Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Hydrogen Energy 31 (2006) 683–692
International Journal of HYDROGEN ENERGY
www.elsevier.com/locate/ijhydene

Acidophilic biohydrogen production from rice slurry

Herbert H.P. Fang*, Chenlin Li, Tong Zhang

Centre for Environmental Engineering Research, Department of Civil Engineering, The University of Hong Kong, Pokfulam Road, Hong Kong SAR, China

Available online 25 August 2005

Abstract

Batch experiment results showed that hydrogen production from rice slurry was found most effective at pH 4.5, 37 °C treating a slurry containing 5.5 g-carbohydrate/L. An anaerobic digester sludge was used as seed after a 100 °C heat treatment for 30 min. After a 36 h acclimation period, the sludge had a maximum specific hydrogen production rate of 2.1 L/(g-VSS d) and a hydrogen yield of 346 mL/g-carbohydrate, corresponding to 62.6% of stoichiometric yield. The effluent was composed mostly of acetate (28.3–43.0%) and butyrate (51.4–70.9%). Based on the 16S rDNA analysis, the 28 clones developed from this acidophilic hydrogen-producing sludge may be classified into nine OTUs, all of which are affiliated with the genus *Clostridium*. Phylogenetic analysis shows that eight OTUs (96.4% of population) form a distinct group with *Clostridium* sp. 44a-T5zd. Results indicate the acidophilic hydrogen-producing bacteria found in this study are unknown, and warrant further studies.

Keywords: Acidophilic; *Clostridium*; Fermentation; Food waste; Hydrogen; Phylogenetic analysis; Rice

1. Introduction

Hydrogen is a high-value industrial commodity with a wide range of applications. It is an ideal fuel, producing only water upon combustion. It may be converted into electricity via fuel cells or directly used in internal combustion engines. It can also be used for the syntheses of ammonia, alcohols and aldehydes, as well as for the hydrogenation of edible oil, petroleum, coal and shale oil [1]. Many believe that hydrogen will replace fossil fuels as the next generation of energy supply [2]. A hydrogen-based economy will impose no risk of global warming, and will significantly improve the urban air quality [3].

Hydrogen is traditionally generated by hydrocarbon reformation or electrolysis of water [1]. It is, however, technically feasible to harvest hydrogen from carbohydrates in waste and wastewater through biological process by fermentative microbes [4]. The hydrogen production characteristics of a number of pure cultures have been studied, including *Clostridia* [5–7] and *Enterobacteria* [8–10]. However, from an engineering point of view, producing hydrogen by mixed cultures is generally preferred because of lower cost, ease of control, and the possible use of organic wastes as substrate. Furthermore, production of hydrogen from organic wastes is a one-stone-two-birds paradigm; it not only cleans up the environment but also produces a clean and readily usable energy in a sustainable fashion [3]. Most studies of bio-hydrogen production so far, however, have been limited to using pure carbohydrates, such as glucose, sucrose and starch. Little information is available on the feasibility of using the carbohydrate-rich agricultural and food wastes.

Fermentative hydrogen production is affected by many parameters such as pH, temperature and feedstock concentration as well as the nature of the microbial community. pH is crucial due to its effects on hydrogenase activity [11], metabolism pathways [12], and microbial communities [13]. Hydrogen production is usually accompanied by the

* Corresponding author. Tel.: +852 2859 2660; fax: +852 2559 5337.
E-mail address: hrechef@hkucc.hku.hk (H.H.P. Fang).

0360-3199/$30.00
doi:10.1016/j.ijhydene.2005.07.005

production of volatile fatty acids (VFA) and alcohols. Some found that lowering the pH to 4.5 or below may shift the VFA-producing pathway to an alcohol-producing pathway [14,15]. Lay et al. [16] found that pH 5.6 was optimal for hydrogen production, and was also the threshold pH for the VFA- and alcohol-producing pathways. Fang and Liu [13] reported an optimal pH of 5.5, and their analysis based on polymerase chain reaction (PCR) and denatured gradient gel electrophoresis (DGGE) indicated that the diversity of microbial community increased with pH. Morimoto et al. [17] demonstrated that the production of hydrogen, which was started at neutral pH, was reduced with lowering pH and ceased at pH 4. Khanal et al. [15] also found the variations in acetate/butyrate ratio, which implied a metabolic alteration due to environmental changes, such as pH, etc. So far in the literature, pH of 5.5 is regarded as the optimal value and little information is known about the hydrogen production under a more acidophilic environment.

Fermentation reactions can be conducted at mesophilic (25–40 °C), thermophilic (40–65 °C), extreme thermophilic (65–80 °C), or hyperthermophilic (>80 °C) temperatures [18]. Most of the studies on hydrogen production by dark fermentative bacteria were conducted at 25–40 °C, with a few exceptions at 60 °C [19] and 55–70 °C [20].

This study was conducted initially to investigate the feasibility of producing hydrogen from rice slurry which is a common carbohydrate-rich food waste. It was soon found that, unlike reported in most studies, acidophilic condition was favored for rice slurry. The focus of this study was then shifted to investigate the performance, optimal operational conditions and microbial community of acidophilic hydrogen-producing sludge, using 16S rDNA-based molecular techniques, including PCR–DGGE, cloning, sequencing and phylogenetic analysis.

