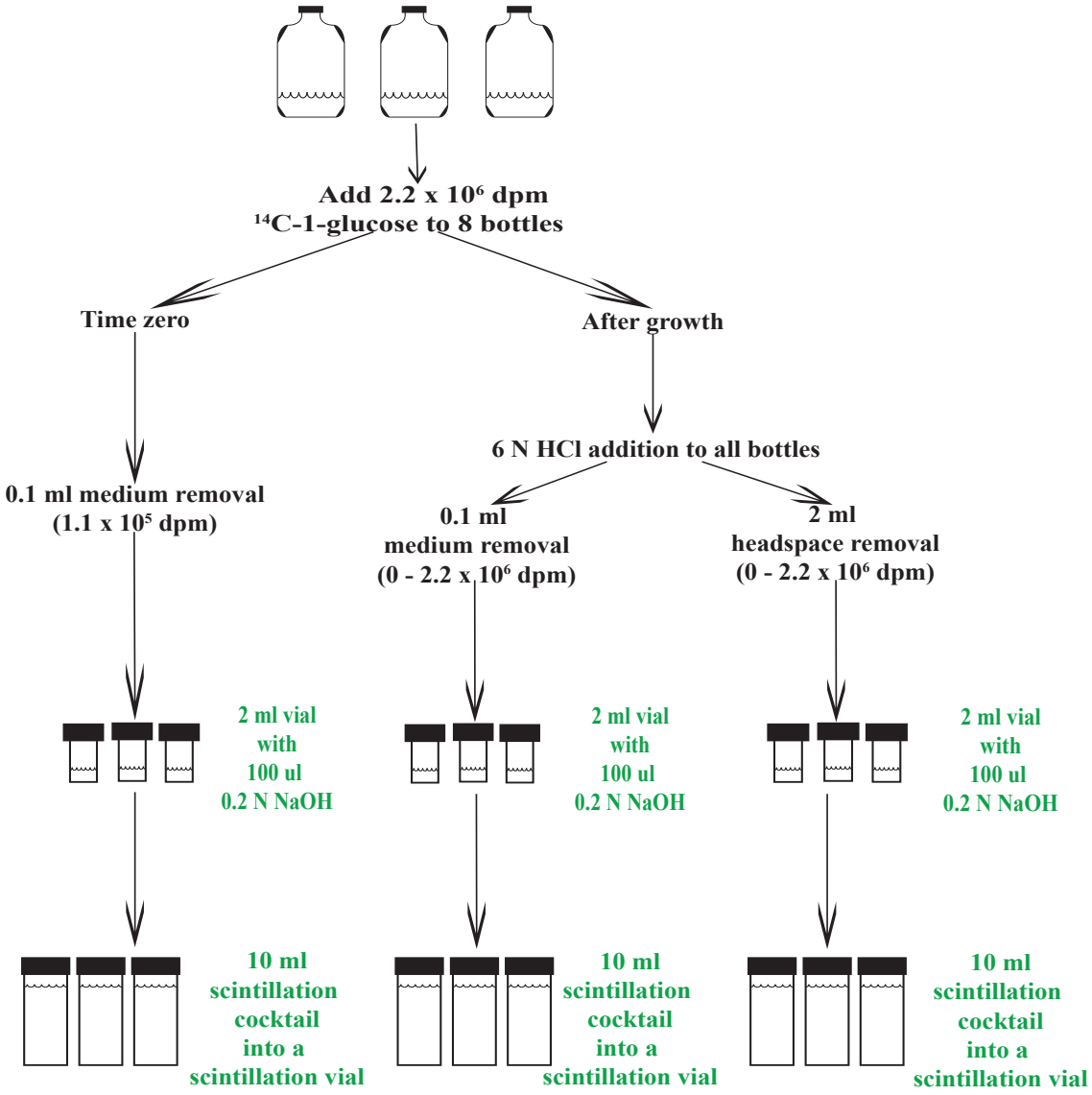
^{13}C-glucose analysis

^{13}C-1-glucose and ^{13}C-3-glucose were purchased from Cambridge Isotope Laboratories. ^{13}C-bicarbonate, ^{13}C-1-acetate and ^{13}C-2-acetate were purchased from Sigma-Aldrich. Anaerobic stock solutions were made for each stable isotope and added to the medium after autoclaving. The mineral medium previously described was used as the growth medium using the labeled glucose as the carbon source, when looking for labeled products, and unlabeled glucose in the presence of labeled bicarbonate and both positions of acetate when looking for exchange reactions.

Headspace gases were analyzed by GC-mass spectrometry (GC-MS) with an Agilent 7890 gas chromatograph **(**Agilent Technologies, Lexington, MA) equipped with an Agilent 5975C mass spectra detector (MSD) **(**Agilent Technologies) and a 27 m 320 um x 20 um column PoraPlotQ column **(**Agilent Technologies). The oven temperature was initially held at 50 °C for 8 min and then raised at a rate of 30 °C/min to 220 °C. Ions corresponding to mass units of 44 and 45 amu were monitored throughout analysis.

Liquid samples were also analyzed by nuclear magnetic resonance (NMR). Cultures were centrifuged at 4 °C for 30 min at 6,000 x g to remove cell material. The culture supernatant was then treated with the appropriate amount of Chelex resin (BioRad), as per the manufacturer's suggestion, for 1 hr at 23 °C to remove any

FIGURE 20. Depiction of the methods used after growth was observed and glucose was oxidized in cultures of *A. hydrogeniformans*, *L. lactis* and *L. mesenteroides* for the ^{14}C-1-glucose assay.



remaning metals. The samples were then freeze dried by lyophylization and resuspended in 1 ml of D_2O, and held at 4 °C until analysis could be completed.

^{13}C NMR using a UNITY INOVA 400 MHz spectrometer at a frequency of 100.529 MHz using a broadband probe. Spectra were collected at 23.5 °C using a single pulse experiment with pulse width (7.35 ms), a delay time of 10 s, an acquisition time of 1.30 s, a spectral width of 24125.5 Hz, and 256 acquisitions. A long delay time was employed to ensure adequate relaxation of carboxylic acid and carbonyl carbon nuclei.

Chemical shifts were calibrated by the addition of the internal standard, sodium 3-trimethylsilylpropionate-2,2,3,3-d_4 (TSP-d_4) (MSD Isotropic Products), that produces a reference signal at 0 ppm.

To ensure there was no exchange reactions taking place during fermentation and the acetate label was correct, *A. hydrogeniformans* was grown in the presence of ^{13}C-bicarbonate, ^{13}C-1-acetate or ^{13}C-2-acetate with either a 100 % N_2 or N_2:CO_2 (80:20) headspace. Each of liquid samples subjected to ^{13}C NMR were prepared and analyzed as stated above. When N_2:CO_2 was used as the gas phase 0.3 g L^{-1} sodium bicarbonate was added after pH adjusting to balance out any acidification due to CO_2 formation of carbonic acid.

Metabolite analysis:

A. hydrogeniformans was grown with the aforementioned medium using glucose as the substrate at a concentration of 3 g L^{-1}. Time points were taken at time zero and subsequently as glucose was 1/3, 2/3 and totally oxidized. Glucose loss was monitored

by the PGO enzyme assay (Sigma). At each of the time points the samples were rapidly cooled in an ice water bath to stop metabolic activity. The cells and culture medium were separated by centrifugation (6,000 x g, 20 min, 4 °C). And frozen at -80 °C until analysis.

The metabolic platforms used in this study were the same as previously described (21, 82). Briefly, the platform consisted of three independent analyses: ultrahigh performance liquid chromatography/tandem mass spectroscopy (UHLC/MS/MS) that was optimized for basic chemical species, UHLC/MS/MS that was optimized for acidic chemical species, and gas chromatography/mass spectroscopy (GC/MS). The major components of the process including the extraction protocol, analysis and metabolite identification were done as previously described (131).

Results

Enzyme Assays

Glycolytic enzymes of A. hydrogeniformans

Enzyme data points to the EM pathway of glycolysis (Table 11). Enzyme activities for phosphoglucose isomerase (1548 nmol min^{-1} mg $protein^{-1}$), phosphofructokinase (87 nmol min^{-1} mg $protein^{-1}$), fructose-1,6-bisphosphate aldolase (296 nmol min^{-1} mg $protein^{-1}$), 2-phosphoglycerate enolase (314 nmol min^{-1} mg $protein^{-1}$) and pyruvate kinase (79 nmol min^{-1} mg $protein^{-1}$) were all detected when assaying for enzyme activity using cell free extracts of *A. hydrogeniformans*. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was not be detected in *A. hydrogeniformans* using NAD^+ or $NADP^+$ as electron acceptors.

Pentose Phosphate Pathway

Low levels of 6-phosphogluconate dehydrogenase (14 nmol min^{-1} mg $protein^{-1}$ with NAD^+, 15 nmol min^{-1} mg $protein^{-1}$ with NADP+), transketolase (18 nmol min^{-1} mg $protein^{-1}$), and transaldolase (19 nmol min^{-1} mg $protein^{-1}$) activities of the pentose phosphate pathway were detected. Glucose-6-phosphate dehydrogenase (using NAD^+, $NADP^+$, or methyl viologen as electron acceptor) or 6-phosphogluconolactonase activities were not detected (oxidative branch enzymes of the PPP). These results suggest that *A. hydrogeniformans* does not have all of the enzymes for the oxidative portion of the PPP, but it does have the enzymes of the non-oxidative branch.

Non-phosporylated ED (nED)

The use of the nED pathway was tested in cell free extracts of *A. hydrogeniformans* by assaying glucose oxidoreductase, glucose dehydrogenase, glyceraldehyde oxidoreductase and glyceraldehyde dehydrogenase. The presence of glyceraldehyde oxidoreductase (GAOR) (413 nmol min^{-1} mg $protein^{-1}$) and low levels of glucose dehydrogenase (14 nmol min^{-1} mg $protein^{-1}$ and 13 nmol min^{-1} mg $protein^{-1}$ with NAD^+ and $NADP^+$ as respective electron acceptors) suggested the potential use of the nED path for glucose oxidation. However, no pyruvate or glyceraldehyde production from gluconate was detected.

Pyruvate decarboxylation

Cell free extracts of *A. hydrogeniformans* showed activity for pyruvate: ferredoxin oxidoreductase (2150 nmol $min^{-1}mg^{-1}$), phosphotransacetylase (758 nmol min^{-1} mg^{-1}) and acetate kinase (1270 nmol $min^{-1}mg^{-1}$) (Table 11). There was no detectable activity found under the assay conditions stated above for pyruvate dehydrogenase using NAD^+ or $NADP^+$ (Table 11). Activity for pyruvate:formate lyase was not detected, nor was formate dehydrogenase.

Hydrogenase

Table 13 shows the enzymatic activities of hydrogenases that were found in the membrane fraction as well as the soluble fraction of cell free extracts of *A. hydrogeniformans.* Enzyme activity for a membrane bound ferredoxin dependent hydrogenase was observed. The soluble fraction of cell free extracts displayed

activity for ferredoxin, NADH and NADPH hydrogenases. There was no observable NAD(P)H hydrogenase activity in the membrane fraction.

