Available at www.sciencedirect.com journal homepage: www.elsevier.com/locate/he

Dark fermentation of xylose and glucose mix using isolated *Thermoanaerobacterium thermosaccharolyticum* W16

Nanqi Ren^{a,*}, Guangli Cao^a, Aijie Wang^a, Duu-Jong Lee^{a,b},
Wanqian Guo^a, Yuhong Zhu^a

^aState Key Laboratory of Urban Water Resource and Environment, Harbin Institute of Technology, Harbin 150090, China

^bDepartment of Chemical Engineering, National Taiwan University, Taipei, Taiwan

ARTICLE INFO

Article history:

Received 11 June 2008

Received in revised form

18 July 2008

Accepted 19 July 2008

Available online 18 September 2008

Keywords:

Thermoanaerobacterium

thermosaccharolyticum

Hydrogen production

Xylose

Fermentation

ABSTRACT

Hydrolyzed sugars from lignocellulosic biomass are primarily glucose and xylose. Efficient dark fermentation of isolated strains of both glucose and xylose to generate hydrogen is of considerable practical and academic importance. This study utilized a newly isolated, moderately thermophilic bacterium, W16, to produce hydrogen from xylose, glucose, and mixed xylose and glucose. The strain W16 was identified and designated as *Thermoanaerobacterium thermosaccharolyticum* W16. Physiology of strain W16 strain with numerous carbon sources and at pH of 4–7.5 and temperature of 30–70 °C was demonstrated. The maximum cumulative H₂ yield and H₂ production rate obtained using the W16 strain in pure glucose and pure xylose tests were 2.42 mol H₂ mol^{−1} glucose and 12.9 mmol H₂ L^{−1} h^{−1}, and 2.19 mol H₂ mol^{−1} xylose and 10.7 mmol H₂ L^{−1} h^{−1}, respectively. The strain W16 also uptakes mixed xylose and glucose at comparable rates of individual pure sugar tests to produce biohydrogen via an acetate–butyrate-type fermentation. The isolated strain W16 was noted to effectively degrade reducing sugars in hydrolysate of corn stover collected in field.

© 2008 International Association for Hydrogen Energy. Published by Elsevier Ltd. All rights reserved.

1. Introduction

Hydrogen is a promising alternative to fossil fuels as it is CO₂-neutral, renewable and has a high energy yield. Lignocellulosic biomass, which primarily contains agricultural by-products and wood chips, is an ideal inexpensive, renewable and abundant resource for hydrogen [1]. Direct fermentation of raw lignocellulosic feedstock is typically inefficient. Hence, pre-treatments are commonly needed to saccharify lignocellulosic material into monomeric sugars, primarily glucose and xylose, prior to biological conversion. Glucose is easily biodegraded during hydrogen production by

fermentation [2]. Conversely, fermentative hydrogen production from xylose is generally inefficient [3–8].

Hydrogen fermentation at high temperatures using thermophilic bacteria has garnered considerable attention due to the high hydrogen production rate, suppression of H₂-consuming bacteria, and ability to utilize numerous substrates [9,10]. Particularly, hyper-thermophiles have higher hydrogen production potentials and yield fewer by-products in dark fermentation compared with mesophiles [7,11,12].

Glucose and xylose are the dominant reducing sugars when converting lignocellulosic biomass to hydrogen. Strains

* Corresponding author. Harbin Institute of Technology, School of Municipal and Environmental Engineering, No. 202 Haihe Road, 2nd Campus of HIT box 2614, Harbin, Hei Longjiang 150090, China. Tel./fax: +86 451 86282008.

E-mail address: rnq@hit.edu.cn (N. Ren).

Table 1 – H₂ yields and production rates reported in xylose fermentation studies

Microorganism	Substrate	Culture mode	Temperature (°C)	pH	HPR (mmol L ⁻¹ h ⁻¹)	H ₂ yield (mol H ₂ mol ⁻¹ substrate)	Reference
Sewage sludge	Xylose	Batch	35	6–7	5.94–8.93	1.92–2.25	[13]
	Xylose	Continuous	35	7.1	4.15	0.7	
Sewage sludge	Xylose	Continuous	40	6.5	25.4	0.8	[8]
<i>Clostridium</i> sp. No. 2	Xylose	Continuous	25	6.0	21.0	2.36	[14]
<i>Clostridium tyrobutyricum</i> ATCC 25755	Xylose	Batch	37	6.3	8.35	0.77	[4]
<i>Clostridium butyricum</i> CGS5	Xylose	Batch	37	7.5	9.64	0.73	[6]
<i>Enterobacter cloacae</i> IIT-BT 08	Xylose	Batch	36	6.0	15.5	0.95	[15]
Microflora	Xylose	Batch	75	7.3	–	0.54	[5]
Hydrogen-producing sludge	Xylose	Continuous	50	7.0	5.76	1.4	[7]
Compost	Xylose	Fed-batch	55	5.0	0.27	1.7	[16]
<i>C. saccharolyticus</i>	Xylose	Batch	70	7.2	11.3	2.24	[3]
	Mix	Batch	70	7.2	9.2	2.32	
	Glucose	Batch	70	7.2	10.7	2.5	
<i>T. thermosaccharolyticum</i> W16	Xylose	Batch	60	6.5	10.7	2.19	This study
	Mix	Batch	60	6.5	11.2–12.7	2.23–2.37	
	Glucose	Batch	60	6.5	12.9	2.42	