2. Materials and methods

2.1. Batch experiments of hydrogen production

Rice, the most common dietary food worldwide, was chosen as the model of carbohydrate-rich food waste after steaming at 100 °C for 30 min. The rice was composed of carbohydrate (78.3%), protein (6.6%), lipid (3.2%) and water (11.9%). An anaerobic digester sludge, sampled from a local municipal wastewater treatment plant, was used as seed. The sludge was pre-heated at 100 °C for 30 min to deactivate the hydrogenotrophic methanogens before it was used to seed the reactors. Three series of batch experiments were conducted in duplicate in 280 mL serum bottles. The wastewater was prepared using the following nutrients (all in mg/L): $NaHCO_3$ 1250; NH_4Cl 2500; KH_2PO_4 250; K_2HPO_4 250; $CaCl_2$ 500; $NiSO_4$ 32; $MgSO_4 \cdot 7H_2O$ 320; $FeCl_3$ 20; $Na_2BO_4 \cdot H_2O$ 7.2; $Na_2MoO_4 \cdot 2H_2O$ 14.4; $ZnCl_2$ 23; $CoCl_2 \cdot 6H_2O$ 21; $CuCl_2 \cdot H_2O$ 10; $MnCl_2 \cdot 4H_2O$ 30; yeast extract 50.

Series I was to investigate the effect of pH on hydrogen production. Experiments were conducted from pH 4.0 to 7.0 with 0.5 increments at 37 °C and 5.5 g-carbohydrate/L. Series II was to compare hydrogen production at 37 and 55 °C treating the rice slurry at pH 4.5 and 5.5 g-carbohydrate/L. After the optimal pH and temperature were identified as 4.5 and 37 °C, respectively, Series III was to further investigate the effect of feedstock concentration at 2.7, 5.5, 8.3, 11.0, 13.8 and 22.1 g-carbohydrate/L. In all batches, 150 mL rice slurry was treated with 85 mg of sludge as measured by volatile suspended solids (VSS). The mixed liquor was first purged with nitrogen for 20 min and capped tightly with butyl rubber to ensure anaerobic conditions. These reactors were then placed in a reciprocating shaker at 100 rpm at controlled temperatures. The mixed liquor pH was periodically adjusted using either 1 M HCl or 1 M NaOH to maintain the desired values.

2.2. Chemical analysis

The amount of biogas produced in each reactor was measured using a glass syringe. Biogas of 50 μL was sampled to analyze the contents of hydrogen, carbon dioxide and methane by a gas chromatograph (GC) (Hewlett–Packard 5890II, USA) equipped with a thermal conductivity detector and a 2 m × 2 mm (inside diameter) stainless steel column packed with Porapak N (80–100 mesh). Injector, detector and column temperatures were kept at 57, 180 and 50 °C, respectively. Argon was used as the carrier gas at a flow rate of 30 mL/min.

Concentrations of VFA and alcohols in the mixed liquor were analyzed by a second GC of the same model equipped with a flame ionization detector and a 10 m × 0.53 mm HP-FFAP fused-silica capillary column. The VFA analyzed included acetate, propionate, butyrate, i-butyrate, valerate, i-valerate and caproate, whereas alcohols included methanol, ethanol, propanol and butanol. Mixed liquor sample of 1 mL was first filtered through a 0.2 μm membrane, acidified by formic acid and measured for free acids. The initial temperature of the column was 70 °C for 3 min followed with a ramp of 10 °C/min and a final temperature of 180 °C for 4.5 min. The temperatures of the injector and detector were 200 and 250 °C, respectively. Helium was used as the carrier gas at a flow rate of 25 mL/min.

The biomass concentration was measured by VSS according to the Standard Methods [21].

2.3. Kinetic modeling

The cumulative hydrogen production in the batch experiments followed the modified Gompertz equation [22,23].

$$H = P \exp\left\{-\exp\left[\frac{R_m e}{P}(\lambda - t) + 1\right]\right\}, \quad (1)$$

where H is the cumulative hydrogen production (mL), λ the lag time (h), P the hydrogen production potential (mL),

R_m the maximum hydrogen production rate (mL/h), e the 2.718281828.

The values of P, R_m and λ for each batch were estimated using the solver function in Excel (version 5.0, Microsoft) with a Newtonian algorithm. The maximum specific hydrogen production rate (L/(g-VSS d)) was calculated by dividing R_m by the initial VSS in the reactor. The hydrogen yield (mL/g-carbohydrate) was calculated by dividing P by the carbohydrate content of feedstock.

2.4. DGGE, cloning, sequencing and phylogenetic analysis

The sludges at pH of 4.5–6.5 were sampled at the 130 h when hydrogen production was ceased for microbial analysis. In addition, the sludge at pH 4.5 was sampled from 0 to 130 h for analysis of microbial population shift. DNA was extracted from the sludge, followed by PCR amplification using the primer set of 341FGC (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGC ACGGGGGGCCTA CGGGAGGCAGCAG-3′) and 518R (5′-ATTACCGCGGCTGCTGG-3′) at the annealing temperature of 55 °C in an automated thermal cycler (GenAmp® PCR 9700, Perkin–Elmer, Foster City, CA) [24,25]. The PCR-amplified products were then screened using DGGE to investigate the microbial population variations under different pH and time intervals using 8% gel with 30–70% denaturant gradients [24,25]. Electrophoresis was conducted in a 1 × TAE buffer solution at 200 V and 60 °C for 4 h. The 16S rDNA bands on the gel were then stained with silver nitrate [26].

The DNA of sludge sample of pH 4.5 at 130 h was further analyzed. Firstly, the DNA was amplified using the primer set of EUB8F (5′-AGAGTTTGATCMTGGCTCAG-3′) and UNIV1392R (5′-ACGGGCGGTGTGTRC-3′) at the annealing temperature of 54 °C. The PCR-amplified product was then used to build a clone library with the TA Cloning Kit (Invitrogen, Carlsbad, CA) as described previously [25]. A total of 28 clones were selected to recover the DNA insert in the plasmids by the whole-cell PCR using the primer set of M13F (5′-CAGGAAACAGCTATGAC-3′) and M13R (5′-GTTTGATCCTGGCTCAG-3′) at the annealing temperature of 54 °C. The PCR product was used as the template for further PCR with the primer set of 341FGC/518R. The PCR products using the primer set of 341FGC/518R were used for DGGE screening to identify the unique sequence based on the band positions. Finally nine unique sequences, i.e. operational taxonomy units (OTUs), were sequenced using primers 1392R and EUB8F, an auto sequencer (ABI model 377A, Perkin–Elmer), and the dRhodamine Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin–Elmer).