Other enzyme activities

Alcohol dehydrogenase using ethanol as electron donor and $NADP^+$ as electron acceptor (38 nmol min^{-1} mg $protein^{-1}$), NADH:methyl viologen oxidoreductase (305 nmol min^{-1} mg $protein^{-1}$), NADPH:methyl viologen oxidoreductase (568 nmol min^{-1} mg $protein^{-1}$) and 2-ketogluconate kinase (39 nmol min^{-1} mg $protein^{-1}$) were also detected. The following enzyme activities could not be detected in cell free extracts of *A. hydrogeniformans* using the assay conditions described: hexokinase, glucose:PEP phosphotransferase, glucose dehydrogenase, glucose-6-phosphate dehydrogenase, gluconate dehydratase, gluconate kinase and adenylate kinase.

Labeling experiments

Spectra of the medium alone

NMR analysis of the growth medium without the addition of labeled glucose or cells of A. hydrogeniformans was conducted. As seen in FIGURE 21, NMR spectra showed there were four signals present at 39.5, 52.7, 62.3 and 64.7. Subsequent analysis defined these peaks as belonging to the buffer TES used in the culture medium. The peaks were used as a scalar for peak intensity because they were present in all samples.

Table 11. Specific activities, electron donor/acceptor and evidence of gene products from the draft genome of enzymes involved in glucose oxidation to acetate, CO_2 and H_2 in cell extracts of *Anaerobaculum hydrogeniformans* compared between the Embden-Meyerhoff-Parnas Pathway, Pentose Phosphate Pathway, Entner-Doudoroff Pathway and Pyruvate Decarboxylation Pathway.

N/A = not assayed

Pathway/Enzyme	**Genome annotation**	**Donor/Acceptor**	**Activity (nmol min^{-1} mg^{-1})**
Embden Meyerhof Parnas			
Hexokinase	-	ATP	-
		ADP	-
PTS system	+	ATP	-
	+	ADP	-
Phosphoglucose isomerase	+		148
Phosphofructokinase	+	ATP	87
Fructose-1,6-bisphosphate aldolase	+		296
Triosephosphate isomerase	+		N/A
Glyceraldehyde-3-phosphate oxidoreductase	+	Benzyl viologen	41
Glyceraldehyde-3-phosphate dehydrogenase	+	NAD+	-
		NADP+	-
Phosphoglycerate mutase	+		N/A
2-phosphoglycerate enolase	+		314
Pyruvate kinase	+		79
Pentose Phosphate			

Glucose-6-phosphate dehydrogenase	+	NAD+	-
6-phosphogluconolactonase	-		-
6-phosphogluconate dehydrogenase	-	NAD+	14
	-	NADP+	15
Pentose-5-phosphate isomerase	+		N/A
Pentose-5-phosphate epimerase	+		N/A
Transketolase	+		18
Transaldolase	+		19
Entner-Doudoroff			
2-keto-3-deoxy-6-phosphogluconate aldolase	+		-
Pyruvate decarboxylation			
Pyruvate:ferredoxin oxidoreductase	+	Methyl viologen	2150
Pyruvate dehydrogenase	+	NAD+	-
	+	NADP+	-
Phosphotransacetylase	+		785
Acetate Kinase	+		1270

Table 12. Comparison the various specific activities (nmol min^{-1} mg^{-1}) and electron acceptors used to elucidate oxidoreductase and dehydrogenase activities on glucose-grown cell free extracts of *A. hydrogeniformans*.

Enzyme	Acceptor	Specific activity (nmol min^{-1} mg^{-1})
Glyceraldehyde-3-phosphate oxidoreductase	Benzyl viologen	41
Glyceraldehyde-3-phosphate dehydrogenase	NAD^+	-
	$NADP^+$	-
Glyceraldehyde oxidoreductase	Benzyl Viologen	413
Glyceraldehyde dehydrogenase	NAD^+	-
	$NADP^+$	-
Pyruvate:ferredoxin oxidoreductase	Benzyl viologen	2150
Pyruvate dehydrogenase	NAD^+	-
	$NADP^+$	-
NADH:methyl viologen oxidoreductase	Methyl viologen	305
NADPH:methyl viologen oxidoreductase	Methyl viologen	568

Table 13. Hydrogenase activity of cytosolic and membrane fractions of *A. hydrogeniformans.*

* BV=benzyl viologen

	μmol min^{-1} mg $protein^{-1}$ cytosolic	membrane
BV* reduction by H_2	13.4	0.061
NAD reduction by H_2	0.383	0
NADP reduction by H_2	0.228	0

^{13}C-C1 labeled glucose standard spectra

A purity check of the ^{13}C-1 glucose dissolved in the media used in the experiments was evaluated by NMR. Two very prominent signals are detected at 93.71 and 97.41 ppm, corresponding to the C_1-labeled carbons of a-pyranose (93.8 ppm) and b-pyranose (97.4 ppm) respectively (9)

^{13}C-C_3 labeled glucose standard spectra

A purity check of the ^{13}C-3 glucose dissolved in the media used in the experiments was evaluated by NMR. Two very prominent signals are detected at 74.63 and 77.51 ppm, corresponding to the C_3-labeled carbons of a-pyranose (74.5 ppm) and b-pyranose (77.5 ppm) respectively (9).

^{13}C-C_1 labeled glucose experiment spectra

The ^{13}C-1 glucose experiment spectra displayed two very large and prominent signals at 18.9 and 26.2 ppm, resulting from ^{13}C labeled products (FIGURE 22). These two signals were much more intense than the four signals produced by the media. Table 14 lists these two signals and the numerous additional signals present at much lower intensity. Of these less intense signals, two signals of equal intensity at 21.07 and 176.49 ppm correspond to the methyl carbon and the carboxylic carbon, respectively, of acetate. The equal intensity of these two signals of acetate suggests that both carbon atoms are equally labeled with ^{13}C, either from natural abundance or the ^{13}C labeled glucose.

Numerous small signals were observed between 10 and 37 ppm that correspond to saturated carbon atoms: methyl, methylene, methine and quaternary carbons. Due to the very low intensity of these signals relative to the two large signals, it is presumed that their ^{13}C isotope composition results from natural abundance.

^{13}C-C3 labeled glucose samples

FIGURE 23 shows the NMR spectra of the ^{13}C-3-glucose grown cultures of *A. hydrogeniformans*. One noticeable difference from the C_1-labeled glucose experiment spectra is the lack of the numerous small signals between 10 and 37 ppm; this region is devoid of such small signals in the C_3-labeled glucose experiment spectra. However, the C_3-labeled glucose experiment spectra show carboxylic acid signals (178.88 ppm) of greater intensity than displayed in the C_1-labeled glucose experiment spectra. If the medium peaks are used as a scalar, the peak intensities were much smaller in the ^{13}C-3-glucose grown cultures as compared to the ^{13}C-1-glucose grown cultures.

^{13}C-C1 labeled acetate with N_2 headspace

The four media signals described above were present. Also present was a signal, similar in intensity to the media signals, at ~ 181 ppm resulting from the ^{13}C labeled C1 carbon of acetate. The decreased signal intensity illustrates that this C13 labeled acetate is being consumed. Another signal, similar in intensity to the media signals, appeared at ~ 51 ppm. This may be due to ^{13}C labeled methanol. Methanol in D2O has a C-13 NMR signal at 49.5 ppm, so the observed 51 ppm seems a bit high. However the solution pH can cause slight shifts in alcohol chemical shifts (Mark Nanny, personal communication). Methanol was not detected by GC-FID. A third signal is observed at ~ 16.7 ppm which

may result from the C2 carbon of ethanol (in D2O, the C-2 carbon of ethanol is at 17.6 ppm). Ethanol has been detected in spent fermentation broth, however, at very low concentrations. A very, very small signal was observed around 24 ppm and probably results from ^{13}C labeled C2 carbon of acetate. ^{13}C labeled C1 acetate is clearly detected, although in much lower concentration than the starting amount. Possible formation of ^{13}C labeled methanol and ^{13}C labeled ethanol with the label at the C2 position, which is confirmed by alcohol quantification by GC-FID.

^{13}C-C1 labeled acetate with N_2:CO_2 headspace and unlabeled bicarbonate

All four media signals describe above were observed. A peak with similar intensity to the media signals was observed at 181.9 ppm from C13 labeled C1 acetate. Also observed was a prominent signal (approximately 10 to 15x larger than the media signals) at 50.8 ppm which may be ^{13}C labeled methanol. Two smaller signals were observed at 24.0 ppm and 16.7 ppm, the initial being ^{13}C labeled C2 carbon of acetate and the later possibly the C2 carbon of ethanol.

^{13}C-C2 labeled acetate with N_2 headspace

All four media signals described above were observed. Two prominent signals (approximately 5 to 10x greater in intensity than the media signals) were observed at 23.6 ppm and 16.8 ppm. The first signal is unreacted ^{13}C labeled C2 carbon acetate and the later signal at 16.8 ppm is probably the ^{13}C labeled C2 carbon of ethanol.

FIGURE 21. NMR spectra of medium prior to inoculation and ^{13}C-glucose addition. These four medium peaks were present in all NMR spectra recorded. The peak associated with 0.000 ppm is from the addition of the internal standard sodium 3-trimethylsilylpropionate-2,2,3,3-*d*$_4$ (TSP-*d*$_4$). Spectral analysis conducted by Dr. Mark Nanny.

