Characterization of a Hydrogen-Producing Granular Sludge

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Abstract: This study demonstrated that hydrogen-producing acidogenic sludge could agglutinate into granules in a well-mixed reactor treating a synthetic sucrose-containing wastewater at 26°C, pH 5.5, with 6 h of hydraulic retention. A typical matured granule is 1.6 mm in diameter, 1.038 g/mL in density, 11% in ash content, and over 50 m/h in settling velocity. Treating a solution containing 12.15 g/L of sucrose at a volumetric loading rate of 48.6 g/(L · d), the reactor containing 20 g/L of granular sludge degraded 97% of sucrose. Effluent comprised 46% acetate and 49% butyrate and the methane-free biogas comprised 63% hydrogen, 35% carbon dioxide, and 2% nitrogen. Hydrogen production rate was 13.0 L/(L · d), and the yield was 0.28 L/g-sucrose. The granule had multiple cracks on the surface and comprised two morphological types of bacteria: fusiform bacilli and a spore-forming bacterium. Phylogenetic analysis showed that 69.1% of the clones were affiliated with four *Clostridium* species in the family Clostridiaceae, and 13.5% with *Sporolactobacillus racemicus* in the *Bacillus/Staphylococcus* group.
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INTRODUCTION

In conventional anaerobic reactors, organic pollutants in wastewater are converted to methane through interactions of various anaerobes. Full-scale applications of anaerobic wastewater treatment technology have become widely accepted in the past few decades (Fang and Liu, 2001). This is largely due to the successful development of several high-rate reactors. Among them, the upflow anaerobic sludge blanket reactor (Lettinga et al., 1980) has become most popular for the treatment of a variety of industrial effluents (Hulshoff Pol and Lettinga, 1986). In an upflow anaerobic sludge blanket reactor, sludge agglutinates into granules, resulting in the increase of biomass concentration and the reduction of sludge washout. Furthermore, the formation of biogranules also facilitates the syntrophic interactions among various microbes involved in the methanogenic degradation of organic pollutants (Fang, 2000).

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However, some industrial wastewaters containing complex pollutants are better treated by the two-stage anaerobic process (Fang and Yu, 2000; Guerrero et al., 1999). In such a process, pollutants are hydrolyzed and acidified in the first reactor at pH, temperature, and hydraulic retention time (HRT) favored by the acidogenic bacteria. The acidic effluent is subsequently treated in a separate methanogenic reactor. In the acidogenic reactor, hydrogen is often a byproduct. Because hydrogen is a valuable raw material for many industrial applications as well as a clean energy source, harvesting hydrogen from the two-stage anaerobic wastewater treatment process has attracted much research attention recently (Mizuno et al., 2000; Nakamura et al., 1993). In all previous studies, hydrogen was produced by acidogens grown in suspension. However, it was demonstrated in this study that the hydrogen-producing biomass can also develop into granules with high bioactivity.

It was found recently that, in addition to anaerobic sludge, aerobic sludge could also develop into granular form (Beun et al., 1999; Morgenroth et al., 1997). The mechanism of granule formation has not been fully understood. However, microbial population (Schmidt and Ahring, 1996; Wiegant and de Man, 1986) and extracellular polymeric substances (EPS) (Schmidt and Ahring, 1996; Veiga et al., 1997) are likely to play important roles in the granule formation.

Traditionally, microbes are identified by isolating individual cultures and examining their physiological, biochemical, and morphological characteristics. These methods are often not reliable. Some microbes are syntrophically associated with others, and thus cannot be isolated and cultured individually (Wagner et al., 1993). Furthermore, many microbes share similar physiological, biochemical, and morphological characteristics, and thus cannot be distinguished from one another based on these characteristics. However, microbial communities may be analyzed using some of the advanced molecular techniques developed recently. Among them, 16S rDNA-based methods have been extensively applied in various studies, including ocean mats (Muyzer et al., 1993), sediments (Devereux and

Mundform, 1994), activated sludge (Nielsen et al., 1999), biofilm (Zhang and Fang, 2000), anaerobic digester (Godon et al., 1997; Leclerc et al. 200l), etc.