The nearly full length sequences of each OTU were then manually edited with BioEdit [27], checked using the CHECK-CHIMERA program [28] to remove possible chimeric artifacts, if any, and compared with the reference microorganisms available in the GenBank by BLAST search [29]. The DNA sequences obtained and their closest 16S rDNA sequences of reference microorganisms retrieved from the GenBank were aligned and checked manually using the BioEdit [27]. Phylogenetic trees were then constructed using the neighbor-joining method [30] with MEGA 2.1 [31]. Bootstrap re-sampling analysis [32] for 500 replicates was performed to estimate the confidence of tree topologies.

2.5. Accession numbers

The nine nucleotide sequence data reported in this paper have been assigned the following accession numbers for the GenBank, EMBL and DDBJ databases: AY862509-17.

3. Results and discussion

In all experiments, the biogas produced contained hydrogen (45–56%) and carbon dioxide (44–55%), excluding the residual nitrogen from the initial purging. The biogas was free of methane due to lack of methanogenic activities in the sludges. Eq. (1) correlates hydrogen production data well with $R^2 > 0.98$ in all series.

3.1. Effect of pH on hydrogen production at 37 °C

Fig. 1 illustrates that plots based on Eq. (1) fit well the cumulative hydrogen production data for pH ranging from 4.0 to 7.0 treating a rice slurry containing 5.5 g-carbohydrate/L at 37 °C. Table 1 further summarizes the three kinetic parameters, plus hydrogen yield and maximum specific hydrogen production rate. Results show that the maximum hydrogen yield of 346 mL/g-carbohydrate occurred at pH 4.5. Under this pH, hydrogen evolved after a long lag phase (36 h), and cumulative yield increased with time before reaching

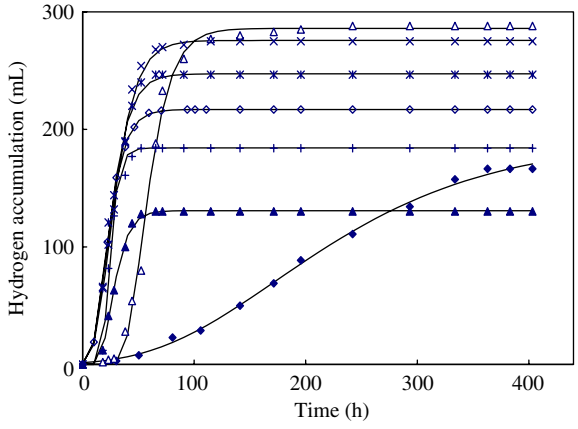

Fig. 1. Cumulative hydrogen production at pH 4.0–7.0: ♦ pH = 4.0; △ pH = 4.5; × pH = 5.0; ∗ pH = 5.5; ◇ pH = 6.0; + pH = 6.5; ▲ pH = 7.0.

Table 1
Kinetic parameters for hydrogen production at pH 4.0--7.0

pH	λ (h)	R_m (mL/h)	P (mL)	Maximum specific hydrogen production rate (L/(g-VSS d))	Hydrogen yield (mL/g-carbohydrate)
4.0	40	0.7	175	0.2	212
4.5	36	7.3	286	2.1	346
5.0	12	9.0	277	2.5	336
5.5	12	11.0	248	3.1	300
6.0	11	8.5	220	2.4	264
6.5	18	14.0	185	4.0	223
7.0	18	8.0	132	2.3	160

Table 2
Comparison of hydrogen yield

Feedstock	pH	Temperature (°C)	Hydrogen yield (mL/g-carbohydrate)	Yield[c] (%)	Reference
Rice	4.5	37	346	62.6	This study
Starch	6.0[a]	55	92	16.6	[33]
Cellulose	7.0[a]	37	72	13.0	[34]
Cellulose	7.0[a]	60	193	34.9	[35]
Sucrose[b]	5.5	36	280	53.4	[36]
Glucose[b]	5.5	37	261	52.2	[13]

[a]Initial pH.
[b]Continuous experiments.
[c]Assuming carbohydrate was totally converted into hydrogen and acetate.

Table 3
VFA and alcohol productions at pH 4.0--7.0

pH	VFA and alcohols (mg/L)	Constituents				Acetate/butyrate ratio	
		Acetate (%)	Butyrate (%)	Methanol (%)	Propionate (%)	This study	Ref. [13]
4.0	3134	30.4	69.6	0.0	0.0	0.44	0.37
4.5	3354	27.3	67.1	5.6	0.0	0.41	0.51
5.0	3748	35.4	57.4	3.6	3.6	0.62	0.61
5.5	3517	37.6	54.1	3.3	5.0	0.70	0.83
6.0	3558	43.0	51.4	1.6	4.0	0.84	0.91
6.5	3192	44.5	48.8	1.3	5.4	0.91	1.05
7.0	3678	48.4	42.3	1.5	7.8	1.15	1.09

the maximum. This finding was in close agreement with the results obtained by Khanal et al. [15] based on the studies carried out using both sucrose and starch.

Table 1 shows that the lag time was greatly affected by pH, varying from as low as 11 h at pH 6.0 to 40 h at pH 4.0. The effect of pH on the maximum specific hydrogen production rate was insignificant in the range of pH 4.5–7.0, the highest being 4.0 L/(g-VSS d) at pH 6.5. However, the maximum specific hydrogen production rate of 0.2 L/(g-VSS d) at pH 4.0 was much lower than the 2.1–4.0 L/(g-VSS d) found at high pH values. Table 1 also shows the hydrogen yield increased sharply from 212 mL/g-carbohydrate at pH 4.0 to 346 mL/g-carbohydrate at pH 4.5, and then decreased sharply with the further increase of pH to 160 mL/g-carbohydrate at pH 7.0.