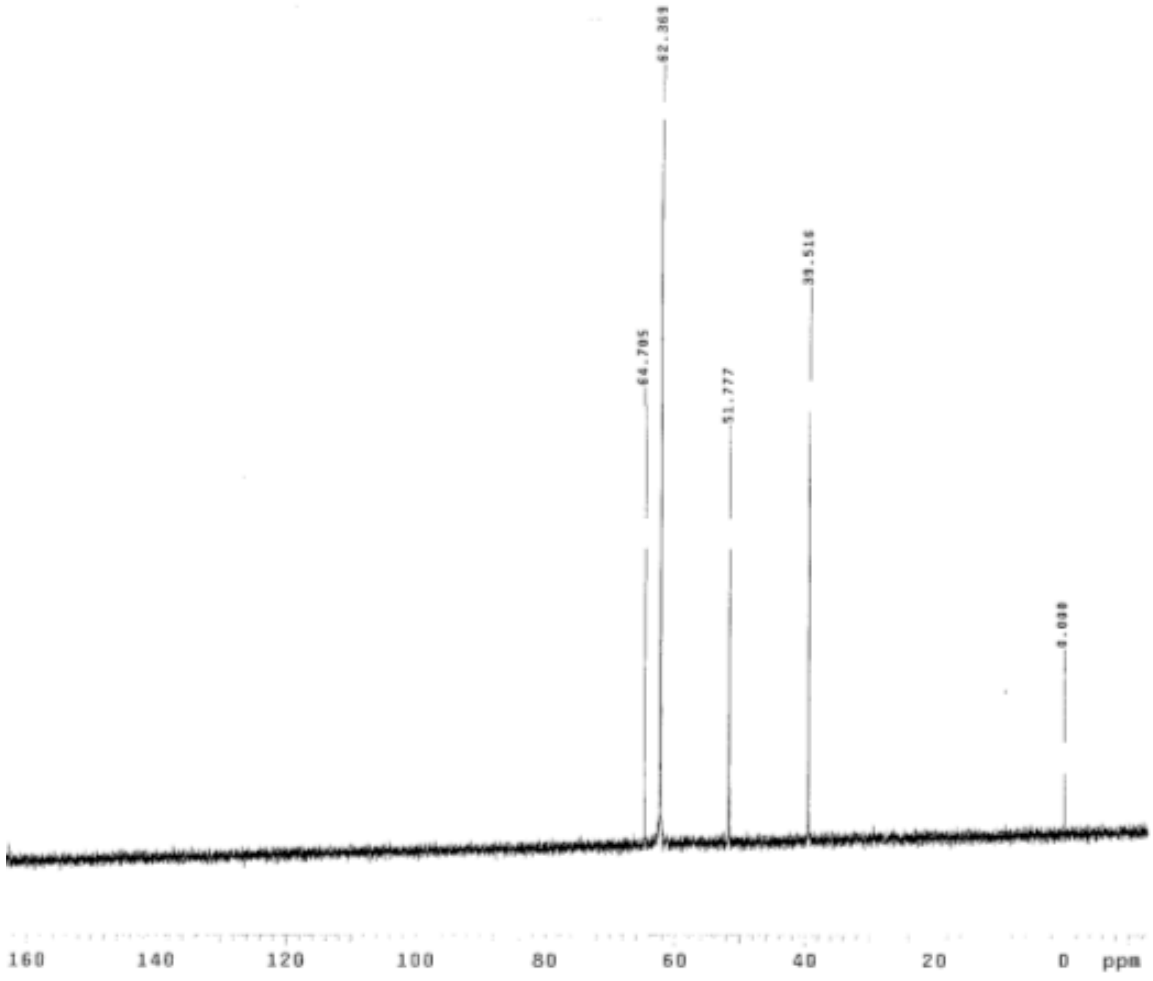


FIGURE 22. NMR spectra of ^{13}C-1-glucose grown *A. hydrogeniformans*. Peaks are labeled according to functional group annotation conducted by Dr. Mark Nanny.

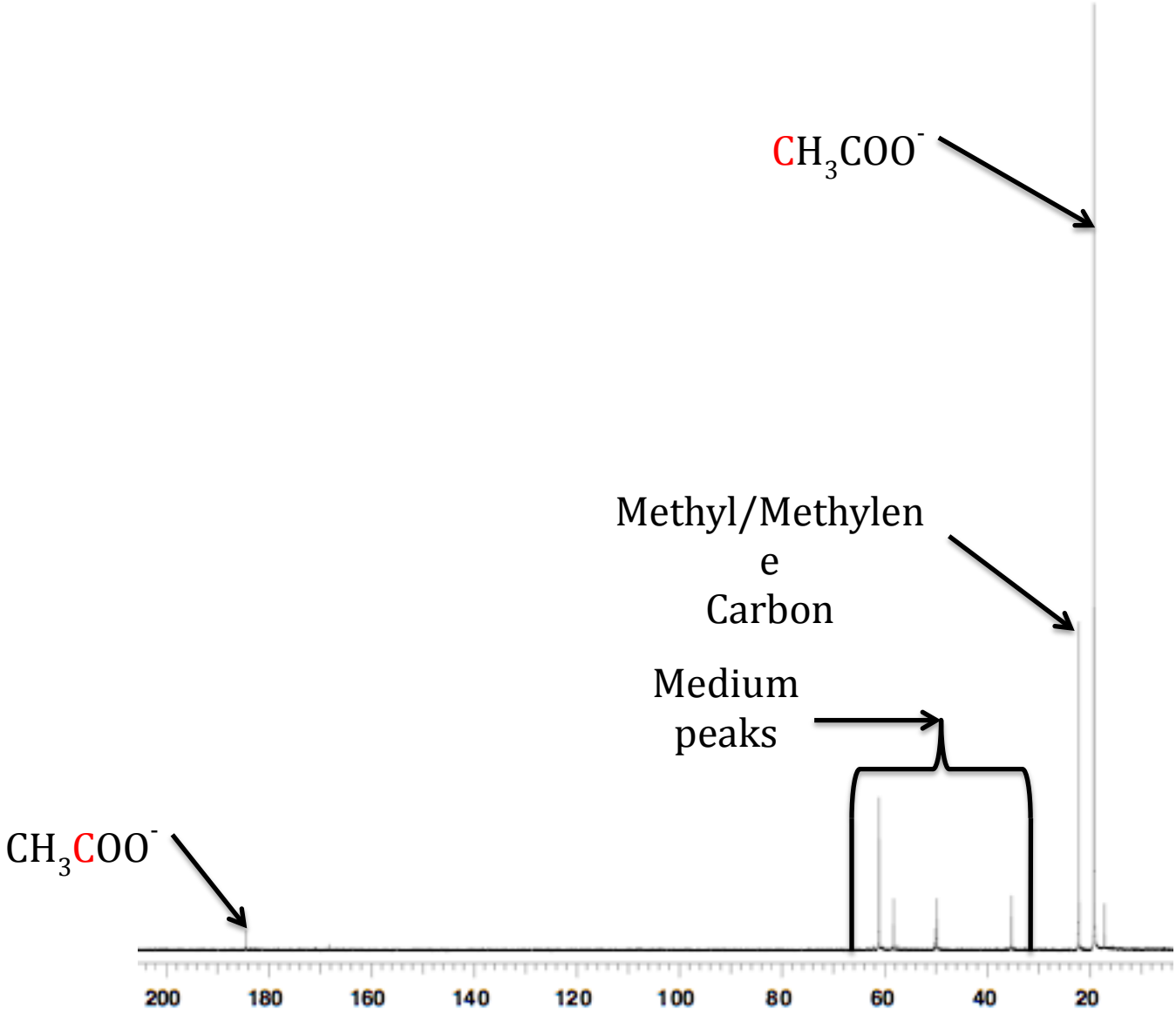
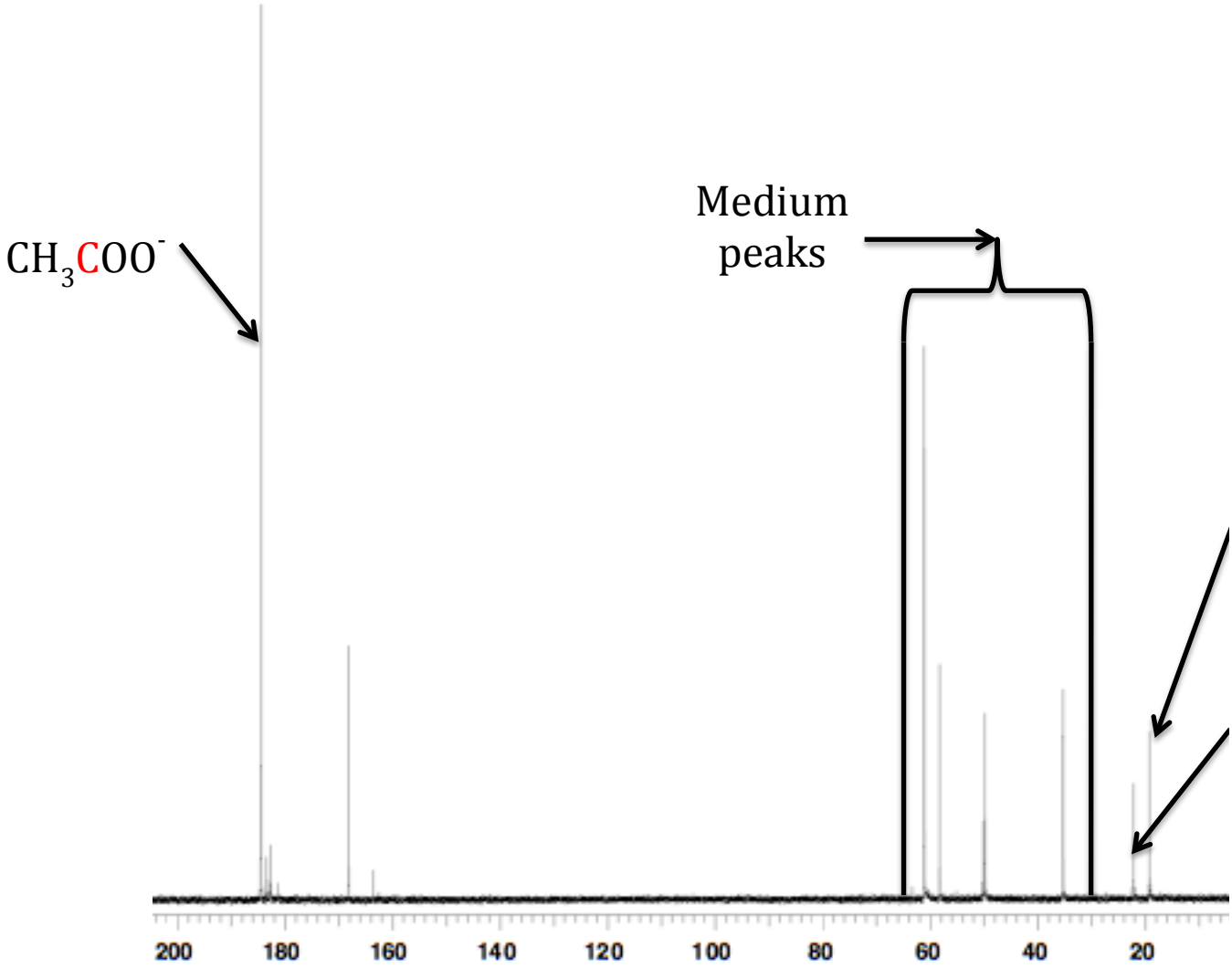


FIGURE 23. NMR spectra of ^{13}C-1-glucose grown *A. hydrogeniformans*. Peaks are labeled according to functional group annotation conducted by Dr. Mark Nanny.



^{13}C-C2 labeled acetate with N_2:CO_2 headspace + unlabeled bicarbonate

All four media signals were observed. At much greater intensity (20 to 50x) than these media signals, two signals at 24.1 ppm and 16.8 ppm were observed. As previously mentioned, these signals result from the ^{13}C label of the C2 carbon in acetate and probably the ^{13}C labeled C2 carbon of ethanol. Very, very small signals were observed at 20.9 ppm, 50.9 ppm, 176.2 ppm, and 181.9 ppm. The 50.9 ppm and 181.9 ppm signals are probably trace amounts of ^{13}C labeled methanol and ^{13}C labeled C1 acetate.

^{13}C labeled bicarbonate with N_2 headspace

All four media signals are present. Four small signals (approximately one tenth the media signals): 16.8 ppm which could be the ^{13}C labeled C2 carbon of ethanol; 24.0 ppm which is probably the ^{13}C labeled C2 carbon of acetate; 51.0 ppm which could be from ^{13}C labeled methanol; and 176.3 ppm which is an unidentified carboxylic acid (which was observed in sample set 4).

