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Biohydrogen production from starch in wastewater under thermophilic condition

Tong Zhang, Hong Liu, Herbert H.P. Fang*

Department of Civil Engineering, Centre for Environmental Engineering Research, University of Hong Kong, Pokulam Road, Hong Kong, China

Abstract

Batch experiments were conducted to convert starch in wastewater into hydrogen at 55 °C at various wastewater pH (4.0–9.0) and starch concentrations (9.2–36.6 g/l). The maximum hydrogen yield of 92 ml/g of starch added (17% of the theoretical value) was found at wastewater pH 6.0, and the maximum specific hydrogen production rate of 365 ml/(g-VSS·d) was at wastewater pH 7.0. The methane-free biogas contained up to 60% of hydrogen. The mixed liquor was composed mostly of acetate (40.2–53.4%) and butyrate (26.0–40.9%). Phylogenetic analysis based on 16S rDNA sequences of the 72 clones developed from the sludge at pH 6.0 shows that 85.7% of the clones were closely affiliated with genus *Thermoanaerobacterium* in family *Thermoanaerobacteriaceae*; the remaining 14.3% were with an uncultured *Saccharococcus* sp. clone ETV-T2.

Keywords: Fermentation; Hydrogen; Starch; *Thermoanaerobacterium*; Thermophilic; Wastewater

1. Introduction

In conventional anaerobic treatment processes, organic pollutants are first converted to fatty acids, which are then further converted to acetate and hydrogen, both of which are eventually converted into methane. Only limited studies have been conducted to explore the feasibility of harvesting the intermediate hydrogen, instead of methane. Hydrogen is an important industrial commodity (Kirk et al., 1985). It is widely used for the syntheses of ammonia, alcohols and aldehydes, as well as for the hydrogenation of edible oils, petroleum, coal and shale oil. In addition, hydrogen is an ideal fuel, producing only water upon combustion. Many believe that hydrogen may replace fossil fuel as the next generation of energy supply. Hydrogen is traditionally produced by hydrocarbon reformation or electrolysis of water (Hart, 1997), but it is technical feasible to harvest hydrogen produced by microorganisms.

Fermentative microorganisms, such as *Clostridium* and *Thermoanaerobacterium*, are able to produce hydrogen from carbohydrates (Ueno et al., 1995; Lay, 2000; Fang and Liu, 2002; Fang et al., 2002a,b; Cann et al., 2001). A few studies have been conducted to produce hydrogen from carbohydrate-rich wastewater under mesophilic condition (Roychowdhury et al., 1988; Ueno et al., 1996; Majizat et al., 1997; Lin and Chang, 1999; Fang and Liu, 2002). However, many industrial effluents, such as those from food processing, are often discharged at elevated temperatures. Treating these starch-laden effluents under conventional mesophilic condition requires costly precooling, and has the risk of losing the biomass activity should the cooling system breaks down. It is thus natural to treat these effluents under thermophilic condition, which presumably also favored the degradation kinetics and the killing of pathogens.

This study was thus conducted to study the effects wastewater pH and starch concentration in wastewater on hydrogen production at the thermophilic condition of 55 °C. The microbial population of the thermophilic hydrogen-producing sludge was analyzed using 16S rDNA-based techniques and fluorescence in situ hybridization (FISH) method.

2. Material and methods

2.1. Experimental conditions

Three series of batch experiments were conducted in 280 ml glass reactors. A hydrogen-producing sludge was obtained from a completely stirred fermentor treating a sucrose-containing wastewater, which was seeded with

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

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sludge obtained from the secondary sedimentation tank of a local wastewater treatment plant. With 8 h of hydraulic retention at pH 5.5 and 37 °C, this sludge had a hydrogen yield of 230 ml/g-sucrose, and each gram of sludge, as measured by volatile suspended solids (VSS), produced 3800 ml of hydrogen daily. In all experiments, the sludge obtained from the completed stirred fermentor was used as seed without acclimation. The wastewater was prepared using starch (Farco Chemical, China) as the sole substrate, plus the following nutrients (in mg/l): $NaHCO_3$ 1000; NH_4Cl 500; KH_2PO_4 250; $MgSO_4{\cdot}7H_2O$ 50; $Na_2MoO_4{\cdot}2H_2O$ 5; $CaCl_2$ 5; $MnCl_2$ 5; $FeSO_4$ 1.5.