Stoichiometrically, each gram of polysaccharides produces a maximum of 553 mL hydrogen assuming acetate being the sole by-product. The maximum hydrogen yield of 346 mL/g-carbohydrate represents 62.6% of the theoretical yield. Table 2 shows that, such a yield was much higher

Table 4
Kinetic parameters at pH 4.5 and various rice concentrations

Rice concentration (g-carbohydrate/L)	λ (h)	R_m (mL/h)	P (mL)	Maximum specific hydrogen production rate (L/(g-VSS d))	Hydrogen yield (mL/g-carbohydrate)
2.7	38	1.0	115	0.3	278
5.5	36	7.3	286	2.1	346
8.3	36	2.1	302	0.6	244
11.0	40	1.8	291	0.5	176
13.8	16	1.6	325	0.4	157
22.1	12	1.6	510	0.4	154

than the 280 mL/g-sucrose reported at 36 °C in a continuous stirred tank reactor [13] and the 193 mL/g-cellulose by the microflora from sludge compost at 60 °C in batch experiments [33]. Overall, pH 4.5 was identified in this series of experiments as the optimal pH for hydrogen production. This is more acidic than the reported optimal pH of 5.5 for hydrogen production from wastewaters containing sucrose and glucose [13,36]. The discrepancy is unclear, but could be attributed to the choice of seed sludge.

Table 3 summarizes the distribution of key VFA/alcohols produced in batches of various pH. It shows that acetate (27.3–48.4%) and butyrate (42.3–69.6%) were the two main by-products in all batches, followed by little amounts of methanol and propionate. In a hydrogen-producing process the pathway may shift from VFA-producing to alcohol-producing when the pH was lowered 4.5 or below [14,15]. However, in these batch experiments, only a small amount of methanol was detected. Results show that the increase of pH has no significant effect on the total VFA and alcohols concentrations. However, increase of pH resulted in the increase of acetate from 30.4% at pH 4.0 to 48.4% at pH 7.0 and the decrease of butyrate from 69.6% to 42.3%. Table 3 shows that the acetate/butyrate ratio increased from 0.41 at pH 4.5 to 1.15 at pH 7.0, suggesting a shift of metabolism pathway as pH changed. This result is consistent with a previous work

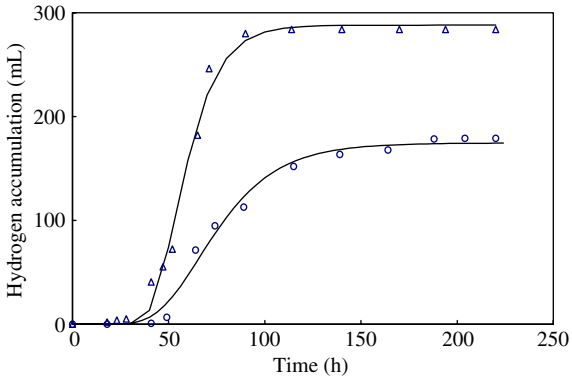

Fig. 2. Cumulative hydrogen production at two temperatures (△ 37 °C; ◯ 55 °C).

[13], in which the ratio of acetate/butyrate increased from 0.51 at pH 4.5 to 1.09 at pH 7.0 for hydrogen production from sucrose at 36 °C in continuous experiments. However, this result is different from another previous work [15], in which the acetate/butyrate ratio was 3–4 at pH 5.5–5.7. The byproducts, which were mostly composed of VFA, can be converted to methane or hydrogen by further fermentation.

Table 5
VFA and alcohol production at pH 4.5 and various rice concentrations

Rice concentration (g-carbohydrate/L)	VFA and alcohols (mg/L)	Constituents						
		Acetate (%)	Butyrate (%)	Methanol (%)	Propanol (%)	Propionate (%)	Valerate (%)	Caproate (%)
2.7	1772	31.2	55.8	0.0	5.1	0.0	0.6	7.2
5.5	3771	43.0	51.4	1.6	0.0	4.1	0.0	0.0
8.3	4734	29.2	62.8	0.0	2.0	0.0	0.3	5.8
11.0	6556	28.3	67.4	0.0	1.4	0.3	0.1	2.4
13.8	7394	34.2	64.8	0.0	0.9	0.1	0.0	0.0
22.1	10587	28.8	70.9	0.0	0.0	0.0	0.0	0.4

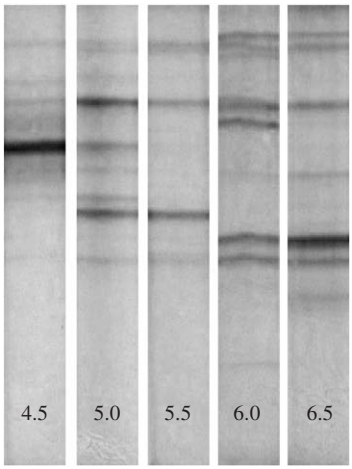

Fig. 3. DGGE profiles for hydrogen-producing communities at pH 4.5–6.5.

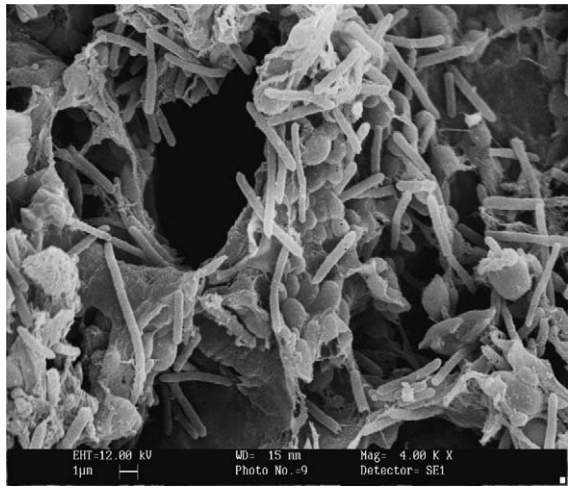

Fig. 5. SEM image of the hydrogen-producing bacteria at pH 4.5.