EPS are metabolic products of bacteria that accumulate on the bacterial cell surface (Morgan et al., 1990). They form a protective layer for the cells against the harsh external environment, and also serve as carbon and energy reserves during starvation. EPS were found to be crucial to the flocculation of activated sludge (Frølund et al., 1996; Rudd et al., 1984) and to the microstructure of methanogenic granular sludge (Kosaric and Blaszczyk, 1990; Schmidt and Ahring, 1996). EPS are composed of a variety of substances, including carbohydrates, protein, humic substance, uronic acid, and DNA (Frølund et al., 1996).

In this study, the hydrogen-producing capacity of the granular sludge was first evaluated. The microbial community of the hydrogen-producing granular sludge (HPG) was then analyzed using the 16S rDNA-based method, and its EPS contents were characterized.

MATERIALS AND METHODS

Experimental Conditions

Hydrogen was produced in a 3-liter fermentor (Biostat B, B. Braun Biotech, Germany) using sucrose as the sole substrate in the synthetic wastewater. The fermentor was operated at 26°C and 6 h HRT for 90 days. The seed sludge was taken from a secondary sedimentation tank of a local wastewater treatment plant. The sucrose concentration in the synthetic wastewater increased gradually from the initial 2.00 g/L to 12.15 g/L by day 20 and kept at that level subsequently throughout the experiment; the corresponding loading rate was 48.6 g/(L · d). Each liter of the synthetic wastewater consisted of the following nutrients (mg): NH_4Cl 500, KH_2PO_4 250, K_2HPO_4 250, $MgSO_4 \cdot 7H_2O$ 320, $FeCl_3$ 30, $NiSO_4$ 32, $CaCl_2$ 50, $Na_2BO_7 \cdot H_2O$ 7, $(NH_4)_6Mo_7O_{24} \cdot H_2O$ 14, $ZnCl_2$ 6, $CoCl_2 \cdot 6H_2O$ 5, $CuCl_2 \cdot 2H_2O$ 4, and $MnCl_2 \cdot 4H_2O$ 5. The pH was kept at 5.5 automatically by two peristaltic pumps feeding NaOH (6 *M*) and HCl (4 *M*) solutions, respectively. The fermentor was mixed continuously at 200 rpm. A level probe and a harvest pump were used to keep the mixed liquor volume at a constant 1.7 L.

Biogas and Effluent Analyses

The amount of biogas produced was recorded daily using the water displacement method. The contents of hydrogen, carbon dioxide, methane, and nitrogen were analyzed by a gas chromatograph (GC) (Model 5890II, Hewlett Packard) 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°C, 180°C, and 50°C, respectively. Argon was the carrier gas at a flow rate of 30 mL/min. The concentrations of volatile fatty acids 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 (Yu and Fang, 2000). Contents of volatile suspended solids and ashes in the granular sludge were determined according to the Standard Methods (American Public Health Association, 1992)

Physical and Microscopic Characteristics of Granule

The hydrogen-producing sludge gradually agglutinated into granules that became visible by day 15. From that point, the average granule diameter was analyzed from the digital photographic images using the UTHSCSA Image Tool 2.00 software (Wilcox et al., 1999). The settling velocity was measured in a plexiglas column (inside diameter 200 mm). The granules were sampled on day 60 for microscopic examination when the reactor was operated under steady-state conditions at the loading rate of 48.6 g/(L · d). The microstructure of the granules was examined using scanning electron microscopy (SEM, Stereoscan 360, Cambridge, MA). The sample preparation procedures were as reported previously (Fang et al., 1994). The presence of endospores (Hippe et al., 1989) was examined using the conventional staining method (Schaeffer and Fulton, 1933).