Series 1 was conducted to compare the hydrogen production at 37 and 55 °C treating a wastewater containing 4.6 g/l of starch at pH 7.0. Experiments in Series 2 examined the effect of wastewater pH (from 4.0 to 9.0 with 1.0 increments) treating a wastewater containing 4.6 g/l of wastewater at 55 °C. Experiments of Series 3 investigated the effect starch concentration in wastewater from 9.2 to 36.6 g/l with 9.2 g/l increments at the initial wastewater pH of 6.0 and 55 °C. In all batches, 200 ml of wastewater was treated with 125 mg of sludge, as measured by VSS. The mixed liquor in the reactor was first purged with nitrogen for 20 min to ensure anaerobic condition prior to each run.

2.2. Biogas and effluent analyses

The amount of biogas produced in each reactor was measured using a glass syringe. The contents of biogas were analyzed by a gas chromatograph (GC) (Model 5890II, Hewlett Packard, 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 the carrier gas at a flow rate of 30 ml/min. The concentrations of volatile fatty acids (VFA) and alcohols in the effluent were determined by a second GC of same model, which was equipped with a flame ionization detector and a 10 m × 0.53 mm HP-FFAP fused-silica capillary column, following the procedures described previously (Fang and Liu, 2002).

2.3. DGGE, cloning, sequencing and phylogenetic analysis

The thermophilic sludge treating 4.6 g/l of starch in wastewater at pH 6.0 was sampled at the end of the batch experiment for microbial analysis. DNA were extracted from 5 ml of sludge, followed by polymerase chain reactions (PCR) amplification (Zhang and Fang, 2000, 2001). After screening using denatured gradient gel electrophoresis (DGGE), the PCR-amplified products were cloned, and the DNA of the major clones were sequenced for phylogenetic analysis. The primer used for sequencing in this study was EUB1509R. A phylogenetic tree was constructed using the neighbor-joining method with MEGA 2.1 (Kumar et al., 1993). Bootstrap re-sampling analysis for 500 replicates was performed to estimate the confidence of tree topologies. Details of these procedures were reported previously (Zhang and Fang, 2000, 2001).

2.4. Fluorescence in situ hybridization

The microbial community of the sludge was also analyzed by FISH (Wagner et al., 1994; Franks et al., 1998) using two oligonucleotide probes: (a) a *Eubacteria*-targeting probe, EUB338, with the 6-carboxy-fluorescein (FAM) label at the 5′-end, and (b) a Chis150 probe targeting for most species of the *Clostridium histolyticum* group (Franks et al., 1998) with the Cy3 label at the 5′-end (Integrated DNA Technologies, Coralville, IA).

The sludge was gently washed with a pH 7.2 phosphate-buffered saline solution (PBS; 0.13 M NaCl, plus 10 mM Na_2HPO_4), fixed in a PBS containing 4% (w/v) paraformaldehyde at 4 °C overnight. After washing three times with PBS (Manz et al., 1992), sludge was added onto the glass slide, air-dried at 46 °C for 10 min, and dehydrated sequentially in 50, 80 and 96% ethanol solutions for 3 min each. The dehydrated sludge was hybridized (Amann et al., 1995) in with probes in a buffer for 90 min at 46 °C. After incubating in a washing buffer for another 30 min at 48 °C, the slide was rinsed briefly with distilled water and air dried. The hybridized sludge sample was then examined using a Zeiss LSM 5 Pascal confocal laser scanning microscope (Zeiss, Jena, Germany), which was equipped with Argon and He–Ne lasers. The FISH images were captured using the multi-track mode, which excited the sample sequentially with lasers at 488 and 543 nm.