3.2. Effect of temperature on hydrogen production at pH 4.5

Fig. 2 illustrates that plots based on Eq. (1) satisfactorily fit the cumulative hydrogen production data at pH 4.5 treating 5.5 g-carbohydrate/L of rice slurry at 37 and 55 °C. The best-fit parameters were: λ 36 h, R_m 7.3 mL/h and P 286 mL for 37 °C; λ 44 h, R_m 2.9 mL/h and P 174 mL at 55 °C. The lengthy lag-phase times were likely needed by the bacteria to adjust their physiological state for the new acidic environment of pH 4.5. Based on the P and R_m values, the maximum specific hydrogen production rates and hydrogen yields at 37 °C were calculated as 2.1 L/(g-VSS d) and 346 mL/g-carbohydrate. The corresponding values at 55 °C were 0.8 L/(g-VSS d) and 210 mL/g-carbohydrate. These results show that the mesophilic sludge was more effective than the thermophilic sludge in treating carbohydrate-rich food waste.

3.3. Effect of feedstock concentration on hydrogen production at pH 4.5 and 37 °C

Table 4 summarizes the kinetic parameters λ, R_m and P, plus maximum specific hydrogen production rate and hydrogen yield for experiments conducted at pH 4.5 and 37 °C treating rice slurries at concentrations from

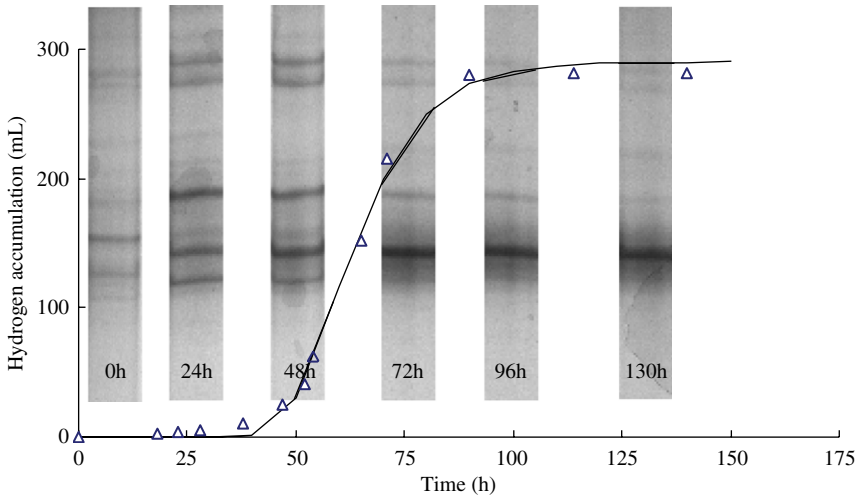

Fig. 4. DGGE profiles at pH 4.5 over 130 h and the corresponding hydrogen accumulation.

Table 6
Phylogenic affiliation of operational taxonomic units

OTU	Phylogenetic relationship		No. of clones	Abundance (%)
	Closest species in GenBank	Similarity (%)		
HPB-R-1	*Clostridium* sp. 44a-T5zd	96	1	3.6
HPB-R-2	*Clostridium* sp. 44a-T5zd	97	17	60.7
HPB-R-4	*Clostridium* sp. 44a-T5zd	96	2	7.0
HPB-R-16	*Clostridium* sp. 44a-T5zd	97	3	10.7
HPB-R-21	*Clostridium* sp. 44a-T5zd	98	1	3.6
HPB-R-25	*Clostridium* sp. 44a-T5zd	97	1	3.6
HPB-R-26	*Clostridium* sp. 44a-T5zd	96	1	3.6
HPB-R-46	*Clostridium* sp. 44a-T5zd	98	1	3.6
HPB-R-55	*Clostridium roseum*	99	1	3.6
Total			28	100.0

2.7 to 22.1 g-carbohydrate/L. It shows that the lag time was sensitive to the concentration, decreasing from 38 h at 2.7 g-carbohydrate/L to 12 h at 22.1 g-carbohydrate/L. Whereas the maximum specific hydrogen production rate increased from 0.3 L/(g-VSS d) at 2.7 g-carbohydrate/L to 2.1 L/(g-VSS d) at 5.5 g-carbohydrate/L and then decreased to 0.4 L/(g-VSS d) at 22.1 g-carbohydrate/L. The concentration of 5.5 g-carbohydrate/L also gave the highest hydrogen yield of 346 mL/g-carbohydrate. Hydrogen yield decreased steadily with concentration above 5.5 g-carbohydrate/L, probably resulting from the increased concentration of VFA and alcohols. Table 5 shows the total concentration VFA and alcohols increased significantly with the increase of feedstock concentration, from 1772 mg/L at 2.7 g-carbohydrate/L to 10,587 mg/L at 22.1 g-carbohydrate/L. It has been known that increased concentration of VFA and alcohols may inhibit the further production of hydrogen [37]. Acetate (28.3–43.0% of total VFA and alcohols) and butyrate (51.4–70.9%) were two main products in all batches, followed by low amounts of caproate, propionate, methanol and propanol. Increase of rice concentration resulted in a slight decrease of acetate and an increase of butyrate.

3.4. Microbial community analyses of sludge

Fig. 3 is the DGGE image illustrating the variation of microbial populations under different pH values. It shows that the biodiversity decreased considerably as pH lowered from pH 6.5 to 4.5, as indicated by the reduced numbers of the major bands. As pH 4.5 is the optimal pH for hydrogen production from rice slurry, the microbial population of sludge at pH 4.5 was further analyzed. Fig. 4 illustrates the microbial population shift over a 130 h period at pH 4.5. It shows that the microbial diversity decreased with time and one band became predominant after 48 h. Fig. 4 also illustrates the hydrogen accumulation over the same time period. It shows that hydrogen production increased substantially after 38 h of lag time for gradually leveled off after 90 h as substrate became depleted. These results strongly suggest that the predominant population shown in Fig. 4 was mainly responsible for the hydrogen production. Fig. 5 illustrates the morphology of the hydrogen-producing bacteria at pH 4.5. The bacteria cells have the typical rod-shaped morphology of *Clostridium* species.