16S rDNA-Based Microbial Analysis

To identify the microbial community of the HPG, DNA was firstly extracted, followed by polymerase chain reactions (PCR) amplification. After screening by denaturing gradient gel electrophoresis (DGGE), the PCR-amplified products were cloned, and the DNA of the major clones were sequenced for the phylogenetic analysis. Details of these procedures are below.

A 10-mL sludge sample was repeatedly frozen (−80°C) and thawed (37°C) four times. The sample was then centrifuged at 1800 g for 5 min. Pellets were resuspended in 1 mL of a pH 8 lysis buffer (0.75 *M* sucrose, 100 m*M* EDTA, 100 m*M* Tris), and homogenized in a Mini-Beadbeater™ (Biospec Products, Bartlesville, OK) with 0.2 g of 0.1 mm glass beads at 5000 rpm for 10 s. The homogenized sample was added with 4 mL of lysis buffer before the DNA was extracted using the method described previously (Chan et al., 2001).

The extracted DNA was amplified by PCR in a 30-μL buffer (Pharmacia Biotech Inc., Piscataway, NJ). The amplification was conducted in an automated thermal cycler (GeneAmp PCR 9700, Perkin–Elmer, Foster City, CA) using the following three-step program: (1)

initial denaturation (94°C for 7 min); (2) 25 or 35 cycles of denaturation (92°C for 1 min), annealing (54°C for 1 min), and extension (72°C for 1 min); and (3) a final extension (72°C for 10 min). In step (2), 35 cycles were applied for DGGE screening but only 25 cycles for cloning. All PCR-amplified products were stored at 4°C.

For DGGE screening, DNA fragments of 478 base pairs were amplified by PCR using the primer set of EUB968F (5′-AACGCGAAGAACCTTAC) plus GC-clamp (5′-CGCCCGGGGCGCGCCCCGGGCGGG-GCGGGGGCACGGGGGG) and UNIV1392R (5′-ACGGGCGGTGTGTRC) (Zhang and Fang, 2000). For cloning, DNA fragments of 1501 base pairs were amplified using the primer set of GM3F (5′-AGAGTTTGATCMTGGCTCAG) and GM4R (5′-GGTTACCTTGTTACGACTT). The PCR products were then cloned using the TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA) following manufacturer's instructions. A total of 52 clones selected for the plasmid recovery (Sambrook et al., 1989) were classified into 12 operational taxonomy units (OTUs) based on the DGGE profiles. The DNA of six major OTUs was then sequenced using an auto sequencer (ABI model 377A, Perkin–Elmer Ltd.) and dRhodamine Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin–Elmer Ltd.). The DNA sequences of the other six OTUs, each of which comprised only one clone, were not analyzed.

Each DNA sequence was checked using the CHECK-CHIMERA program (Maidak et al., 1997) to remove possible chimeric artifacts, if any, and compared with the reference microorganisms available in the GenBank by BLAST search (Altschul et al., 1990). The obtained DNA sequences and their closest 16S rDNA sequences of reference microorganisms retrieved from the GenBank were aligned with the Clustal W (Thompson et al., 1994) to construct phylogenetic trees using the neighbor-joining method (Saito and Nei, 1987) by MEGA 2.1 (Kumar et al., 1993). Bootstrap resampling analysis (Felsenstein, 1985) for 500 replicates was performed to estimate the confidence of tree topologies.

EPS extraction and Analysis

EPS were extracted using formaldehyde and NaOH from the day-60 granules following the procedures reported previously (Fang and Jia, 1996). To remove low molecular-weight metabolites, and substrate and formaldehyde residues, the extracted EPS solution was filtered first by a 0.2-μm membrane and then by a dialysis membrane (Pierce, Rockford, IL) with molecular weight cut-off of 3,500 Dalton. The extracted EPS were then lyophilized at −50°C for 48 h. The contents of carbohydrate, protein, humic substance, uronic acid, and DNA were analyzed.