Marked are thermophilic tests.

that can effectively degrade glucose and xylose and a glucose–xylose mix are of considerable academic and practical interest. However, according to summary in Table 1, a limited number of hyper-thermophiles that convert hydrogen from xylose and glucose have been identified and tested. This work isolates a hydrogen-producing strain that effectively converts xylose, glucose and a xylose–glucose mix into hydrogen via thermophilic fermentation. A particular strain, which was acquired from a hot spring, may generate high hydrogen yields from xylose, glucose or a xylose–glucose mix. Optimal solution temperature, pH and substrate concentrations to maximize H₂ production from these sugars were identified. Moreover, the isolated strain was tested using a hydrolyzed solution obtained from corn stover feedstock collected in the field.

2. Experiment

2.1. Strain isolation and identification

Sludge sample was collected from a hot spring in Anshan, Liaoning Province, China. The temperature and pH of the hot spring were at 67 °C and 7.0, respectively. In total, 1 g diluted (1:10) sludge sample was incubated at 60 °C for 2 h in sterilized, modified MB medium [17], consisting of (per liter): 1.0 g NH₄Cl, 0.3 g K₂HPO₄, 0.3 g KH₂PO₄, 0.5 g MgCl₂·6H₂O, 2.0 g NaCl, 0.2 g KCl, 0.05 g CaCl₂, 0.5 g cysteine–HCl, 2.0 g yeast extract, 2.0 g tryptone, 10 g xylose, 1 mL trace element solution, 1 mL vitamin solution [18], and 1 ml 0.1%(w/v) resazurin. The pH of the oxygen-free medium was increased to 7.0 with 2 M KOH, poured into the Hungate tubes and then autoclaved. Strain enrichment was achieved anaerobically in resting 100-mL glass bottles containing 50 mL modified MB medium and 5 mL incubated sludge samples at 60 °C for 5 d. Suspensions acquired were plated on MB agar plates. Strain purification

using the Hungate roll-tube procedure was repeated a few times until pure cultures were obtained at 60 °C in modified MB agar medium (2%, w/v, agar) [19]. Capability to utilize 0.5% v/v carbon substrates by isolates was observed in batch tests. Isolates with high hydrogen production potential from xylose (described later) were identified and tested.

Gram staining of isolate cells was performed following the Hucker method [20]. The morphology and motility of cells in their exponential-growth phase in MB medium were examined by light microscopy and electron microscopy.

The genomic DNA of selected isolates was extracted and purified using the bacterial DNA mini kit (Watson, Shanghai, China) according to manufacturer instructions. The 16S rRNA was amplified by PCR using a pair of universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGATCCAGCC GCA-3'), which correspond to positions 8–27 and 1541–1525 [21]. The PCR products were purified using a UNIQ-10 PCR product purification kit (Sangon, Shanghai, China) and cloned into vector pMD19-T using the pMD19-T vector system I kit according to manufacturer instructions (Takara, Dalian, China). The 16S rRNA sequence of the isolate was aligned

Table 2 – Hydrogen yields and metabolites from xylose with different strains

Strain	Y _{H₂} (mL L ⁻¹ culture)	H ₂ content (%)	Metabolites (mg L ⁻¹)			
			Ethanol	Butanol	Acetate	Butyrate
W16	2020	64	487	80.5	2050	1880
W4	1890	60	590	ND	1840	1980
W17	1870	59	636	97.4	1940	1790
D26	1540	53	1020	126	1680	1548
F3	1580	55	888	102	1510	1950

ND: not detectable.