^{13}C labeled carbonate with N_2:CO_2 headspace

These samples were identical to samples from the ^{13}C labeled bicarbonate with N_2 headspace except the signal at 176 ppm was barely observable.

^{13}C-GC-MS analysis

Cultures of *A. hydrogeniformans* grown with [1-^{13}C]-D-glucose or [3-^{13}C]-D-glucose were subjected to GC-MS analysis. Cell grown with [^{13}C-1]-D-glucose showed an acetate peak corresponding to a one mass unit shift. This conflicts with earlier studies

using ^{13}C-NMR in which a labeled carboxyl position was detected when cells were grown on [3-^{13}C]-D-glucose. However, the peaks observed in the NMR spectra were not much more substantial than the peaks resulting from the medium blank. This shows that NMR is more sensitive to the detection of ^{13}C labeled product when compared to GC-MS and was only used as a qualitative tool for metabolite detection (Mark Nanny, personal communication). There were no labeled intermediates were detected on cells grown on [3-^{13}C]-D-glucose.

^{13}C-CO_2 GC-MS analysis

Headspace ^{13}C-CO_2 produced from either ^{13}C-1 or ^{13}C-3 glucose-grown cultures of *A. hydrogeniformans* were analyzed by GC-MS, in which ion 44 was monitored for the presence or absence of a labeled CO_2. When *A. hydrogeniformans* was grown with [1-^{13}C]-D-glucose no ^{13}C-CO_2 was detected by monitoring ion 44 (FIGURE 24). When grown with [3-^{13}C]-D-glucose, *A. hydrogeniformans* produced about 30 % ^{13}C-CO_2 in the headspace (FIGURE 25). Theoretically there should have been 50 % ^{13}C-CO_2 in the headspace. The CO_2 could have been trapped as carbonates or incorporated into other compounds.

^{14}C-CO_2 analysis

Cultures grown with 1-^{14}C-D-glucose had only small amounts of radio labeled CO_2 in the headspace after near depletion of glucose. Control cultures of *Lactococcus lactis* (a homofermentative lactic acid bacterium) and *Leuconostoc mesenteriodes* (a heterofermentative lactic acid bacterium) were used to ensure the method of CO_2 trapping was effective. A homofermentative lactic acid bacterium only produced lactic acid as an

FIGURE 24. GC-MS profile of ^{13}C-1 glucose grown cultures of *A. hydrogeniformans*.

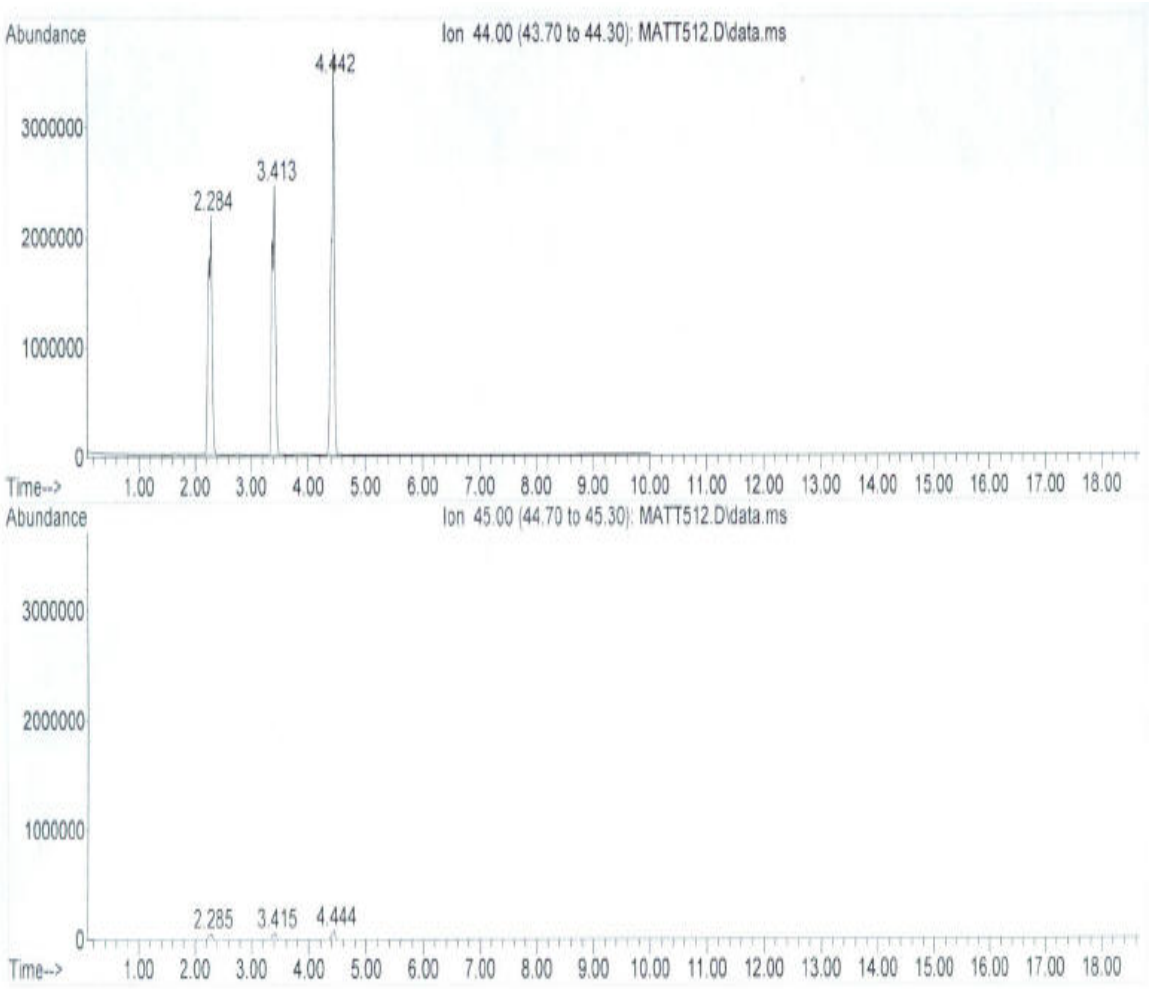
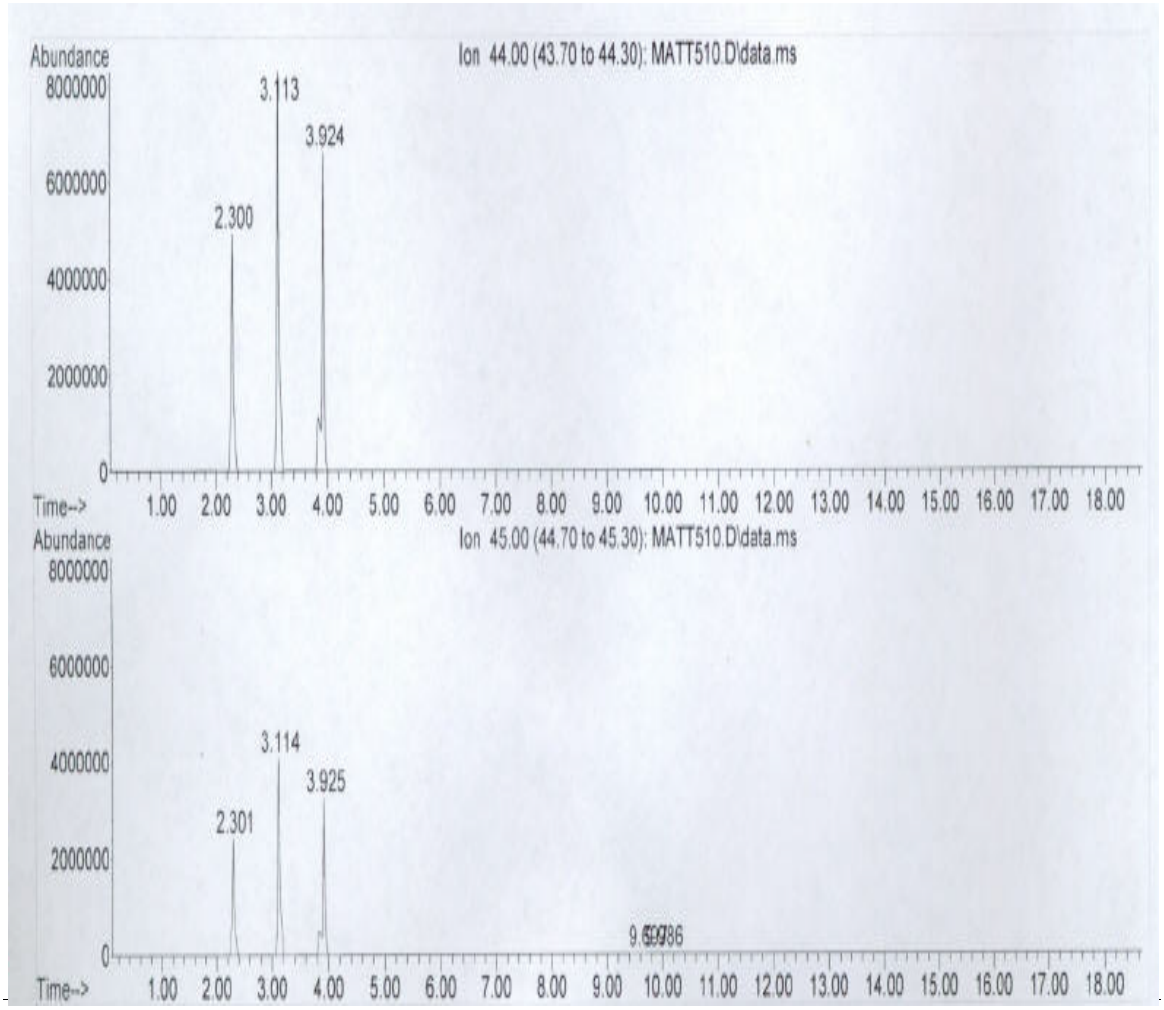


FIGURE 25. GC-MS profile of ^{13}C-3 glucose grown cultures of *A. hydrogeniformans*.



end-product, thus would not produce CO_2 from the C-1 of glucose so the label would remain in the culture medium. A heterofermentative lactic acid bacterium, however, produces lactic acid and CO_2 as the products of fermentation. CO_2 is produced from the C-1 of glucose, thus making it a good positive control. Over 95 % of the label remained in the liquid phase after acidified cultures of *A. hydrogeniformans* were analyzed by liquid scintillation counting (Table 14), further supporting the ^{13}C-CO_2 data. Both control cultures and the medium control performed as expected (Table 14).

Metabolite screening

Metabolites were screened for glucose grown cultures of *A. hydrogeniformans* and time points were taken as glucose was depleted. FIGURE 26 shows how the data was qualified by Metabolon and fitted into Box and Whiskers plots. This form of data analysis was used to determine if the metabolites were present in all three of the time points. FIGURE 27 shows the metabolites detected during the time course experiment.

Table 14. % ^{14}C observed from the liquid and gas phase and total % ^{14}C recovery that resulted from ^{14}C-1-glucose grown cultures of *A. hydrogeniformans*, *L. lactis*, *L. mesenteriodes* and an uninoculated control.