The carbohydrate content in EPS was analyzed using the anthrone method (Gaudy, 1962) using glucose as standard. Protein and humic substance were analyzed following the modified Lowry method (Frølund et al., 1995) using bovine serum albumin and humic acid (Pract., Fluka, Switzerland) as respective standards. Uronic acid was analyzed by the *m*-hydroxydiphenyl sulfuric acid method (Blumenkrantz and Asboe-Hansen, 1973) modified by Kintner and Van Buren (1982) using glucuronic acid as standard. To ensure that there was no cell lysis during formaldehyde–NaOH extraction, the DNA contents in EPS and the sludge sample were analyzed following the diphenylamine colorimetric method (Sun et al., 1999) using *Escherichia coli* DNA as standard.

Accession Numbers

The nucleotide sequence data reported in this article have been submitted to the GenBank, EMBL, and DDBJ databases and assigned the following accession numbers: AY029589 (HPG-S-31), AY029590 (HPG-S-10), AY029591 (HPG-S-13), AY029592 (HPG-S-15), AY029593 (HPG-S-3), and AY029594 (HPG-S-1).

RESULTS

Sludge Granulation and Hydrogen Production Performance

Hydrogen production was detected starting at day 2. The seed sludge was originally dark in color due to the presence of sulfide produced by the sulfate-reducing bacteria in the digester. The dark sludge gradually whitened and became creamy white by day 6. This was due to the washout of the sulfate-reducing bacteria and the low sulfate concentration in the synthetic wastewater. As a result, production of hydrogen sulfide was ceased, and the residual sulfide precipitates in the seed sludge were gradually washed out from the reactor. The sludge soon began to agglutinate forming granules, which became visible by day 15. Figure 1 illustrates the increase of average granule diameter during the startup. It shows that the size of granules increased slowly in the initial stage but grew rapidly after day 20 and then leveled off after day 60. The size of mature hydrogen-

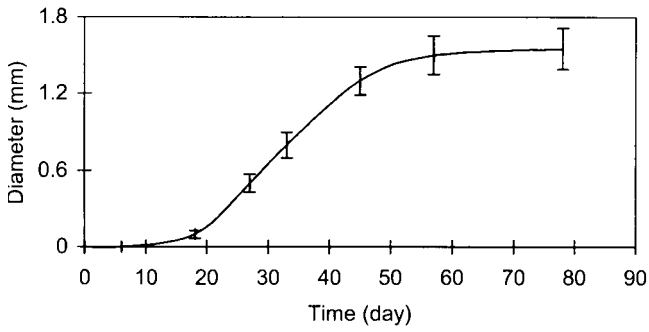

Figure 1. Average granular sizes during startup.

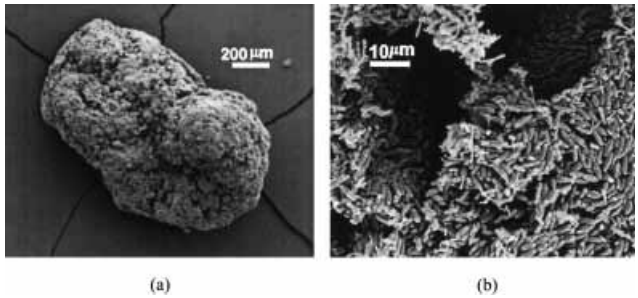

Figure 2. SEM images of (a) a typical HPG and (b) its porous structure.

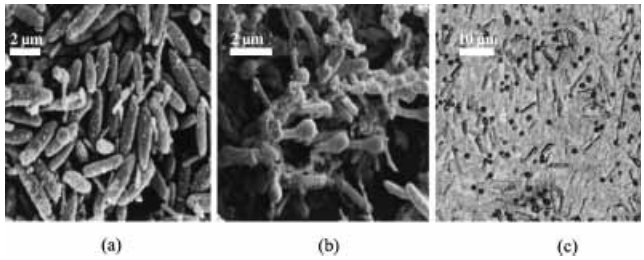

Figure 3. Micrographs of (a) fusiform bacilli, (b) spore-forming rod shape bacteria, and (c) stained spores.

producing granules varied from 0.9 mm to 2.5 mm, with 80% within the range of 1.3–1.9 mm.