2.5. Kinetic analysis

The cumulative hydrogen production in the batch experiments followed the modified Gompertz equation (Lay and Noike, 1999):

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

where H represents the cumulative volume of hydrogen produced (ml), P the hydrogen production potential (ml), R_m the maximum production rate (ml/h), and λ the lag time (h). The values of P, R_m and λ for each batch was determined by best fitting the hydrogen production data for Eq. (1) using the Matlab 6.0 with Optimization Toolbox 2.1 (MathWorks, Inc, 2000). The maximum specific hydrogen production rate (ml/(g-VSS·d)) was calculated by dividing R_m by the initial sludge VSS. The hydrogen yield (ml/g-starch) was calculated by dividing P by the quantity of starch added in wastewater.

2.6. Accession numbers

The seven nucleotide sequence data reported in this paper have been assigned the following accession numbers

for the GenBank, EMBL and DDBJ databases: AY185509-AY185515.

3. Results and discussion

In all three series of experiments, the produced biogas contained hydrogen (45–60%) and carbon dioxide (40–55%), excluding the residual nitrogen from the initial purging. The biogas was free of methane, indicating the lack of methanogenic activities in the sludges. Eq. (1) correlates hydrogen production data well with $R^2 > 0.95$ in all series.

3.1. Effect of temperature on hydrogen production

Fig. 1 illustrates the cumulative hydrogen production from starch in wastewater at pH 7.0 and two temperatures: 37 and 55 °C. The two best-fit curves based on Eq. (1) fits the data satisfactorily. The best-fit kinetic parameters for the 37 °C data were: λ 3 h, P 43 ml and R_m 2.5 ml/h; those for the 55 °C data were: λ 54 h, P 71 ml and R_m 1.9 ml/h. Based on the P and R_m values, the hydrogen yield at 37 °C was calculated as 47 ml/g-starch and the maximum specific hydrogen production rate was 480 ml/(g-VSS·d). The corresponding values at 55 °C were 78 ml/g-starch and 365 ml/(g-VSS·d). These results show that the mesophilic sludge was effective in treating wastewater at 55 °C without acclimation. At 55 °C, sludge required a longer lag time for hydrogen production, converted more starch into hydrogen although at a slower rate. The longer lag time was likely due to the need for bacteria to modify their physiological state for the new environment.

3.2. Effect of wastewater pH on hydrogen production at 55 °C

Table 1 summarizes the three kinetic parameters, plus hydrogen yield and maximum specific hydrogen production rate, for conversion of 4.6 g/l of starch into hydrogen at 55 °C and pH ranging 4.0–9.0. Results show that no hydrogen was produced at pH 4.0, indicating that hydrogen production from starch was inhibited at low pH. The lag time was greatly affected by the initial pH. It varied from as low as 21 h at pH 8.0 up to 72 h at pH 5.0.

The hydrogen yield increased with initial pH from 5.0 to 6.0, and then decreased as pH further increased from 6.0 to 9.0. The maximum hydrogen yield of 92 ml/g-starch, which is 17% of the theoretical yield, occurred at pH 6.0. Such a yield was substantially higher than the 49 ml/g-cellulose at 37 °C (Lay and Noike, 1999) and 45 ml/g-cellulose at 60 °C by digester sludge, but lower than the 193 ml/g-cellulose by the microflora from sludge compost at 60 °C (Ueno et al., 1995). According to stoichiometry, each gram of starch produces a maximum of 553 ml hydrogen with acetate as by-product. Such efficiency was much lower than the 52.2% from glucose (Fang and Liu, 2002) and 53.4% from sucrose (Fang et al., 2002a). This is due to the incomplete hydrolysis of starch (McCarty and Mosey, 1991). The maximum specific hydrogen production rate was 365 ml/(g-VSS·d) at pH 7.0, higher than the 50–250 ml/(g-VSS·d) produced from cellulose under mesophilic condition (Lay, 2001). Overall, pH 6.0–7.0 was identified in this study as the suitable pH range for hydrogen production from starch under the thermophilic condition.