Fig. 1 – Phylogenetic tree showing the relationships between strain W16 and related species based on 16S rRNA gene sequences. The sequence of *T. elfii* served as an outgroup sequence. Bar represents 2% sequence divergence.

manually using the BLAST algorithm with all nucleotide sequences deposited in the GenBank database. Multiple alignments and calculation of degrees of sequence similarity were performed via Clustal X [22]. The phylogenetic

relationship among isolates was constructed by the neighbor-joining method [23] using MEGA 3.1 from the evolutionary distance data corrected by two-parameter model developed by Kumar et al. [24], and evaluated by bootstrap resampling [25] with 1000 replicates. The accession numbers of reference strains used in sequence comparisons were as follows: *Thermoanaerobacterium thermosaccharolyticum* (M59119), *Thermoanaerobacterium aciditolerans* (AY350594), *Thermoanaerobacterium islandicum* (EF088330), *Thermoanaerobacterium thermosulfurigenes* (L09171), *Thermoanaerobacterium saccharolyticum* (L09169), *Thermoanaerobacterium xylanolyticum* (L09172), *Thermoanaerobacterium polysaccharolyticum* (U40229), *Thermoanaerobacterium zeae* (U75993), *Thermoanaerobacter sulfurigenens* (AF234164), *Thermoanaerobacter ethanolicus* (AF542519), *Thermoanaerobacter Brockii* (L09165), *Thermoanaerobacter pseudethanolicus* (L09164), *Thermoanaerobacter uzonensis* (EF530067) and *Thermotoga elfii* (X80790).

Table 3 – Growth of *T. thermosaccharolyticum* strains reported in literature and in this work on carbon source utilization

Carbon source	DSM571 [33]	FH1 [34]	PSU-2 [35]	W16 (This work)
Sucrose	+	+	+	+
Arabinose	+	+	+	+
D-xylose	+	+	+	+
Lactose	+	+	+	+
Glucose	+	+	+	+
Fructose	+	+	+	+
Rhamnose	–	ND	+	+
Mannose	+	ND	+	+
Galactose	+	N	+	+
Xylan	+	+	+	+
Cellulose	ND	ND	+	+
Cellubiose	+	+	+	+
Dextrin	–	+	+	+
Yeast extract	ND	+	+	+
Peptone	ND	–	+	+
Salicin	ND	ND	ND	+
Maltose	+	+	+	+
Mannose	+	+	+	+
Hepatin	ND	+	ND	+
Melizitose	ND	ND	ND	+
Trehalose	+	ND	+	+
Raffinose	+, –	+	+	+
Starch	+	+	+	+
Inulin	–	ND	ND	+
Pectin	–	–	–	–
Glycerol	–	–	–	–

ND: not determined.

2.2. Batch test of isolated strain with synthetic medium

Individual isolates were cultivated anaerobically in a medium consisting of (per liter) 1.0 g NH_4Cl , 1.5 g K_2HPO_4 , 0.75 g KH_2PO_4 , 0.5 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 2.0 g NaCl , 0.2 g KCl , 0.5 g cysteine-HCl, 2.0 g yeast extract, 2.0 g tryptone, and 10 g xylose. The inoculum acquired during the exponential-growth phase was added at 2% v/v with 50 mL medium in 100 mL glass serum bottles with pH adjusted to 7.0. In the subsequent tests, the effects of concentrations of different carbon substrates (10 g L^{-1} for sucrose, arabinose, lactose, fructose, rhamnose, mannose, galactose, xylan, cellulose, cellubiose, dextrin, salicin, maltose, mannose, hepatin, melizitose, trehalose, raffinose, starch, inulin, pectin, or glycerol tests, and $0\text{--}25 \text{ g L}^{-1}$ for xylose and glucose tests), yeast extract and tryptone ($0\text{--}5 \text{ g L}^{-1}$), solution pH (4–8) and temperature ($30\text{--}70^\circ\text{C}$) on hydrogen production potential were determined. In the test of the xylose–glucose

Fig. 2 – H₂ production of *T. thermosaccharolyticum* W16 with different carbon sources at 10 g L⁻¹ concentration, 60 °C, initial pH 6.5. (A) Hydrogen production and cell density; (B) metabolites concentrations.

mix, total sugar concentration was maintained at 10 g L⁻¹; the following mixture fractions were used: (xylose/glucose) = 100/0, 80/20, 50/50, 20/80, and 0/100. In batch tests, cell density, pH, residual carbon substrate concentration, quantity and compositions of produced biogas, and metabolic products in liquid phase were determined.

2.3. Batch test of isolated strain with hydrolysate of corn stover feedstock

The corn stover feedstock containing 37.6% w/w cellulose, 21.5% w/w hemicellulose, 19.1% w/w lignin on the dry basis was collected at Harbin city, Heilongjiang Province, China. The hydrolysate of corn stover was prepared based on previously described procedures [26,27]. The corn stover chips (2–10 mm) were hydrolyzed at 121 °C with 2.0% (w:v) sulfuric acid for 3 h,

with an initial liquid to solid ratio of 10:1. The resulting slurry (5%, w/v) was enzymatically hydrolyzed (20 U g⁻¹ cellulose using cellulase from Sigma, >0.3 units mg⁻¹ solid, derived from *Aspergillus niger*.) at pH 5.0, 50 °C, 200 rpm for 72 h. The pH of the hydrolysate, containing 17.3 g L⁻¹ of glucose, 12.6 g L⁻¹ of xylose and 1.9 g L⁻¹ arabinose, was adjusted to neutral with NaOH. The neutralized hydrolysate was centrifuged and then diluted to yield a supernatant of reducing sugar concentrations of 5.8 g L⁻¹ glucose, 4.2 g L⁻¹ xylose and 0.6 g L⁻¹ arabinose.