During days 20–90, the reactor was operated at 26°C, pH 5.5, and 6 h of HRT and treated synthetic wastewater containing 12.15 g/L of sucrose, corresponding to a loading rate of 48.6 g/(L · d). The reactor reached steady state by day 60. Under the steady-state conditions, the reactor contained 20 g/L of the granular sludge degrading 97% of sucrose. The effluent was mainly composed of butyrate (49%) and acetate (46%), plus smaller quantities of methanol, ethanol, propionate, i-butyrate butanol, and undegraded, sucrose, whereas the biogas was composed of 63% of hydrogen, plus carbon dioxide (35%), and nitrogen (2%). The biogas was free of methane, indicating the complete suppression of methanogenic activity in the granular sludge. Under this condition, degrading each gram of sucrose produced 0.28 L of hydrogen. The specific hydrogen production rate was 0.7 [L/(g-VSS · d)], and the sludge yield was 0.16 g - VSS/g sucrose degraded.

Microscopic Examination of HPG

Figure 2(a and b) shows the SEM images of a typical HPG sampled on day 60. Figure 2a illustrates that the HPG had multiple cracks on the surface, and Figure 2b illustrates the highly porous inner structure of the granule. A close examination revealed that there were two predominant bacterial morphologies in the HPG: the fusiform bacilli (3–5 μm in length and 1–2 μm in width), as illustrated in Figure 3a, and the spore-forming, rod-shaped bacteria (4–6 μm in length and 0.4–0.6 μm in width), as illustrated in Figure 3(b and c). Figure 3c illustrates the presence of endospores, a characteristics of some hydrogen-producing bacteria (Hippe et al., 1989), as evidenced by the bright green image after staining.

Microbial Species in the Hydrogen-Producing Granule

Based on DGGE profiles, 12 OTUs were identified from the microbial community, of which six major OTUs comprised 46 clones (88.5%), and the remaining 6 OTUs comprised only one clone each. Table I lists the sequence length, number of clones, and relative abundance of each of the six major OTUs, plus the closest species found in the GenBank and the degree of similarity obtained using Blast analysis. Results in Table I show that all the sequenced OTUs were affiliated with the low G+C, G^+ bacteria. A total of 36 clones (69.1%) were affiliated with four *Clostridium* species in the family Clostridiaceae and seven clones (13.5%) with the species *Sporolactobacillus racemicus* in the *Bacillus*/*Staphylococcus* group. One OTU (HPG-S-10) comprising three clones (5.8%) had no close relative. It was remotely related to *Papillibacter cinnamivorans* (88% similarity) and an uncultured bacterium clone UASB_brew_B86 (91% similarity) obtained from the microbial community in

Table I. Phylogenetic affiliation of key OTUs.

Phylogenetic affiliation	OTU	Sequence length (base pairs)	Phylogenetic relationship		No. of clones	Abundance (%)
			Closest species in GenBank	Similarity		
Clostridiaceae	HPG-S-31	502	*Clostridium pasteurianum*	96	19	36.5
	HPG-S-3	493	*Clostridium tyrobutyricum*	98	10	19.2
	HPG-S-15	478	*Clostridium pasteurianum*	94	5	9.6
	HPG-S-13	460	*Clostridium acidisoli*	92	2	3.8
Bacillus/*Staphylococcus* group	HPG-S-1	472	*Sporolactobacillus racemicus*	98	7	13.5
None	HPG-S-10	337	*Papillibacter cinnamivorans*	88	3	5.8
Unchecked	Six	n/a	n/a	n/a	6	11.5
Totals					52	100

n/a: not applicable.