The final pH in all batches was consistently within the range of pH 4.0–4.6, regardless of the starting wastewater pH. Table 2 summarizes the distribution of key VFA/alcohols produced in batches of various wastewater pH. It shows that acetate (50.0–53.4% of total VFA/alcohols) and butyrate (26.0–31.6%) were two main products of fermentation in all batches, followed by ethanol, methanol and propionate, plus traced amounts of propanol, butanol, i-butyrate, i-valerate, valerate and i-caproate.

Table 2 shows that small amounts of VFA and alcohols were produced in treating wastewater at pH 4.0, even though hydrogen was undetected under this condition. It also shows that contents of acetate and butyrate in mixed liquor were insensitive to the wastewater pH; however, increase of pH did result in the increase of propionate, from 1.5% of the total VFA/alcohols at pH 4.0–8.1% at pH 9.0, and the decrease of ethanol, from 12.1% at pH 4.0–3.9% at pH 9.0. Methanol was undetected in all batches, except at

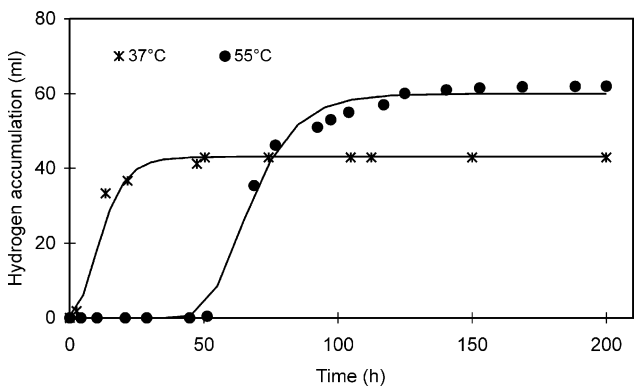

Fig. 1. Cumulative hydrogen production from starch at thermophilic and mesophilic conditions.

Table 1
Kinetic parameters for hydrogen production from starch at 4.6 g/l, 55 °C and various initial pH

Initial pH	λ (h)	R_m (ml/h)	P (ml)	Maximum specific H_2 production rate (ml/(g-VSS·d))	Hydrogen yield (ml/g-starch)
4.0	–	–	–	–	–
5.0	72	0.7	34	135	37
6.0	37	1.0	84	194	92
7.0	51	1.9	60	365	67
8.0	21	0.6	55	116	60
9.0	42	0.7	45	135	49

Table 2
Final pH, VFA and alcohols production from starch at various initial wastewater pH

Initial pH	Final pH	VFA and alcohols (mg/l)	Acetate (%)	Butyrate (%)	Propionate (%)	Ethanol (%)	Methanol (%)
4.0	4.0	28.2	50.1	26.0	1.5	12.1	0
5.0	4.1	227.4	52.3	26.5	2.8	10.5	3.9
6.0	4.2	298.5	50.0	28.9	3.0	9.5	3.6
7.0	4.4	272.4	51.7	31.2	5.1	9.0	0
8.0	4.6	270.9	52.0	30.2	6.4	7.0	0
9.0	4.6	227.1	53.4	31.6	8.1	3.9	0

pH 5.0 and 6.0. These results differed from observations of hydrogen production from municipal solid wastes. Lay and Noike (1999) found that increasing pH would lead to the metabolic shift from VFA to alcohols production, resulting in a decrease of hydrogen yield. Such a discrepancy could be due to many factors, including differences of microbial population and mixing condition (Lamed et al., 1988).