The inoculum acquired during the exponential-growth phase was also added at 2% v/v with 50 mL neutralized corn stover hydrolysate in 100 mL glass serum bottles with pH adjusted to 7.0. The tested hydrolysate was supplemented with the following nutrients (per liter) NH₄Cl 1.0 g, K₂HPO₄ 1.5 g, KH₂PO₄ 0.75 g, MgCl₂·6H₂O 0.5 g, NaCl 2.0 g, KCl 0.2 g,

Table 4 – Effect of yeast extract and/or tryptone on the growth and hydrogen production of *T. thermosaccharolyticum* W16

Medium components (g L ⁻¹)			Consumed (m mol L ⁻¹)		Produced (m mol L ⁻¹ medium)		
Xylose	Yeast extract	Tryptone	OD _{600nm}	Xylose	Hydrogen	Acetate	Butyrate
0	5	2	0.15	–	3.2	3.4	0.5
0	5	5	0.15	–	3.2	3.3	0.6
0	2	0	0.06	–	2.0	1.7	0.4
0	2	2	0.10	–	2.4	2.2	0.5
5	5	2	1.85	33.3	64.3	16.1	8.5
5	5	5	2.05	33.3	57.9	13.8	10.0
5	2	0	1.33	30.5	45.8	11.5	5.2
5	2	2	1.71	31.7	47.4	12.8	7.3
5	0	0	0.73	29.5	40.2	11.3	4.2

Fig. 3 – Effect of process parameters on hydrogen production using strain W16. 25-h batch test. (A) Effects of temperature, initial pH 6.5, xylose concentration 10 g L⁻¹. (B) Effects of initial pH, 60 °C, xylose concentration 10 g L⁻¹. (C) Effects of xylose concentration, 60 °C, initial pH 6.5.

cysteine–HCl 0.5 g, yeast extract 2.0 g, and tryptone 2.0 g. In a parallel test synthetic medium with the same levels of principal reducing sugars (5.8 g L⁻¹ glucose, 4.2 g L⁻¹ xylose and 0.6 g L⁻¹ arabinose) and of other micronutrients was tested with the isolated strain.

2.4. Analytical methods

Cell density in the liquid medium was monitored by measuring turbidity at 600 nm. The xylose, arabinose and glucose concentrations in filtered samples were measured using high-

performance liquid chromatography (HPLC) (Thermo Electron Corporation, Boston, MA, USA). Volatile fatty acids and alcohols were detected by gas chromatography (GC-SC2) (Shanghai Analytical Apparatus, Shanghai, China) equipped with a hydrogen flame-ionization detector and a 2.0-m stainless-steel column packed with GDX103 (60/80 mesh). Gases were measured by GC-SC2 equipped with a thermal conductivity detector and a 2.0-m stainless-steel column packed with TDS-01 (60/80 mesh) and N₂ at a flow rate of 70 mL min⁻¹ as the carrier gas. Hydrogen yield (Y_{H_2}) was calculated as $Y_{H_2} = (\text{accumulated biogas production quantity}) \times (\text{hydrogen content in gas}) / (\text{consumed sugar under quantity})$. In the glucose–xylose mix culture, hydrogen yield was calculated as $Y_{H_2} = (\text{accumulated biogas production quantity}) \times (\text{hydrogen content in gas}) / (\text{consumed glucose quantity} + \text{consumed xylose quantity})$.

3. Experimental results

3.1. Strain screening

Isolated strains were tested for hydrogen production potential using the medium described in Section 2.2 at 60 °C. Five strains, W16, W4, W17, D26 and F3, had high hydrogen yields from xylose with acetate, butyrate and ethanol as the principal metabolites (Table 2). Strain W16 had higher hydrogen yield (2020 mL L⁻¹ culture) than the other four strains (1540–1890 mL L⁻¹ culture). Hence, strain W16 was examined further.

3.2. Identification and characterization of strain W16

Morphological and physiological tests demonstrate that strain W16 was rod-shaped, Gram-positive, strictly anaerobic, and spore-forming (data not shown for brevity). Cells were motile, as observed under light microscopy, and flagella were observed.