	Liquid phase (% ^{14}C)	Gas phase (% ^{14}C)	Recovery (%)
Medium	94.1	1.1	95.2
A. hydrogeniformans	94.4	4.3	98.7
L. lactis	96.2	3.4	99.6
L. mesenteroides	16.7	101.6	118.3

FIGURE 26: An example of the metabolite ribose as qualified by Metabolon using a Box and Whiskers plot for data interpretation, which is based on the scaled intensity value.

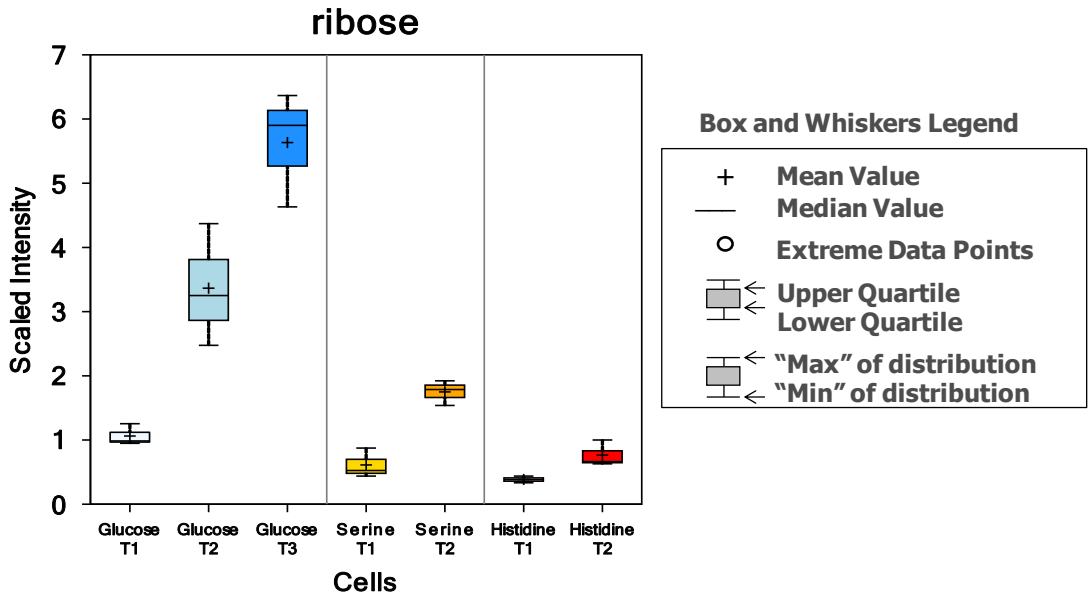
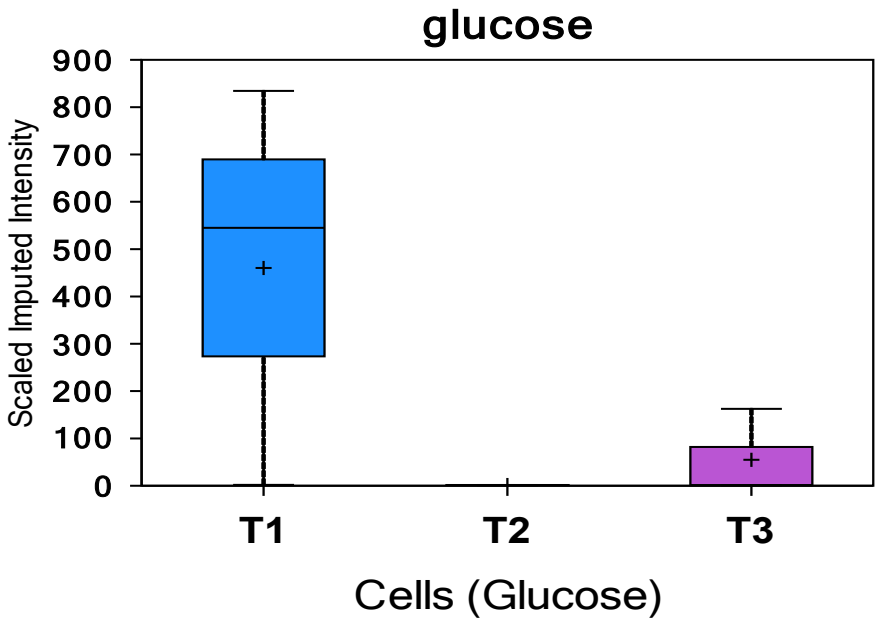


FIGURE 27. Data compiled from glycolytic intermediate metabolite detection (presented in Box and Whiskers plots) by Metabolon Inc. All metabolites reported were detected from the extraction procedure carried out on cell material and are labeled accordingly: A) glucose; B) glucose-6-phosphate; C) fructose-6-phosphate; D) fructose-1,6-bisphosphate/glucose-1,6-bisphosphate isobar; E) dihydroxyacetone phosphate; F) 3-phosphoglycerate, G) 2-phosphoglycerate; H) pyruvate; I) acetyl-CoA; J) acetylphosphate and K) ribulose-5-phosphate/xylulose-5-phosphate isobar.

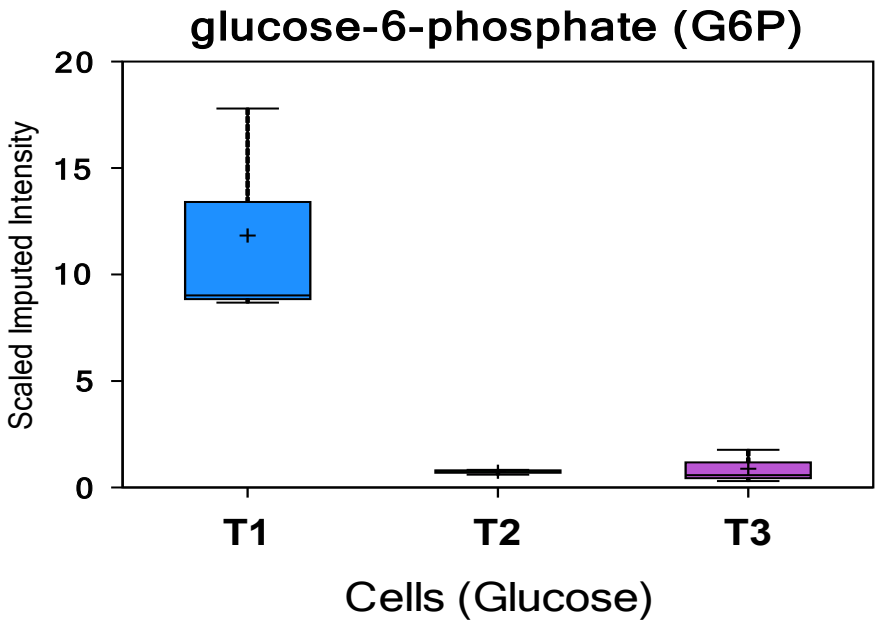
Data from the metabolites were collected from three distinct time points in which *A. hydrogeniformans* oxidized 1/3 (T_1), 2/3 (T_2) and all (T_3) of the initial glucose added to the medium.

Metabolite abundance is reported as a scaled intensity value, which is described in the materials and methods.

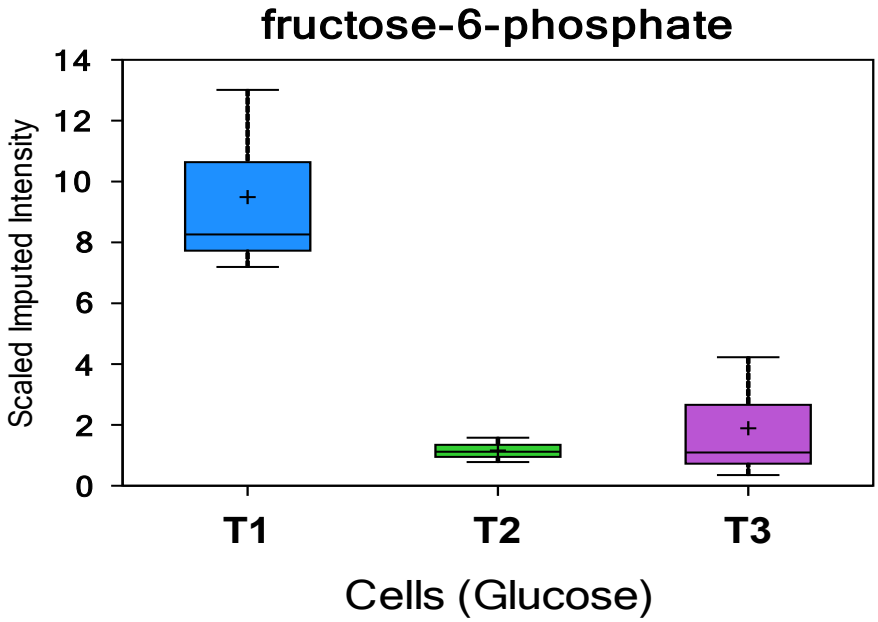
A)


glucose
900
800
700
600
500
400
300
200
100
0
Scaled Imputed Intensity
T1
T2
T3
Cells (Glucose)

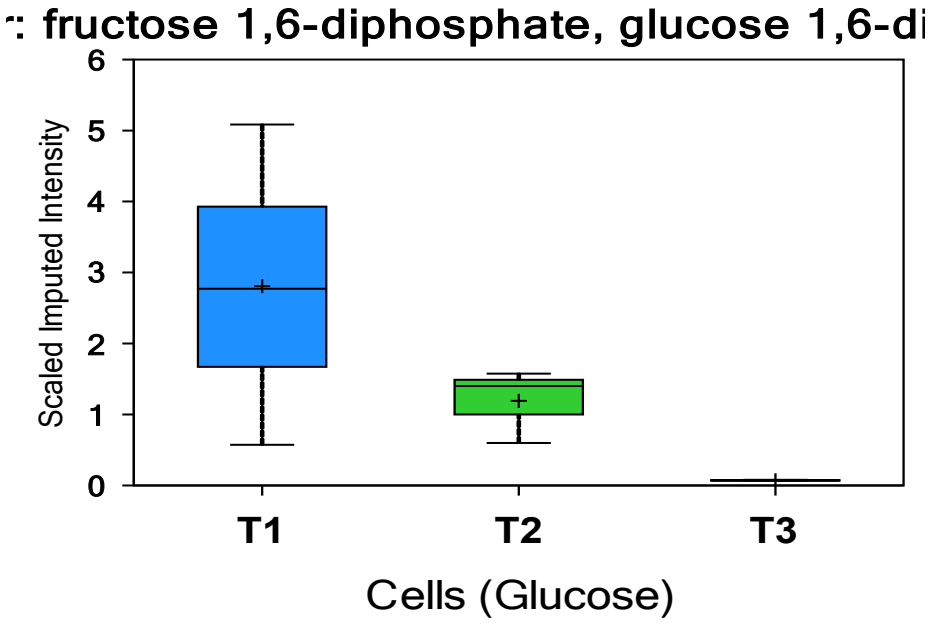
B)


glucose-6-phosphate (G6P)
20
15
10
5
0
Scaled Imputed Intensity
T1
T2
T3
Cells (Glucose)