3.3. Effect of starch concentration on hydrogen production at 55 °C

Table 3 summarizes the results of Series 3 experiments treating wastewaters containing starch (9.2–36.6 g/l) at the initial wastewater pH of 6.0 and 55 °C. It shows that the lag time (11–33 h) was relatively short. For comparison, using the digester sludge as inoculant, a lag time of over three days was needed for hydrogen production (Lay and Noike, 1999). Table 3 further shows that the maximum hydrogen production rate increased with starch concentration in wastewater, from 97 ml/(g-VSS·d) at 9.2 g/l to 271 ml/(g-VSS·d) at 27.5 g/l, and then decreased to 232 ml/(g-VSS·d) at 36.6 g/l. The maximum rate in study was found in treating 27.5 g/l of starch. This was similar to the observations of Lay (2001), in which the maximum rate for cellulose conversion to hydrogen was found in treating 25 g/l of cellulose in wastewater. Table 3 also shows that hydrogen yield decreased steadily with the increase of starch concentration from 67 ml/g-starch at 9.2 g/l to 24 ml/g-starch to 36.6 g/l. The decrease in hydrogen yield could be resulted from the increase of total VFA/alcohols, which may inhibit the further production of hydrogen (Van Ginkel et al., 2001).

Table 3
Kinetic parameters at various initial starch concentrations

Concentration (g/l)	λ (h)	R_m (ml/h)	P (ml)	Maximum specific H_2 production rate (ml/(g-VSS·d))	Hydrogen yield (ml/g-starch)
9.2	33	0.5	121	97	67
18.3	11	0.7	179	135	49
27.5	13	1.4	222	271	40
36.6	21	1.2	179	232	24

Table 4 shows that the total VFA/alcohols concentration increased only slightly with the increase of starch concentration, from 323.1 mg/l at 9.2 g/l to 440.5 mg/l at 36.6 g/l. The composition of VFA/alcohols was only slightly dependent on the starch concentration in wastewater. Increase of starch concentration resulted in a slight decrease of acetate production (from 46.3 to 40.2%), but increases of butyrate (from 35.6 to 40.9%) and ethanol (from 7.9 to 10.3%); it has little effect on the contents of propionate (1.0–1.4%) and methanol (2.1–2.9%)

3.4. Microbial characteristics of sludge

DGGE images in Fig. 2 show that a shift of microbial population from the mesophilic seed sludge to the sludge at the end of the thermophilic batch experiment. The shift was due to the changes of temperature and substrate.

Fig. 3 illustrates the FISH images of the mesophilic seed sludge (Fig. 3A and B) and the thermophilic starch-degrading sludge (Fig. 3C and D). Images in Fig. 3A and C hybridized with EUB338 illustrate the abundance of *Eubacteria* in both the mesophilic seed sludge and the thermophilic starch-degrading sludge. Chis150-hybridized image in Fig. 3B illustrates the abundance of *C. histolyticum* group in the mesophilic sludge. However, image in Fig. 3D illustrates that the *C. histolyticum* group was nearly absent.

Table 4
Final pH, VFA and alcohols production at various initial starch concentrations

Starch concentration (g/l)	Final pH	VFA and alcohols (mg/l)	Acetate (%)	Butyrate (%)	Propionate (%)	Ethanol (%)	Methanol (%)
9.2	4.5	323.1	46.3	35.6	1.1	7.9	2.1
18.3	4.3	352.1	45.2	38.8	1.4	8.7	2.9
27.5	4.5	398.4	46.3	39.4	1.4	9.5	2.4
36.6	4.2	440.5	40.2	40.9	1.0	10.3	2.6

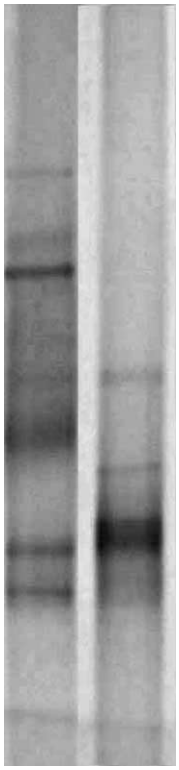

Fig. 2. DGGE images of the mesophilic seed (M) and the thermophilic communities (T). (The denaturant gradient was from 40 to 60%).