The complete 16S rRNA gene sequence (1509 bp) of the W16 strain was deposited at GenBank and EMBL (accession number, EU563362). Based on the similarity analysis of the 16S rRNA gene, a phylogenetic tree for the W16 strain rooted with *T. elfii* was constructed (Fig. 1); the closest relationship of the gene was with *T. thermosaccharolyticum* DSM571 (formerly *Clostridium thermosaccharolyticum*) at a similarity of 98.4%. The phenotypical characteristics of the W16 isolate were also consistent with *T. thermosaccharolyticum*; thus, the isolated strain was characterized as *T. thermosaccharolyticum* and designated as *T. thermosaccharolyticum* W16.

The strain *T. thermosaccharolyticum* is a thermophilic saccharolytic microorganism that effectively produces hydrogen from acetate/butyrate fermentation of carbohydrates (but not xylose) [10,28,29]. This strain was examined in a thermophilic, anaerobic sequencing batch reactor [30], trickling biofilter reactors [31] and continually stirred tank reactors [29,32]. The W16 strain and other *T. thermosaccharolyticum* strains reported in literature can grow on a wide variety of complex and simple carbohydrates (Table 3). However, no study has demonstrated that *T. thermosaccharolyticum* can utilize xylose to generate hydrogen.

Fig. 4 – Growth of strain W16 on glucose, xylose and their mix. Initial total sugar concentration = 10 g L^{-1} . 2% v/v inoculum.

3.3. Effects of process parameters on hydrogen production via strain W16

3.3.1. Effects of carbon sources

At 60°C , strain W16 effectively utilizes starch (soluble starch and potato starch), disaccharides (cellobiose, lactose and sucrose) and monosaccharides (glucose, fructose and xylose) to generate hydrogen (Fig. 2). A strong correlation existed between hydrogen production quantity and cell density ($\text{OD}_{600\text{nm}}$). The predominant metabolites are acetate, butyrate, and ethanol. Degradation of cellulose and xylan was poor using strain W16.

Table 4 lists hydrogen yield, xylose consumption rate, and metabolite concentrations for W16 at different xylose, yeast extract and tryptone loadings. With no added xylose, production of hydrogen and other liquid metabolites by W16 was limited ($2.0\text{--}3.2 \text{ mmol H}_2 \text{ L}^{-1}$). Corresponding cell growth was restricted ($\text{OD}_{600\text{nm}} = 0.06\text{--}0.15$). Hence, with yeast extract concentration up to 5 g L^{-1} , hydrogen production by W16 was not affected significantly. Conversely, the W16 strain degraded effectively xylose without yeast extract or tryptone ($40.2 \text{ mmol H}_2 \text{ L}^{-1}$). However, with 5 g L^{-1} yeast extract and 2 g L^{-1} tryptone, the W16 strain increased its hydrogen production ($64.3 \text{ mmol H}_2 \text{ L}^{-1}$); additionally, corresponding cell growth also increased ($\text{OD}_{600\text{nm}} = 1.85$).

3.3.2. Effect of solution temperature, pH, and initial xylose concentration

Hydrogen yield, hydrogen production rate, and cell mass were functions of solution temperature, pH, and xylose concentration following 25 h batch fermentation (Fig. 3). Fermentation tests were performed using the same medium as described in Section 2.2. Concentrations of all components except for xylose were fixed in all experiments.

Generally, hydrogen yield, hydrogen production rate, and cell mass increased first, then decreased after exceeding a maximum when increasing solution temperature, pH or initial xylose concentration. Particularly, using the test conditions in Fig. 3 as an example, maximum hydrogen yield ($2.0\text{--}2.2 \text{ mol H}_2 \text{ mol}^{-1}$ xylose), hydrogen production rate ($5.0\text{--}5.5 \text{ mmol H}_2 \text{ L}^{-1} \text{ h}^{-1}$), and cell mass ($1.1\text{--}1.3 \text{ g L}^{-1}$) occurred at 60°C , pH of 6.5 and $10\text{--}12.5 \text{ g L}^{-1}$ xylose. Additionally, the W16 strain degraded xylose with a satisfactory efficiency at 55--

60°C , pH of 5.5–7.0, and xylose concentration $<15 \text{ g L}^{-1}$. This observation demonstrates the importance of properly controlling reaction temperature and solution pH to achieve high activity of the W16 strain. Moreover, reduction of W16 activity at high xylose concentration may be yielded by pH change or product inhibition. Continuous flow reactor can easily remove this restriction in practice.

3.4. Production of hydrogen from xylose/glucose mix

The W16 strain can grow on glucose, xylose, and a glucose–xylose mix (Fig. 4); the strain prefers glucose over xylose. With a fixed total sugar quantity (10 g L^{-1}), the log phase growth of strain W16 was 4–13 h for the pure glucose test and 6–16 h for the pure xylose test.