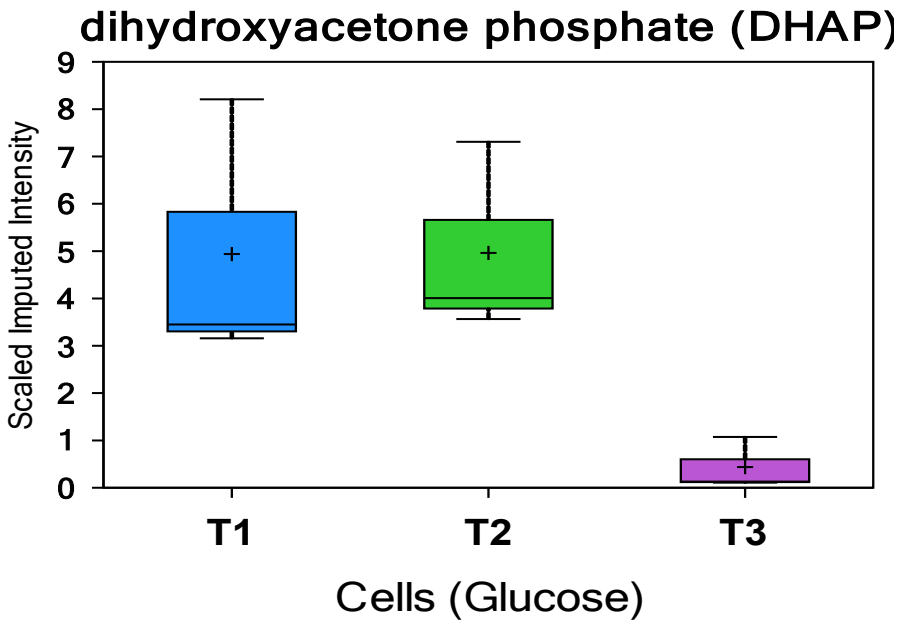
C)


fructose-6-phosphate
Scaled Imputed Intensity
14
12
10
8
6
4
2
0
T1
T2
T3
Cells (Glucose)

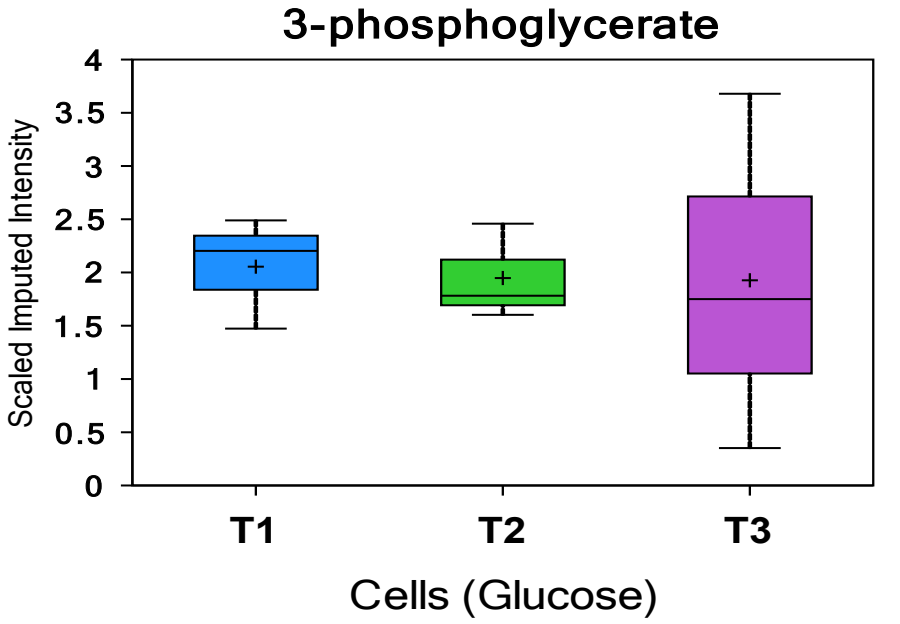
D)


: fructose 1,6-diphosphate, glucose 1,6-di
Scaled Imputed Intensity
6
5
4
3
2
1
0
T1
T2
T3
Cells (Glucose)

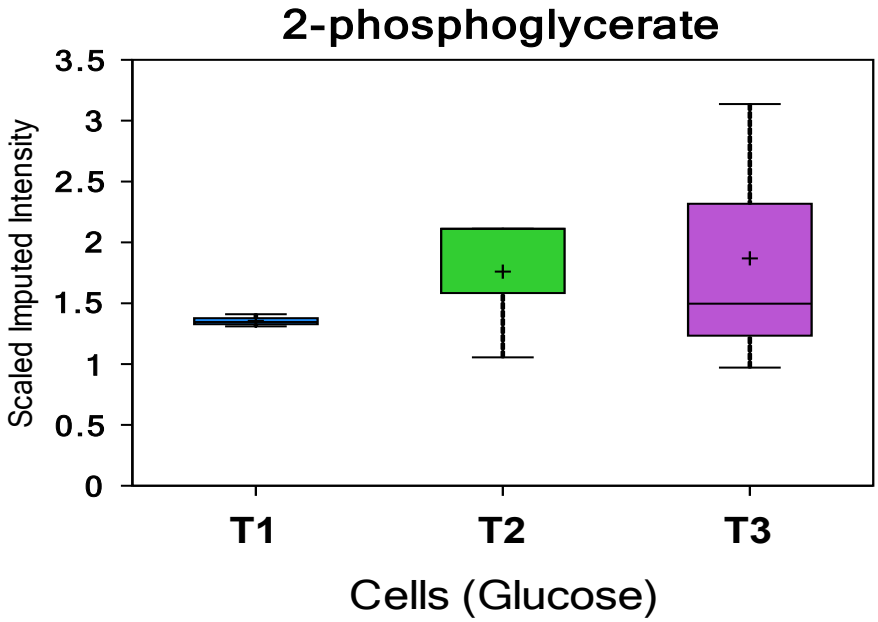
E)


dihydroxyacetone phosphate (DHAP)
Scaled Imputed Intensity
9
8
7
6
5
4
3
2
1
0
T1
T2
T3
Cells (Glucose)

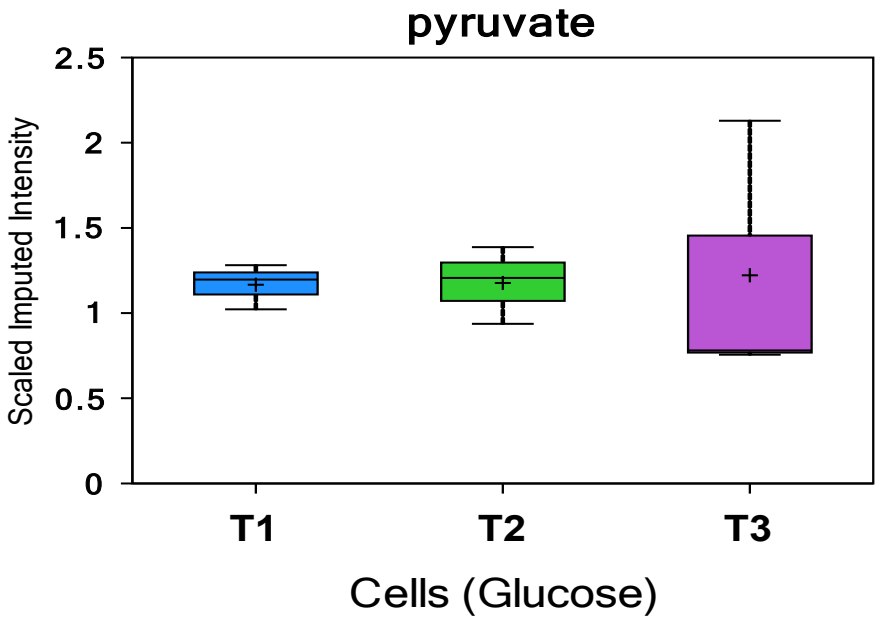
F)


3-phosphoglycerate
Scaled Imputed Intensity
4
3.5
3
2.5
2
1.5
1
0.5
0
T1
T2
T3
Cells (Glucose)

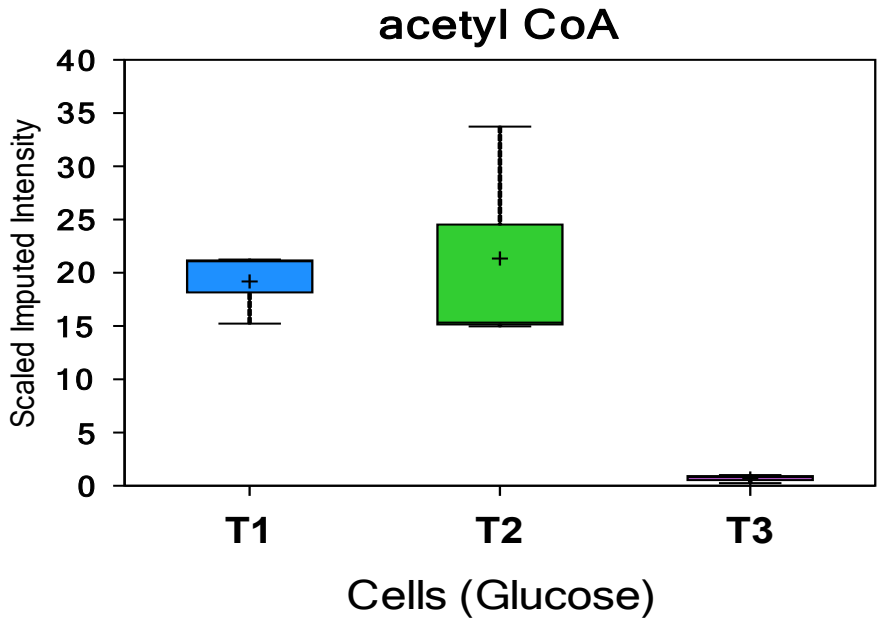
G)


2-phosphoglycerate
Scaled Imputed Intensity
3.5
3
2.5
2
1.5
1
0.5
0
T1
T2
T3
Cells (Glucose)