This is due to the population shift during the thermophilic degradation process.

Seven operational taxonomy units (OTUs) were classified from the 72 clones developed from this thermophilic hydrogen-producing sludge. The phylogenetic tree illustrated in Fig. 4 shows that six out of the seven OTUs (85.7% of 72 clones) were affiliated with a thermophilic genus *Thermoanaerobacterium* in family *Thermoanaerobacteriaceae*, even though the seed sludge was mesophilic. These six OTUs form a cluster with 88% bootstrap value with all the eight known species of *Thermoanaerobacterium*, plus *Clostridium thermoamylolyticum* and two uncultured thermophiles tbr1–8 (accession number: AF280823) and tbr1–78 (accession number: AF280836). The six OTUs have 98% similarity with *C. thermoamylolyticum* (Collins et al., 1994), *T. saccharolyticum* (Lee et al., 1993), *T. thermosulfurigenes* (Lee et al., 1993), *T. aotearoense* (Liu et al., 1996) and *T. xylanolyticum* (Lee et al., 1993). They also have 97% similarity with *T. lactoethylicum* (also known as *Thermoanaerobacter lactoethylicum* and *Thermoanaerobium lactoethylicum*), *T. thermosaccharolyticum* (also known as *C. thermosaccharolyticum)*, and the uncultured tbr1–8 and tbr4–78, but low similarity of 93% with other two *Thermoanaerobacterium* species, *T. zeae* and *T. polysaccharolyticum*.

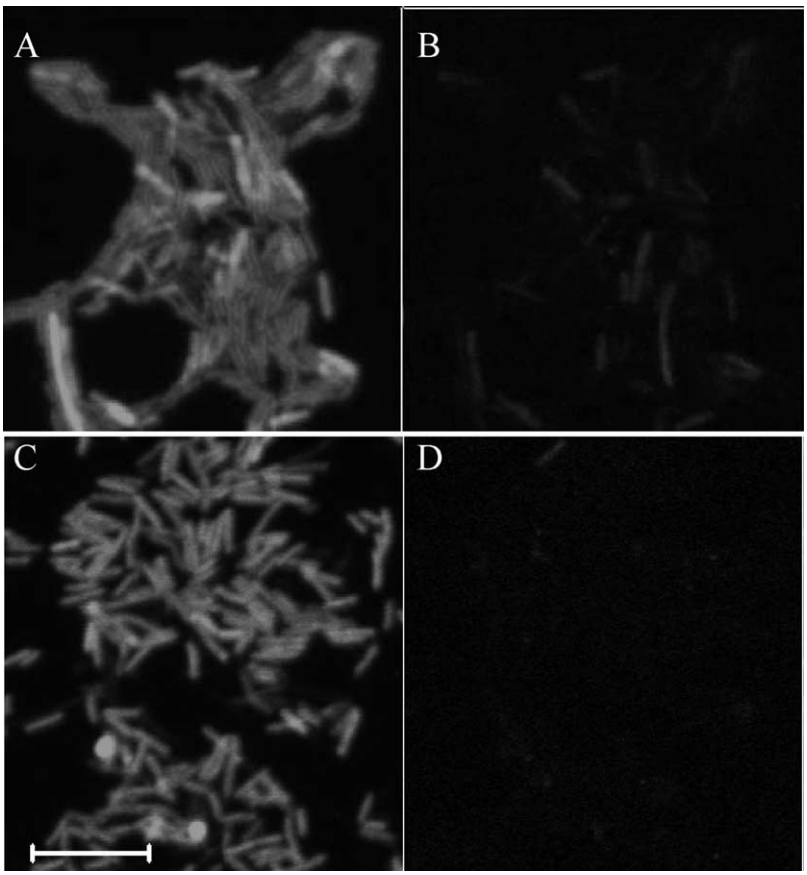

Fig. 3. FISH images of the mesophilic seed (A and B) and the thermophilic sludges (C and D). (A and C are images hybridized with EUB338; B and D are images hybridized with Chis150; Bar = 10 μm).