Based on the cloning-sequencing analysis, nine OTUs were classified from the 28 clones developed from this acidophilic hydrogen-producing sludge. Table 6 shows the number of clones and relative abundance of each OTUs, plus the closest species found in the GenBank and the similarity by BLAST analysis. The phylogenetic tree illustrated in Fig. 6 shows that all the OTUs are affiliated with the genus *Clostridium*, and fell into the 16S rDNA Cluster I [38]. Eight OTUs, HPB-R-1, −2, −4, −16, −21, −25, −26 and −46, (totaling 96.4% of relative abundance) form a group at 100% bootstrap value with two known species, *Clostridium acetobutylicum*, *Clostridium felsineum*, plus several uncultured clones found in acidic conditions, i.e. *Clostridium* sp. FA3/2 and *Clostridium* sp. L1/6 involved in the water retting process [39], and *Clostridium* sp. 44a-T5zd (AY082483) in an acid mine drainage system. The eight OTUs form a group with *Clostridium* sp. 44a-T5zd at a bootstrap value of 100%. This uncultured group may be divided into two subgroups. The similarity within each subgroup is over 97% whereas the similarity between two subgroups is below 96%. Fig. 6 illustrates that species of *Clostridium* genus, for example, *C. felsineum* and *C. roseum*, *C. thiosulforeducens* and *C. subterminale*, *C. argentinense*, *C. botulinum*, have shorter interspecies evolution distances as compared to these eight OTUs. This implies that these OTUs likely represent eight new species with characteristics different from any known species of *Clostridium*.

The remaining HPB-R-55 (3.6% abundance) is closely related to *C. roseum*, *C. beijerinckii* and *C. diolis* with 99% similarity. *C. beijerinckii* and *C. roseum* are well-known fermentative and hydrogen-producing bacteria while little information is available on the hydrogen production of *C. diolis*.

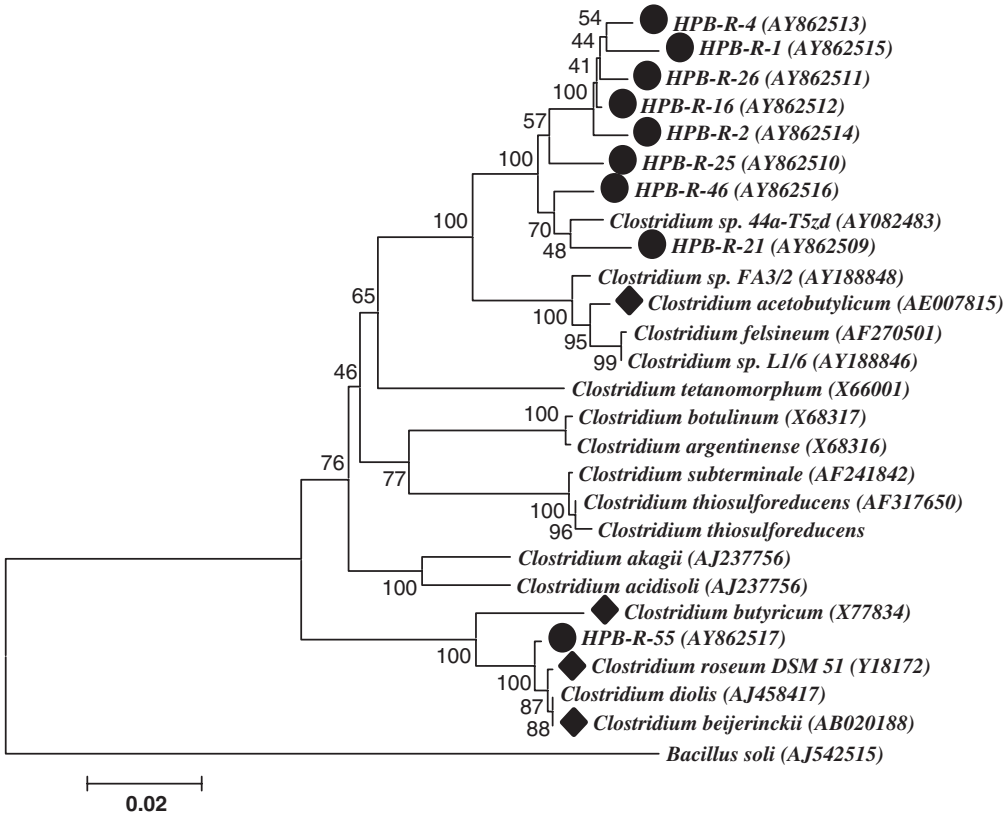

Fig. 6. Phylogenetic tree of the nine OTUs and their close relatives based on almost fully length 16S rDNA. The tree based on Jukes-Cantor distance was constructed using neighbor-joining algorithm with 1000 bootstrappings. *Bacillus soli* was selected as the outgroup species. The scale bar represents 0.02 substitution per nucleotide position. Numbers at the nodes are the bootstrap values. • OTUs obtained in this study, ♦ known hydrogen-producing bacterium species.