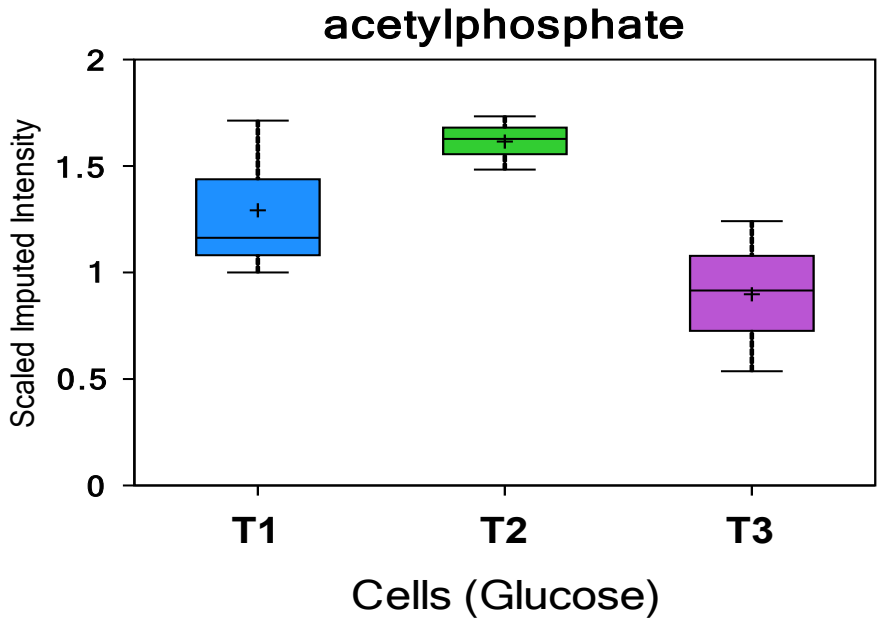
H)


pyruvate
Scaled Imputed Intensity
2.5
2
1.5
1
0.5
0
T1
T2
T3
Cells (Glucose)

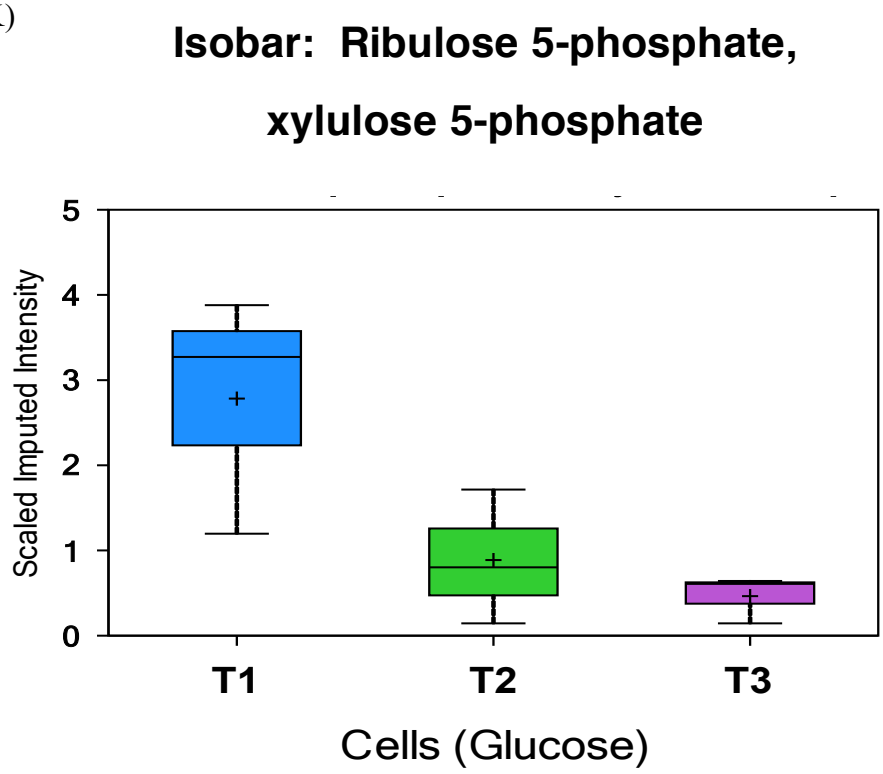
I)


acetyl CoA
Scaled Imputed Intensity
40
35
30
25
20
15
10
5
0
T1
T2
T3
Cells (Glucose)

J)


acetylphosphate
Scaled Imputed Intensity
2
1.5
1
0.5
0
T1
T2
T3
Cells (Glucose)

K)


Isobar: Ribulose 5-phosphate,
xylulose 5-phosphate
Scaled Imputed Intensity
5
4
3
2
1
0
T1
T2
T3
Cells (Glucose)

Discussion

Entner-Doudoroff Pathway

The use of the traditional phosphorylated-Endtner-Doudoroff (ED) pathway of glycoclysis was assessed by assaying enzyme activities in cell free extracts of *A. hydrogeniformans*. The key enzymes of the ED pathway, glucose-6-phosphate dehydrogenase and KDPG aldolase, were not detected under the assay conditions established in this study. Hence, the traditional ED pathway is not used by *A. hydrogeniformans* for central carbon metabolism. However, other modifications of the ED pathway have been described in the literature and could be used by *A. hydrogeniformans*, such as the nonphosphorylated ED (nED) pathway (97).

The nED pathway is characterized as having nonphosphorylated intermediates prior to the metabolite 2-keto-3-deoxygluconate. Gluconate is dehydrated to 2-keto-3-deoxygluconate by gluconate dehydratase, and the 2-keto-3-deoxygluconate is further phosphorylated by a saecial kinase to form KDPG (132). It was originally thought that glucose oxidation by *P. furiosus* was done so by a modified or nED pathway (97) and this has been shown in other thermophilic archaeal species, such as *Sulfolobus acidocaldarius* (13) and *Thermoplasma acidophila* (99). This was first hypothesized because cell free extracts of *P. furiosus* possessed activity for a glucose oxidoreductase that converted glucose to gluconate (49). The gluconate was oxidized further to pyruvate in the nonphosphorylated forms of the intermediates (49). There were detectable activities of glyceraldehyde oxidoreductase (GAOR) (413 nmol min^{-1} mg $protein^{-1}$) and low levels of glucose dehydrogenase (14 nmol min^{-1} mg $protein^{-1}$ and 13 nmol min^{-1} mg $protein^{-1}$ with NAD^+ and $NADP^+$ as respective electron acceptors) in cell free extracts of *A.*

hydrogeniformans. There was no detectable gluconate dehydratase activity and KDPG aldolase. The use of gluconate dehydratase is by *A. hydrogeniformans* unlikely. Glucose oxidoreductase and glyceraldehyde dehydrogenase were also assayed. However no activity was detected in this study.

Another function of the GAOR was described in a modification of the traditional Embden Meyerhof (EM) pathway in which fructose-1-phosphate was cleaved by fructose-1-phosphate aldolase to produce dihydroxyacetone phosphate and glyceraldehyde. The glyceraldehyde was then converted to glycerate by GAOR, which could then be transformed to 2-phosphoglycerate by glycerate kinase and enter the later portion of the EM pathway (97). The use of this pathway by *A. hydrogeniformans* is unlikely because no glycerate kinase activity was observed under the conditions tested.

Key metabolites of the ED pathway, such as 6-phosphogluconate dehydrogenase or 2-keto-3-deoxy-6-phosphogluconate (KDPG) were not detected. The lack of metabolites is not surprising because there was no measurable KDPG aldolase activity, which is a key enzyme of the ED pathway (99). Labeling studies with ^{13}C-1 and ^{13}C-3 glucose as the substrate for *A. hydrogeniformans* did not show the C-1 of glucose incorporated in CO_2 or the C-3 of glucose in the methyl position of acetate, which is indicative of the ED and nED pathways (99).

Pentose Phosphate Pathway

The potential use of the Pentose Phosphate Pathway (PPP) by *A. hydrogeniformans* was addressed by conducting enzyme assays looking at both the

oxidative and nonoxidative branches of the PPP. In order for the PPP to be considered for central carbon metabolism both branches of the PPP need to be active. Enzyme assays showed that *A. hydrogeniformans* lacked activity for glucose-6-phosphate dehydrogenase and suggest that *A. hydrogeniformans* does not have all of the enzymes for the oxidative portion of the PPP, but it does have the enzymes of the nonoxidative branch. This could be indicative of the use of the PPP for the generation of pentoses for DNA synthesis similar to that reported for *E. coli* (93) and *T. maratima* (123).

If the PPP is used for central carbon metabolism, then the C-1 of glucose is converted to CO_2 and the C-3 will be incorporated into the carboxyl position of acetate (99). NMR analysis did show some incorporation of the C-3 of glucose into the carboxyl position of acetate, but the signal intensity was much lower than that observed in the methyl position of acetate from C-1 glucose grown cultures. Additionally, there was no labeled CO_2 from the C-1 of glucose. If the PPP is being used as the dominant central metabolic process there would have been detectable levels of labeled CO_2 from the C-1 of glucose. Thus, the possible use of the nonoxidative branch of the PPP for production of biosynthetic intermediates was assessed by metabolite analysis.

The nonoxidative branch intermediates ribulose-5-phosphate and xylulose-5-phosphate were detected throughout the time course metabolite screening. No metabolites corresponding to the oxidative portion of the PPP were detected. This supports the labeling data, in that the nonoxidative portion of the PPP is being used, however it is more than likely for fatty acid biosynthesis and/or nucleotide biosynthesis similar to that observed in cultures of *T. maratima* (123).

Embden-Meyerhoff Pathway

Activities were detected for all enzymes of the EM pathway except for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using NAD^+ or $NADP^+$ as electron acceptors. In eubacteria and eukarya, the oxidation of glyceraledehyde-3-phosphate (GAP) is catalyzed by the two-step conversion of GAP to 3-phosophoglycerate by the use of GAPDH and a 3-phsophoglycerate kinase (118). The use of a GAPDH has been described in *T. maritima* (98) and *Caldicellulosiruptor saccharolyticus* (116), which also produce 4 H_2 per glucose using the EM pathway. However, the glycolytic conversion of GAP in the hyperthermophilic archaeon *Pyrococcus furiosus* is performed by a phosphate-independent glycerladehyde-3-phosphate oxidoreductase (GAPOR) (71, 118). Selig *et al*. (99) showed the activity of GAPOR from various other thermophilic archaeal species such as *Desulfurococcus amylolyticus*, *Thermococcus celer* and *Thermococcus litoralis*. Selig *et al*. (99) also confirmed that these species used the EM pathway of glycolysis for the oxidation of glucose to pyruvate. Cell free extracts of *A. hydrogeniformans* did exhibit activity for a phosphate-independent and ferredoxin-dependent GAPOR using benzyl viologen as an electron acceptor. This is the first report of GAPOR activity observed in a eubacterial species, which nullifies the previous hypothesis that the use of GAPOR is a physiological characteristic of archaea exclusively (118).

The use of the EM pathway for central carbon metabolism was further supported by ^{13}C NMR and metabolite analysis data. NMR analysis of C-1 and C-3 glucose grown cultures of *A. hydrogeniformans* showed the incorporation of the C-1 into the methyl position of acetate and CO_2 produced from the C-3 position of glucose, which is a labeling pattern specific to the EM pathway (99).

Metabolites corresponding to the EM pathway were all detected with the exception of glyceraldehyde-3-phosphate and 1,3-bisphosphoglycerate. Glyceraldehyde-3-phosphate has been shown to be an unstable metabolite (71), which makes its detection difficult. The use of a modified EM pathway using GAPOR was shown to not produce 1,3-bisphosphoglycerate when Mukund and Adams (71) assayed cultures of *P. furiosus*, but GAPOR directly oxidized glyceraldehyde-3-phosphate to 3-phosphoglucerate. This further supports the hypothesis that *A. hydrogeniformans* is using GAPOR in a fashion similar to that of *P. furiosus*.