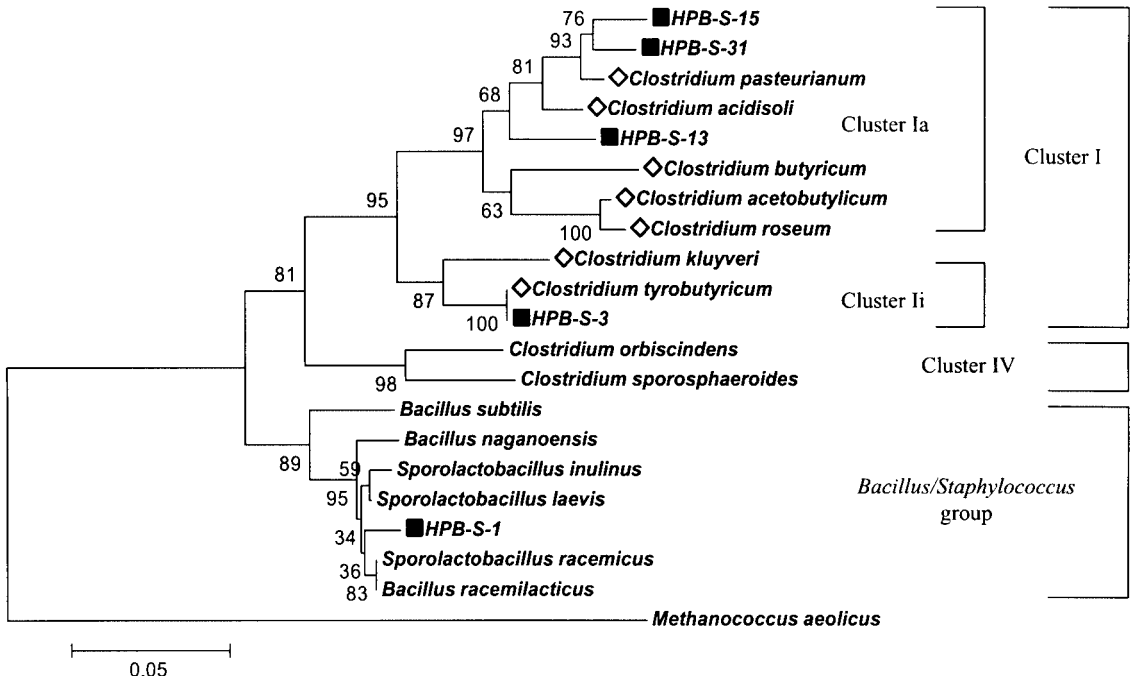

Figure 4. Phylogenetic tree of five key OTUs in hydrogen-producing sucrose-degrading sludge and their close relatives in low G+C Gram-positive bacteria based on 413 nucleotides in 16S rDNA sequence. The Clusters Ia, Ii and IV are the rDNA clusters in the genus Clostridium. The tree based on Jukes-Cantor distance was constructed using neighbor-joining algorithm with 500 bootstrappings. Methanococcus aeolicus was selected as the outgroup species. The scale bar represents 0.05 substitution per nucleotide position. Numbers at the nodes are the bootstrap values. ■, OTUs obtained in this study; ◇, known HPB species.

granular sludge-treating brewery wastewater (Liu et al., in press). The affiliations of the remaining six OTUs, comprising one clone each, were not identified.

Phylogenetic Tree

Microbial population (Schmidt and Ahring, 1996; Wiegant and de Man, 1986) and EPS (Schmidt and Ahring, 1996; Veiga et al., 1997) are likely to play important roles in the formation of granular sludge. Figure 4 illustrates the phylogenetic tree constructed for the five major OTUs and their close relatives using the neighbor-joining algorithm with 500 times of bootstrapping based on 413 nucleotides. Each sequence covered regions V6, V7, and V8 of 16S rDNA.