Hydrogen producing bacteria may be classified into four major groups: strictly anaerobes, facultative anaerobes, aerobes and photosynthetic bacteria [40]. In this study, the strictly anaerobic *Clostridium* was found most abundant in the sludge. Many *Clostridium* species are capable of producing hydrogen, including *C. acetobutylicum* [41], *C. butylicum* [42], *C. butyricum* [43], *C. kluyveri* [44] and *C. pasteurianum* [45], of which some are known acidophilic species. It has been reported that *C. acetobutylicum* can grow at pH 4.3 [46] and *C. butyricum* at pH 4 [47]. Two anaerobic acid-tolerant bacteria, *C. akagii* CK58T and *C. acidisoli* CK74T, have been isolated from acidic beech litter and acidic peat-bog soil, respectively [48]. The growth of *C. akagii* CK58T and *C. acidisoli* CK74T on glucose yielded hydrogen, butyrate, lactate, acetate, formate, and CO_2 at pH 3.7–7.1 and 3.6–6.9, respectively. In addition to *Clostridium*, an acidophilic *Enterobacter aerogenes* strain HO-39 capable of producing hydrogen at the pH of 4.0 has also been isolated [49]. However, none of these acidophilic hydrogen-producing species was detected in this study. This indicates the acidophilic hydrogen-producing bacteria found in this study are unknown. Further study, including isolation and characterization, is warranted.

4. Conclusions

Hydrogen production from rice slurry was found most effective at pH 4.5, 37 °C treating a slurry containing 5.5 g-carbohydrate/L using an anaerobic digester sludge as seed after a 100 °C heat treatment for 30 min. After a 36 h acclimation period, the sludge had a maximum specific hydrogen production rate of 2.1 L/(g-VSS d) and a hydrogen yield of 346 mL/g-carbohydrate, corresponding to 62.6% of stoichiometric yield. The effluent was composed mostly of acetate (28.3–43.0%) and butyrate (51.4–70.9%), plus small amounts of alcohols. Based on the 16S rDNA analysis, the 28 clones developed from this acidophilic hydrogen-producing sludge may be classified into nine OTUs, all of which are affiliated with the genus *Clostridium*. Phylogenetic analysis shows that eight OTUs (96.4% of population)

form a distinct group with *Clostridium* sp. 44a-T5zd. Results indicate the acidophilic hydrogen-producing bacteria found in this study are unknown, and warrant further study.

Acknowledgements

The authors wish to thank the Hong Kong Research Grant Council for the financial support of this study (HKU7007/02E and 7106/04E) and Chenlin Li wishes to thank HKU for the postgraduate studentship.

References

[1] Hart D. Hydrogen power: the commercial future of "the ultimate fuel". London: Financial Times Energy Publishing; 1997.

[2] Rocha JS, Barbosa MJ, Wijffels RH. Hydrogen production by photosynthetic bacteria. In: Zaborsky OR, Benemann JR, Miyake J, Pietro AS, editors. Biohydrogen. New York: Plenum Publishing; 2001. p. 3–30.

[3] Fang HHP, Liu H, Zhang T. Bio-hydrogen production from wastewater. Water Sci Technol: Water Supply 2004;4(1): 77–85.

[4] Roychowdhury S, Cox D, Levandowsky M. Production of hydrogen by microbial fermentation. Int J Hydrogen Energy 1988;13(7):407–10.

[5] Karube I, Urano N, Matsunaga T, Suzuki S. Hydrogen production from glucose by immobilized growing cells of *Clostridium butyricum*. Eur J Appl Microbiol 1982;16(1):5–9.

[6] Taguchi F, Chang JD, Mizukami N, Saito-Taki T, Hasegawa K, Morimoto M. Isolation of a hydrogen-producing bacterium, *Clostridium beijerinckii* strain AM21B. Can J Microbiol 1993; 39(7):726–30.

[7] Taguchi F, Mizukami N, Yamada K, Hasegawa K, Saito-Taki T. Direct conversion of cellulosic materials to hydrogen by *Clostridium* sp. strain no. 2. Enzyme Microbial Technol 1995;17(2):147–50.

[8] Tanisho S, Kamiya N, Wakao N. Hydrogen evolution of *Enterobacter aerogenes* depending on culture pH: mechanism of hydrogen evolution from NADH by means of membrane-bound hydrogenase. Biochem Biophys Acta 1989;973(1): 1–6.

[9] Rachman MA, Nakashinmada Y, Kakizono T, Nishio N. Hydrogen production with high yield and high evolution rate by self-flocculated cells of *Enterobacter aerogenes* in a packed-bed reactor. Appl Microbiol Biotechnol 1998; 49(4):450–4.

[10] Kumar N, Das D. Enhancement of hydrogen production by *Enterobacter cloacae* IIT-BT 08. Process Biochem 2000;35(6):589–93.

[11] Dabrock B, Bahl H, Gottschalk G. Parameters affecting solvent production by *Clostridium pasteurianum*. Appl Environ Microbiol 1992;58(4):1233–9.

[12] Lay JJ. Modeling and optimization of anaerobic digested sludge converting starch to hydrogen. Biotechnol Bioeng 2000;68(3):269–78.

[13] Fang HHP, Liu H. Effect of pH on hydrogen production from glucose by a mixed culture. Bioresource Technol 2002;82(2):87–93.

[14] Byung HK, Zeikus JG. Importance of hydrogen metabolism in regulation of solventogenesis by *Clostridium acetobutylicum* continuous culture system of hydrogen-producing anaerobic bacteria. Proceedings of the eighth international conference on anaerobic digestion, vol. 2, 1985. p. 383–90.

[15] Khanal SK, Chen WH, Li L, Sung S. Biological hydrogen production: effects of pH and intermediate products. Int J Hydrogen Energy 2004;29(11):1123–31.

[16] Lay JJ, Lee YJ, Noike T. Feasibility of biological hydrogen production from organic fraction of municipal solid waste. Water Res 1999;33(11):2579–86.

[17] Morimoto M, Atsuko M, Atif AAY, Ngan MA, Fakhru'l-Razi A, Iyuke SE, Bakir AM. Biological hydrogen production of hydrogen from glucose by natural anaerobic microflora. Int J Hydrogen Energy 2004;29(7):709–13.

[18] Levin DB, Pitt L, Love M. Biohydrogen production: prospects and limitations to practical application. Int J Hydrogen Energy 2004;29(2):173–85.