Table 5 lists the experimental results for batch fermentation of the xylose/glucose mix using strain W16. The presence of 80% xylose reduced the utilization rate of glucose slightly from around $0.36 \text{ g L}^{-1} \text{ h}^{-1}$ to $0.25 \text{ g L}^{-1} \text{ h}^{-1}$. Conversely, the presence of 50% glucose markedly reduced the utilization rate of xylose from around $0.29 \text{ g L}^{-1} \text{ h}^{-1}$ to $0.09 \text{ g L}^{-1} \text{ h}^{-1}$. Hence, the content of glucose in the mixture had great effect on the consumption of xylose, however, the glucose consumption rate remained essentially constant independent of the xylose content. The cumulative hydrogen yield per mole substrate of glucose and xylose was 2.42 mol H_2 and 2.19 mol H_2 corresponding to $13.57 \text{ mmol H}_2 \text{ g}^{-1}$ glucose and $14.40 \text{ mmol H}_2 \text{ g}^{-1}$ xylose, respectively. Apparently, strain W16 exhibited efficient degradation of xylose for hydrogen production as glucose. The final yield of hydrogen in the mixture was not distinctively different from the pure sugar medium. In other words, strain W16 could utilize the xylose–glucose mix for hydrogen production from hydrolyzed lignocellulose.

3.5. Production of hydrogen from corn stover hydrolysate

The strain *T. thermosaccharolyticum* W16 degraded completely the arabinose and glucose and $>55\%$ xylose in the corn stover hydrolysate to hydrogen, acetate, butyrate, and ethanol (Fig. 5). The tests with synthetic medium of the same reducing sugar levels also revealed a similar result. Specifically, the

Table 5 – Fermentation parameters on glucose/xylose mixtures using *T. thermosaccharolyticum* W16

Feed	Maximum H ₂ production rate (m mol H ₂ L ⁻¹ h ⁻¹)	H ₂ yield (mol H ₂ mol ⁻¹ substrate)	Cell yield (g L ⁻¹)	Glucose consumption rate (g L ⁻¹ h ⁻¹)	Xylose consumption rate (g L ⁻¹ h ⁻¹)	Concentration of end metabolites (m mol L ⁻¹)		
						Ethanol	Butanol	Acetate
100% Glucose	12.9 ± 0.10	2.42 ± 0.11	1.33 ± 0.05	0.38 ± 0.02	0	14.6 ± 0.20	0.70 ± 0.01	33.3 ± 0.07
80% glucose + 20% xylose	12.7 ± 0.08	2.37 ± 0.15	1.31 ± 0.05	0.36 ± 0.03	0.03 ± 0.01	11.3 ± 0.10	0.75 ± 0.02	32.1 ± 0.56
50% glucose + 50% xylose	12.5 ± 0.20	2.32 ± 0.20	1.30 ± 0.02	0.36 ± 0.02	0.10 ± 0.01	10.1 ± 0.06	0.83 ± 0.01	31.3 ± 0.17
20% glucose + 80% xylose	11.2 ± 0.20	2.23 ± 0.08	1.24 ± 0.03	0.25 ± 0.03	0.23 ± 0.02	9.8 ± 0.07	0.84 ± 0.01	31.6 ± 0.27
100% xylose	10.7 ± 0.10	2.19 ± 0.12	1.21 ± 0.02	0 ± 0.00	0.29 ± 0.01	9.1 ± 0.02	0.85 ± 0.02	32.3 ± 0.49

Values are means of three incubations ± standard deviations.

strain W16 produced biohydrogen from corn stover hydrolysate and synthetic medium at 2.3–2.4 mol H₂ mol⁻¹ substrate and at 12.0–12.7 mol H₂ L⁻¹ h⁻¹ production rates. Apparently, the components in hydrolysate of corn stover did not inhibit the growth of strain W16. Additionally, the strain W16 produced equally well the biohydrogen from corn stover hydrolysate than that from mix of pure glucose and xylose (2.2–2.4 mol H₂ mol⁻¹ xylose).

4. Discussion

Table 1 compares H₂ yields and production rates reported in xylose fermentation studies. Thermophiles typically generate higher hydrogen yield from xylose (1.7–2.5 mol H₂ mol⁻¹ xylose) than mesophiles (commonly <1 mol H₂ mol⁻¹ xylose). Conversely, with a novel reactor design and cell immobilization technology, the hydrogen production rate from xylose can achieve a high level [8]. Hence, optimizing reactor design should enhance the hydrogen production rate for thermophiles from xylose. Among the thermophiles tested in literature (Table 1), strain W16 and the strain *Caldicellulosiruptor saccharolyticus* [3] obtained peak hydrogen yields and hydrogen production rates from xylose and glucose which are the primary hydrolyzed product from lignocellulose. The hydrogen yield and hydrogen production rate of *T. thermosaccharolyticum* W16 were not distinct from synthetic medium, it showed that the strain could tolerance the inhibit component existed in the hydrolysate. However, the hydrogen production rate of the *C. saccharolyticus* reduced on the hydrolysate [3] indicated the strain was influenced by the components in the hydrolysate. From the point of industrial application, the stains possessed the tolerance of inhibitors is advantageous.