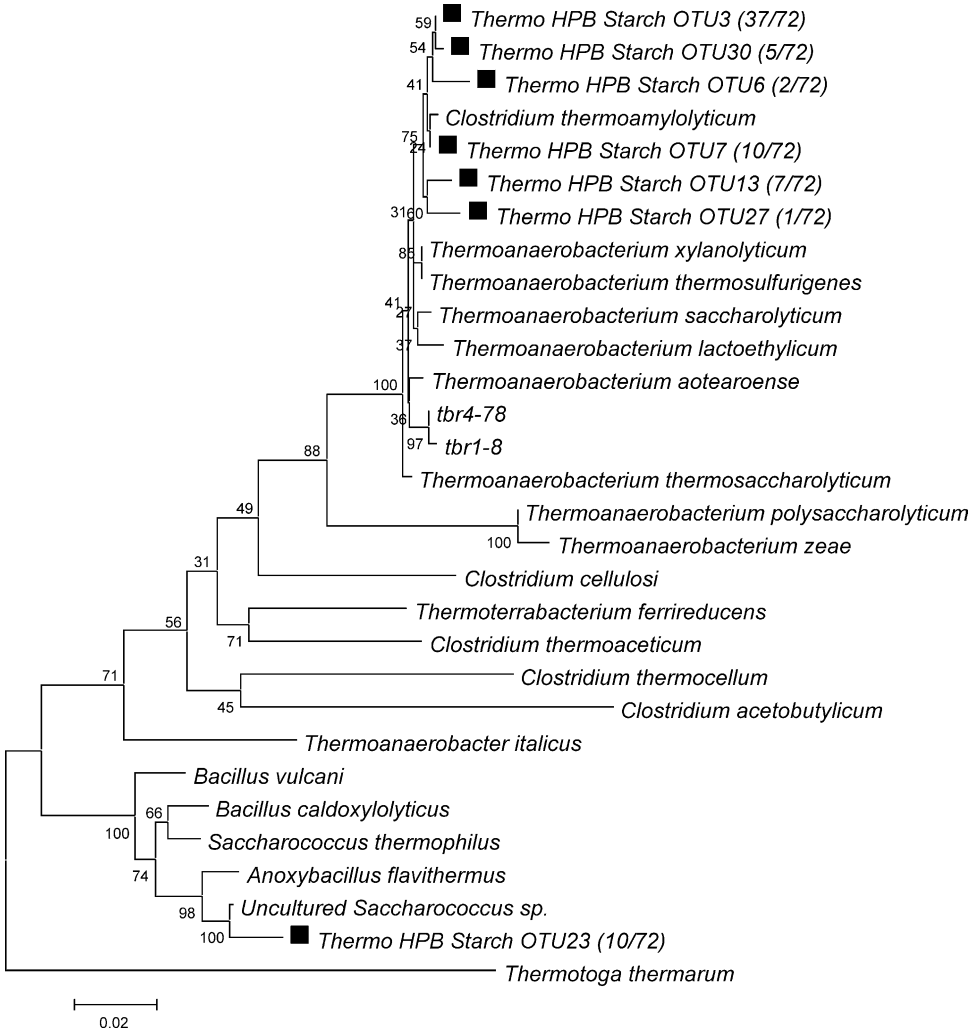

Fig. 4. Phylogenetic tree of the seven thermophilic species and their close relatives based on partial 16S rDNA. The tree based on Jukes–Cantor distance was constructed using neighbor-joining algorithm with 1000 bootstrappings. *Thermotogales thermarum* was selected as the outgroup species. The scale bar represents 0.02 substitution per nucleotide position. Numbers at the nodes are the bootstrap values. The number in the parenthesis indicated the clone number of that OTU in the library.

Fig. 4 illustrates that species of *Thermoanaerobacterium* genus have short interspecies evolution distances as compared to those of OTUs. This implies that the OTUs found in this study likely represent new species with characteristics different from those of *Thermoanaerobacterium*.