In looking at all of the data collected from this study it can be concluded that *A. hydrogeniformans* utilizes the EM pathway for glucose oxidation with a substitution of the traditional eubacterial GAPDH for a GAPOR. The use of a modified EM pathway has been confirmed by labeling, metabolite and genome analysis. The pathway seems to be identical to that used by the archaeon *P. furiosus* (118). This is the first description of this modified EM pathway in a eubacterial species.

Pyruvate decarboxylation

Activities for pyruvate:ferredoxin oxidoreductase (POR), phosphotransacetylase and acetate kinase were detected in cell free extracts of *A. hydrogeniformans*. These results are similar to that found in cell free extracts of *P. furiosus* (94, 96), *T. maratima* (6) and *C. saccharolyticus* (135), which all use a three enzyme system of pyruvate decarboxylation. Draft genome annotation of *A. hydrogeniformans* showed there were gene products for both the pyruvate:ferredoxin oxidoreductase and the pyruvate dehydrogenase. However enzyme activity for pyruvate dehydrogenase was not detected in cell free extracts of *A. hydrogeniformans*. There was also no annotations for either the

pyruvate:formate lyase or formate dehydrogenase, which are enzymes used for pyruvate decarboxylation in mesophilic species such as enterics and *E. coli* (51, 110, 114). The genomic data is supported by the lack of enzymatic activity of those two enzyme in the conditions established in this study.

Hydrogenases

Hydrogenases are oxygen-sensitive enzymes found in all three domains of life that are central to cellular H_2 formation and can be reversed for H_2 utilization. These enzymes contain complex metal cofactors and typically consist of multiple subunits (11). The hydrogenases of most 4 H_2 producing microorganisms remain unstudied (11). The description of a membrane-bound, ferredoxin-dependant hydrogenase has been described for *T. tengcongensis*, and may act as a proton pump to generate a proton motive force (104). The same hydrogenase has been observed in the genome of *C. saccharolyticus* (116), but its functionality has yet to be tested. A soluble NADH dependent hydrogenase has been described in *T. maritima* (127), and the gene sequence of a soluble NADH dependent hydrogenase has been detected in the genome of *C. saccharolyticus* (116). It is thought to regenerate reducing equivalence by the production of H_2 (116). A soluble ferredoxin dependent hydrogenase has been described for the archaeon *Thermococcus kodakaraensis* (44), but has yet to be reported in other microorganisms. NAD(P)H and ferredoxin dependent soluble hydrogenase and a solely ferredoxin dependent membrane bound hydrogenase activities were found in cell free extracts of *A. hydrogeniformans*. *T. maritima,* has a bifurcating hydrogenase system that uses NADPH (produced from glyceraldehyde-3-phosphate dehydrogenase) and ferredoxin (produced form pyruvate oxidoreductase) to synergistically produce H_2 from a bifurcating hydrogenase. The

detection of both NAD(P)H and ferredoxin hydrogenases in cultures of *A. hydrogeniformans* correlates to the *T. maritime* system, but there was no detection of glyceraldehyde-3-phosphate dehydrogenase. Thus a bifurcating system in *A. hydrogeniformans* is unlikely.

Conclusions

In collating the enzyme data, glucose labeled studies and metabolite profile of *Anaerobaculum hydrogeniformans* it can be concluded that it uses the modified Embden Meyerhof (EM) pathway of glycolysis (FIGURE 28). The use of this modified EM pathway has only been detected in archael species. The lack of enzyme activity for the traditional eubacterial glyceraldehyde-3-phosphate dehydrogenase further supports the presence of the modified EM pathway. To the author's knowledge this is the first report of the use of a GAPOR enzyme in a eubacterial species.

This conclusion is supported by the results observed in the metabolite profile for glucose grown cells of *A. hydrogeniformans*. 1,3-bisphosphoglycerate was not detected during analysis, which a key intermediate of the traditional EM pathway. This would only be possible if this intermediate is skipped during the oxidation of glyceraldehyde-3-phospate to 3-phosphoglycerate, which is similar to that observed in *P. furiosus*. None of the intermediates for the Entner-Doudoroff (ED) pathway were detected during metabolite analysis and only a fraction of the enzymes found in the Pentose Phosphate pathway (PPP) were detected. This data supports the conclusion that *A. hydrogeniformans* is using the PPP to maintain certain metabolic precursors needed for other metabolic pathways such as pyrimidine synthesis.

The EM pathway was the only one that had detectable enzyme activities of all the steps in the pathway. The lack of enzymes needed for the oxidative branch of the PPP rules out its use in central carbon metabolism. Also the lack of activity for the KDPG aldolase negates the use of the ED pathway.

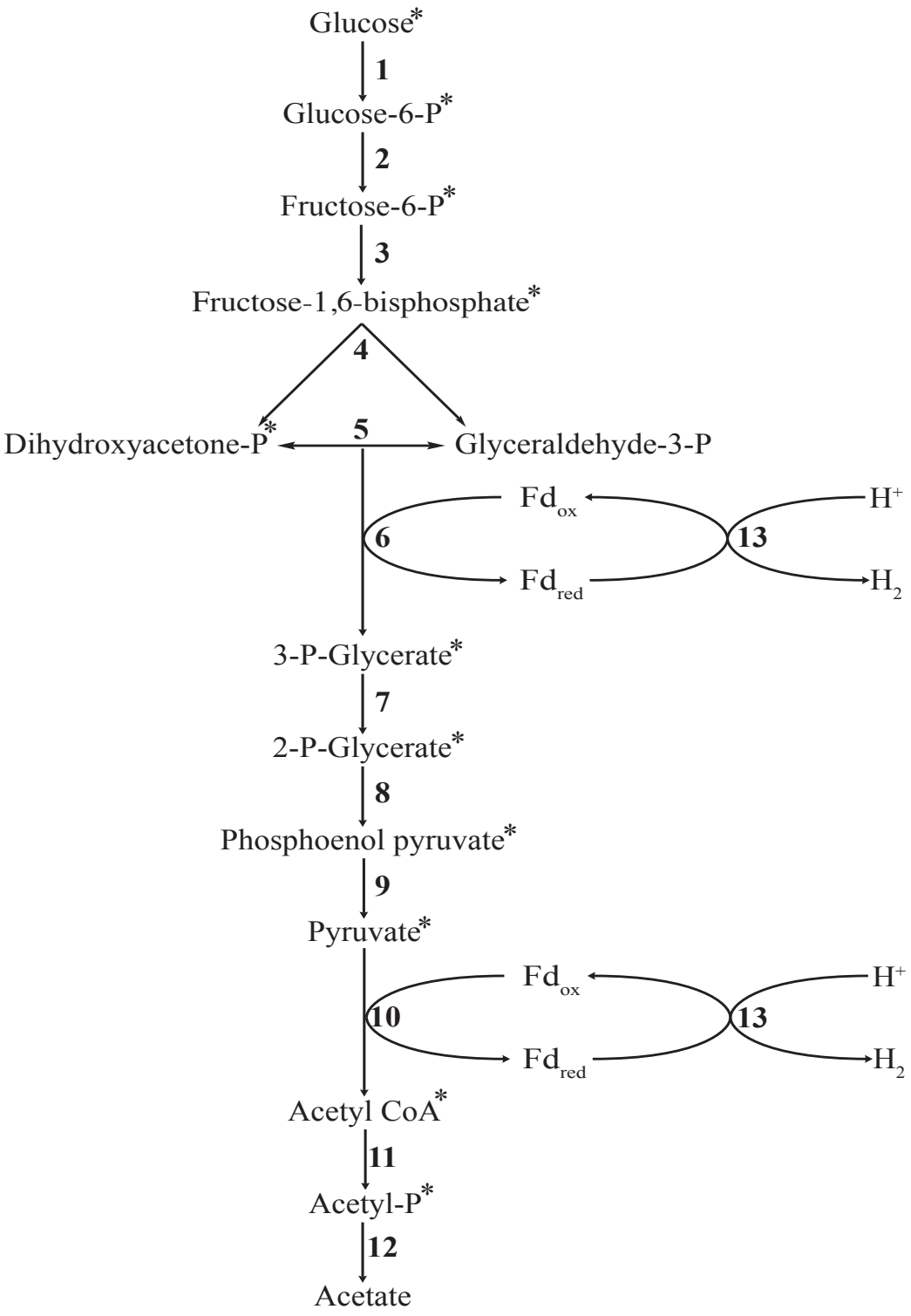
The use of the EM pathway is supported further by the labeled glucose studies. The detection of ^{13}C-CO_2 from the 3 carbon of glucose is only indicative of the EM pathway. The NMR results did show acetate labeled in the methyl group from the C-1 of glucose (FIGURE 25).. This finding needs to be duplicated and the identity of the compound will need to be elucidated in future studies.

Pyruvate decarboxylation is conducted by a 3 enzyme process, in which pyruvate:ferredoxin oxidoreductase (POR) is the initial step. The use of POR is well documented in eubacterial species that are substantially divergent taxonomically. Aside from *P. furiosus*, this is the first report of a glycolytic pathway totally dependent upon a ferredoxin system and that does not rely on NAD^+ or $NADP^+$. The use of this system explains how *A. hydrogeniformans* can produce H_2 at such high concentrations at 55 °C when compared to *P. furiosus* grown at 90 °C. Thermodynamically this gives *A. hydrogeniformans* an advantage when it comes to the production of H_2 because the reaction does not become limited by the lower redox potential of NADH or NADPH versus that of ferredoxin.

A. hydrogeniformans has at least two active hydrogenase systems in the cytosol. One that functions by means of NADH or NADPH and one that is ferredoxin dependent. Membrane analysis provides evidence for the use of a membrane bound ferredoxin dependent hydrogenase system. It has yet to be tested if *A. hydrogeniformans* can generate ATP by producing H_2, as has been seen in *P. furiosus* or if *A. hydrogeniformans* possesses a bifurcating hydrogenase system like that of *T. maritma*, but this will be tested in future research.

FIGURE 28. The proposed pathway for glucose oxidation and H_2 production by *Anaerobaculum hydrogeniformans* including pyruvate decarboxylation. Enzymes: 1, phosphotransferase system; 2, phosphoglucoisomerase; 3, phosphofructokinase; 4, fructose-1,6-bisphosphate aldolase; 5, triosephosphate isomerase; 6. glyceraldehyde-3-phosphate oxidoreductase; 7, phosphoglycerate mutase; 8, 2-phosphoglycerate enolase; 9, pyruvate kinase; 10, pyruvate oxidoreductase; 11, phosphotransacetylase; 12, acetate kinase; 13, ferredoxin oxidizing hydrogenase.

(* indicates intermediates that were detected during metabolite screening)


Glucose*
1
Glucose-6-P*
2
Fructose-6-P*
3
Fructose-1,6-bisphosphate*
4
Dihydroxyacetone-P*
5
Glyceraldehyde-3-P
Fd$_{ox}$
H$^+$
6
13
Fd$_{red}$
H$_2$
3-P-Glycerate*
7
2-P-Glycerate*
8
Phosphoenol pyruvate*
9
Pyruvate*
Fd$_{ox}$
H$^+$
10
13
Fd$_{red}$
H$_2$
Acetyl CoA*
11
Acetyl-P*
12
Acetate

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