5. Conclusions

This study investigates the use of a newly isolated, moderately thermophilic bacterium, W16, to produce hydrogen from xylose, glucose, and mixed xylose and glucose. The bacterium was isolated at a hot spring in northern China, identified based on 16S rRNA gene sequences as *T. thermosaccharolyticum* and designated herein as *T. thermosaccharolyticum* W16. The W16 strain uses numerous carbon sources for H₂ production at a pH of 4–7.5 and temperature of 30–70 °C. The optimal pH and temperature for hydrogen production are 6.5 and 60 °C, respectively, with xylose and starch as the preferred substrates. The yeast extract and tryptone are not prerequisites for the growth of strain W16, but they aid the growth of W16. The maximum cumulative H₂ yield and H₂ production rate obtained using the W16 strain in pure glucose and pure xylose tests were 2.42 mol H₂ mol⁻¹ glucose and 12.9 mmol H₂ L⁻¹ h⁻¹, and 2.19 mol H₂ mol⁻¹ xylose and 10.7 mmol H₂ L⁻¹ h⁻¹, respectively. In the mixed sugar tests, W16 uptakes both xylose and glucose, but prefers glucose over xylose as the substrate. The hydrogen production rate and hydrogen yield for the mixed sugar tests decreased from 12.7 mmol H₂ L⁻¹ h⁻¹ and 2.37 mol H₂ mol⁻¹ substrate to 11.2 mmol H₂ L⁻¹ h⁻¹ and 2.23 mol H₂ mol⁻¹ substrate as the

Fig. 5 – Hydrogen production by *T. thermosaccharolyticum* W16 using (A) corn stover hydrolysate and (B) synthetic medium having the same sugar concentrations as in the corn stover hydrolysate with inoculum 2%(v/v), initial pH 6.5.

xylose fraction increased. The end products were predominantly acetate and butyrate, which generated an acetate-butyrate-type fermentation. The isolated strain degrades as efficient the reducing sugars in hydrolysate of corn stover feedstock as the xylose/glucose mix.

Acknowledgement

This research was supported by National Natural Science Foundation of China (50678049).

REFERENCES

- [1] Chandakant P, Bisaria VS. Simultaneous bioconversion of cellulose and hemicellulose to ethanol. *Crit Rev Biotechnol* 1998;18:295–331.
- [2] Fang HHP, Liu H. Effect of pH on hydrogen production from glucose by a mixed culture. *Bioresour Technol* 2002;82:87–93.
- [3] Kadar Z, Vrije TD, van Noorden GE, Budde MAW, Szengyel Z, Reczey K, et al. Yields from glucose, xylose, and paper sludge hydrolysate during hydrogen production by the extreme thermophile *Caldicellulosiruptor saccharolyticus*. *Appl Biochem Biotechnol* 2004;113–116:497–508.
- [4] Zhu Y, Yang ST. Effect of pH on metabolic pathway shift in fermentation of xylose by *Clostridium tyrobutyricum*. *J Biotechnol* 2004;110:143–57.
- [5] Yokoyama H, Moriya N, Ohmori H, Waki M, Ogino A, Tanaka Y. Community analysis of hydrogen-producing extreme thermophilic anaerobic microflora enriched from cow manure with five substrates. *Appl Microbiol Biotechnol* 2007;77(1):213–22.
- [6] Lo YC, Chen WM, Hung CH, Chen SD, Chang JS. Dark H₂ fermentation from sucrose and xylose using H₂-producing indigenous bacteria: feasibility and kinetic studies. *Water Res* 2008;42:827–42.
- [7] Lin CY, Wu CC, Hung CH. Temperature effects on fermentative hydrogen production from xylose using mixed anaerobic cultures. *Int J Hydrogen Energy* 2008;33:43–50.
- [8] Wu SY, Lin CY, Lee KS, Hung CH, Chang JS, Lin PJ, et al. Dark fermentative hydrogen production from xylose in different bioreactors using sewage sludge microflora. *Energy Fuels* 2008;22:113–9.
- [9] Talabardon M, Schwitzguebel JP, Peringer P. Anaerobic thermophilic fermentation for acetic acid production from milk permeate. *J Biotechnol* 2000;76:83–92.
- [10] Fang HHP, Zhang T, Liu H. Biohydrogen production from starch in wastewater under thermophilic condition. *J Environ Manage* 2003;69:149–56.
- [11] van Niel EWJ, Budde MAW, de Haas GG, van der Walb FJ, Claassenb PAM, Stamsa AJM. Distinctive properties of high hydrogen producing extreme thermophiles *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii*. *Int J Hydrogen Energy* 2002;27:1391–8.
- [12] van Niel EWJ, Claassenb PAM, Stamsa AJM. Substrate and product inhibition of hydrogen production by the extreme thermophile, *Caldicellulosiruptor saccharolyticus*. *Biotechnol Bioeng* 2003;81:255–62.
- [13] Lin CY, Cheng CH. Fermentative hydrogen production from xylose using anaerobic mixed microflora. *Int J Hydrogen Energy* 2006;31:832–40.
- [14] Taguchi F, Mizukami N, Saito-Taki T, Hasegawa K. Hydrogen production from continuous fermentation of xylose during growth of *Clostridium* sp. strain No. 2. *Can J Microbiol* 1995;41:536–40.
- [15] Kumar N, Das D. Enhancement of hydrogen production by *Enterobacter cloacae* IIT-BT 08. *Process Biochem* 2000;35:589–93.
- [16] Calli B, Schoenmaekers K, Vanbroekhoven K, Diels L. Dark fermentative H₂ production from xylose and lactose-effects of on-line pH control. *Int J Hydrogen Energy* 2008;33:522–30.
- [17] Fardeau ML, Ollivier B, Patel BKC, Magot M, Thomas P, Rimbault A, et al. *Thermotoga hypogaea* sp. nov., a xylanolytic, thermophilic bacterium from an oil-producing well. *Int J Syst Bacteriol* 1997;47:1013–9.
- [18] Wolin EA, Wolin MJ, Wolfe RS. Formation of methane by bacterial extracts. *J Biol Chem* 1963;238:2882–6.
- [19] Hungate RE. A roll tube method for cultivation of strict anaerobes. *Methods Microbiol* 1969;3B:117–32.
- [20] Doetsch RN. Determinative methods of light microscopy. In: Gerhardt P, editor. *Manual of methods for general bacteriology*. Washington, DC: American Society for Microbiology; 1981. p. 29–30.
- [21] Winker S, Woese CR. A definition of the domains Archaea, Bacteria and Eucarya in terms of small subunit ribosomal RNA characteristics. *Syst Appl Microbiol* 1991;14:305–10.
- [22] Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 1997;25:4876–82.
- [23] Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 1987;4:406–25.