[19] Ueno Y, Sato S, Morimoto M. Hydrogen production from industrial wastewater by anaerobic microflora in chemostat culture. J Ferment Bioeng 1996;82(2):194–7.

[20] Ueno Y, Haruta S, Ishii M, Igarashi Y. Microbial community in anaerobic hydrogen-producing microflora enriched from sludge compost. Appl Microbial Biotechnol 2001;57(4): 555–62.

[21] APHA. Standard methods for the examination of water and wastewater. 18th ed. Washington, DC, USA: American Public Health Association; 1992.

[22] Lay JJ, Li YY, Noike T. The influences of pH and moisture content on the methane production in high-solids sludge digestion. Water Res 1997;31(6):1518–24.

[23] Zwietering MH, Jongenburge RI, Rombouts FM, Vantriet K. Modeling of the bacterial-growth curve. Appl Environ Microbiol 1990;56(6):1875–81.

[24] Muyzer G, de Waal EC, Uitterlinden AG. Profiling of complex microbial population by DGGE analysis of polymerase chain reaction amplified genes encoding for 16S rRNA. Appl Environ Microbiol 1993;62:2676–80.

[25] Zhang T, Fang HHP. Phylogenetic diversity of a SRB-rich marine biofilm. Appl Microbiol Biotechnol 2001;57(3): 437–40.

[26] Zhang T, Fang HHP. Digitization of DGGE (denaturing gradient gel electrophoresis) profile and cluster analysis of microbial community. Biotechnol Lett 2000;22:399–405.

[27] Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Window 95/98/NT. Nucleic Acids Symp Ser 1999;41:95–8.

[28] Maidak BL, Olsen GJ, Larsen N, Overbeek R, McCaughey MJ, Woese CR. The RDP (ribosomal database project). Nucleic Acid Res 1997;25(1):109–10.

[29] Altschul SF, Gish W, Miller W, Myers E, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215(3):403–10.

[30] Satio N, Nei M. The neighbour-joining method: a new method for constructing phylogenetic trees. Mol Biol Evol 1987;4: 406–25.

[31] Kumar S, Tomura K, Nei M. MEGA: Molecular evolution genetics analysis, version 1.0. Philadelphia: Pennsylvania State University; 1993.

[32] Felsenstein J. Confidence limits of phylogenies: an approach using the bootstrap. Evolution 1985;39(4):783–91.

[33] Zhang T, Liu H, Fang HHP. Biohydrogen production from starch in wastewater under thermophilic condition. J Environ Manage 2003;69(2):149–56.
[34] Lay JJ. Biohydrogen generation by mesophilic anaerobic fermentation of microcrystalline cellulose. Biotechnol Bioeng 2001;74(4):280–7.
[35] Ueno Y, Kawai T, Sato S, Otsuka S, Morimoto M. Biological production of hydrogen from cellulose by mixed anaerobic microflora. J Ferment Bioeng 1995;79(4):395–7.
[36] Fang HHP, Liu H, Zhang T. Characterization of a hydrogen-producing granular sludge. Biotechnol Bioeng 2002;78(1): 44–52.
[37] Van Ginkel S, Sung S, Lay JJ. Biohydrogen production as a function of pH and substrate concentration. Environ Sci Technol 2001;35(24):4726–30.
[38] Stackebrandt E, Rainey FA. Phylogenetic relationships. In: Rood J, editor. The *Clostridia*: Molecular Biology and Pathogenesis. Imprint San Diego, California: Academic Press; 1997. p. 3–19.
[39] Tamburini E, Leon AG, Perito B, Mastromei G. Characterization of bacterial pectinolytic strains involved in the water retting process. Environ Microbiol 2003;5(9): 730–6.
[40] Nandi R, Sengupta S. Microbial production of hydrogen—an overview. Crit Rev Microbiol 1998;24(1):61–84.
[41] Esteso MA, Estrella CN, Podesta JJ. Evaluation of the absorption on mild steel of hydrogen evolved in glucose fermentation by pure cultures of *Clostridium acetobutylicum* and *Enterobacter*. Sensors Actuators BL Chem 1996;32(1): 27–31.
[42] Kataoka N, Miya A, Kiriyama K. Studies on hydrogen-production by continuous cultures system of hydrogen-producing anaerobic bacteria. Water Sci Technol 1997;36 (6–7):41–7.
[43] Karube I, Urano N, Matsunaga T, Suzuki S. Hydrogen production from glucose by immobilized growing cells of *Clostridium butyricum*. Eur J Appl Microbiol 1982;16(1):5–9.
[44] Benemann JR, Berenson JA, Kaplan NO, Kamen MD. Hydrogen evolution by a chloroplast-ferredoxin-hydrogenase system. Proc Natl Acad Sci 1973;70(8):2317–20.
[45] McTavish H. Hydrogen evolution by direct electron transfer from photosystem I to hydrogenases. J Biochem 1998; 123(4):644–9.
[46] Bahl H, Andersch W, Gottschalk G. Continuous production of acetone and butanol by *Clostridium acetobutylicum* in a two-stage phosphate limited chemostat. Eur J Appl Microbiol Biotechnol 1982;15(4):201–5.
[47] Gaudy A, Gaudy E. Microbiology for environmental scientists and engineers. New York: McGraw-Hill; 1980.
[48] Kuhner CH, Matthies C, Acker G, Schmittroth M, Gößner A, Drake HL. *Clostridium akagii* sp. nov. and *Clostridium acidisoli* sp. nov., acid-tolerant, N_2-fixing *clostridia* isolated from acidic forest soil and litter. Int J Syst Bacteriol 2000;50:873–81.
[49] Yokoi H, Tokushige T, Hirose J, Hayashi S, Takasaki Y. Hydrogen production by immobilized cells of aciduric *Enterobacter aerogenes* strain HO-39. J Ferment Bioeng 1997;83(5):481–4.