Several species of *Thermoanaerobacterium* are known for their hydrogen producing characteristics, including *T. thermosaccharolyticum* (Collins et al., 1994), *T. polysaccharolyticum* (Cann et al., 2001), *T. zeae* (Cann et al., 2001), *T. lactoethylicum* (Lee et al., 1993) and *T. aotearoense* (Liu et al., 1996). As compared to other thermophilic anaerobes, the genus *Thermoanaerobacterium* has the unique capability of reducing thiosulfate to elemental sulfur in acidic solutions (Lee et al., 1993; Collins et al., 1994; Liu et al., 1996; Cann et al., 2001). Species of *Thermoanaerobacterium* have been isolated from thermal volcanic spring (Schink and Zeikus, 1983), hot spring (Liu et al., 1996), and a high temperature acidic (pH 5.5) leachate of a waste pile from a canning factory (Cann et al., 2001). All *Thermoanaerobacterium* species have the optimal growth conditions of 55–70 °C and pH 5.2–7.8. All *Thermoanaerobacterium* can degrade starch, except *T. polysaccharolyticum* (Lee et al., 1993; Collins et al., 1994; Liu et al., 1996; Cann et al., 2001). Several thermally stable enzymes have been purified or cloned from *Thermoanaerobacterium*, including endoxylanases (Lee et al., 1993; Liu et al., 1996), which can hydrolyze polysaccharides.

C. thermoamylolyticum was isolated from mud hot springs in Hveragerdi, Iceland (Katkocin, 1985) and the uncultured tbr1–8 and tbr1–78 were detected from thermophilic bioreactors treating a pharmaceutical wastewater (LaPara et al., 2000). The physiological characteristics of these three species are not clear.

Fig. 3 also illustrates that OTU-S23 was the only one not affiliated with *Thermoanaerobacterium*. It is most closely related to an uncultured *Saccharococcus* sp. clone ETV-T2

with 98% similarity, followed by *Anoxybacillus flavithermus* (similarity 96%) and *Saccharococcus thermophilus* (similarity 95%). Both *Saccharococcus* and *Anoxybacillus* are Gram positive facultative anaerobic thermophiles. Species of *Saccharococcus* are often found in sugar refinery effluent. They are capable of producing lactate and other acids from glucose, but cannot degrade starch, and have an optimal temperature of 68 °C (Holt et al., 1994). The optimal growth conditions for *Anoxybacillus* are 62 °C and pH 9.5–9.7. *Anoxybacillus* are known to be capable of producing hydrogen from monosaccharides (Pikuta et al., 2000), but whether *Saccharococcus* can produce hydrogen or not is not clear.

All of the detected species in this study are phylogenetically thermophilic. All members of *Thermotogales*, a thermophilic order, are capable of producing hydrogen from a wide variety of substrates, including complex carbohydrates and proteins. (van Ooteghem et al., 2002). However, no species of *Thermotogales* was detected in this study.

4. Conclusions

More hydrogen can be produced from starch under thermophilic condition (55 °C) than mesophilic condition (37 °C) at 4.6 g/l and pH 7. The optimum wastewater pH range for thermophilic hydrogen production was found as 6.0–7.0. The maximum hydrogen yield of 92 ml/g-starch was found in treating wastewater at pH 6.0, and the maximum specific hydrogen production rate of 365 ml/(g-VSS·d) was at pH 7.0. The biogas produced contained up to 60% of hydrogen and was free of methane. The acidified products were mainly composed of acetate (50.0–53.4%) and butyrate (26.0–31.6). Based on phylogenetic analysis of the 16S rDNA sequences 85.7% of the 72 clones developed from the starch-degrading sludge were closely affiliated with genus *Thermoanaerobacterium* in family *Thermoanaerobacteriaceae*. The remaining 14.3% were most closely related to an uncultured *Saccharococcus* sp. clone ETV-T2.

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