- [24] Kumar S, Tamura K, Nei M. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* 2004;5:150–63.
- [25] Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 1985;39:783–791.
- [26] Esteghlalian A, Hashimoto AG, Fenske JJ, Penner MH. Modeling and optimization of the dilute-sulfuric-acid pretreatment of corn stover, poplar and switchgrass. *Bioresour Technol* 1997;59:129–136.
- [27] Lloyd TA, Wyman CE. Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids. *Bioresour Technol* 2005;96:1967–77.
- [28] Liu H, Zhang T, Fang HHP. Thermophilic hydrogen production from a cellulose containing wastewater. *Biotechnol Lett* 2003;25:365–9.
- [29] Shin HS, Youn JH, Kim SH. Hydrogen production from food waste in anaerobic mesophilic and thermophilic acidogenesis. *Int J Hydrogen Energy* 2004;29:1355–63.
- [30] O-Thong S, Prasertsan P, Intrasingkha N, Dhamwichukorn S, Birkeland NK. Improvement of biohydrogen production and treatment efficiency on palm oil mill effluent with nutrient supplementation at thermophilic condition using an anaerobic sequencing batch reactor. *Enzyme Microb Technol* 2007;41:583–90.
- [31] Ahn Y, Park EJ, Oh YK, Park S, Webster G, Weightman AJ. Biofilm microbial community of a thermophilic trickling biofilter used for continuous biohydrogen production. *FEMS Microbiol Lett* 2005;249:31–8.
- [32] Shin HS, Youn JH. Conversion of food waste into hydrogen by thermophilic acidogenesis. *Biodegradation* 2005;16:33–44.
- [33] Ganghofer D, Kellermann J, Staudenbauer WL, Bronnenmeier K. Purification and properties of an amylopullulanase, a glucoamylase, and an alpha-glucosidase in the amylolytic enzyme system of *Thermoanaerobacterium thermosaccharolyticum*. *Biosci Biotechnol Biochem* 1998;62:302–8.
- [34] Hoster F, Daniel R, Gottschalk G. Isolation of a new *Thermoanaerobacterium thermosaccharolyticum* strain (FH1) producing a thermostable dextranase. *J Gen Appl Microbiol* 2001;47:187–92.
- [35] O-Thong S, Prasertsan P, Karakashev D, Angelidaki I. Thermophilic fermentative hydrogen production by the newly isolated *Thermoanaerobacterium thermosaccharolyticum* PSU-2. *Int J Hydrogen Energy* 2008;33:1204–14.