Contents of Extracted EPS

Each gram of hydrogen-producing granules (in volatile suspended solids) contained 179.1 mg EPS, which included 110.9 mg (61.9%) carbohydrates, 25.8 mg (14.4%) protein, 15.1 mg (8.4%) humic substance, 5.5 mg (3.1%) uronic acid, 0.15 mg (0.1%) DNA, and 21.7 mg (12.1%) of unidentified substances, possibly lipids and/or phenols (Schmidt and Ahring, 1996).

It has been a concern that extracting EPS under harsh conditions might rupture cell membranes, resulting in the release of intracellular polymeric substances. In this study, each gram of sludge contained 13.8 mg of DNA, of which only 1.1% was found in the extracted EPS. This confirms that the EPS extracted by formaldehyde–NaOH were not contaminated by the intracellular polymers.

DISCUSSION

Hydrogen Production

It was demonstrated in this study that hydrogen-producing sludge could develop into granules. The average HPG was 1.6 ± 0.2 mm in size, 1.038 g/mL in density, > 50 m/h in settling velocity, and 11 ± 1% in ash content. Table II shows that HPG in this study was com-

Table II. Physical characteristics of hydrogen-producing and methanogenic granules.

Sludge	Diameter (mm)	Density (g/mL)	Settling velocity (m/h)	Ash (%)	Reference
Hydrogen producing	1.6 ± 0.2	1.038	> 50	11 ± 1	This study
Methanogenic	1–2	n/a	6–60	10	Ross, 1984
Methanogenic	1.5	1.039	52.9	18	Hulshoff Pol, 1989
Methanogenic	1.4	n/a	4.3	19	Noyala and Moreno, 1994
Methanogenic	2.6	1.040	n/a	16	Yan and Tay, 1997

n/a: not applicable.

Table III. Hydrogen production characteristics.

Substrate	Loading rate [g/(L · d)]	VSS (g/L)	Hydrogen (%) Content	Hydrogen Yield (L/g)[a]	Hydrogen Volumetric production rate (L/L · d)	Reference
Sucrose	48.6	20.0	63	0.28	13.0	This study
Starch	6.0	0.7	61	1.37[b]	1.5	Lay, 2000
Glucose	28.0	n/a	12	n/a	1.2	Nakamura et al., 1993
Glucose	0.07	0.001	43	0.21	0.02	Lin and Chang, 1999
Glucose	27.0	1.5	53	0.11	2.9	Mizuno et al., 2000
Glucose	n/a	n/a	n/a	0.27	n/a	Kumar and Das, 2000
Glucose	30.0	n/a	n/a	0.29	n/a	Kataoka et al., 1997

n/a-not available.
[a]L/g-carbohydrates.
[b]Substantially higher than theoretical value for unknown reasons.

parable to methanogenic granular sludge in many physical characteristics, except the ash content. The low ash content in the HPG was likely due to the absence of sulfide precipitates. Table III summarizes the hydrogen production characteristics of the granular sludge developed in this study and the corresponding results in literature using carbohydrate as substrate. All experiments were conducted in continuous reactors under mixing condition, except one using a batch reactor (Kumar and Das, 2000). Five reactors used mixed culture sludge, and two used pure cultures, i.e. *Enterobacter cloacae* (Kumar and Das, 2000) and *Clostridium butyricum* (Kataoka et al., 1997).

Although the reactor in this study was mixed at 200 rpm continuously, the mixed liquor maintained higher concentrations of sludge than the effluent. This was due to the formation of granules of high settleability. During days 60–90, the VSS content in the mixed liquor averaged 20.0 ± 0.5 g/L, whereas that in the effluent averaged only 2 ± 0.5 mg/L. The average sludge yield was 0.16 g-VSS/g-sucrose. The biogas contained 63% hydrogen, which was higher than those reported by others. Degrading each gram of sucrose produced 0.28 L of hydrogen. Such a yield was higher than 0.21 L/g (Lin and Chang, 1999) and 0.11 L/g (Mizuno et al., 2000) for the degradation of glucose by mixed cultures but comparable to the yields reported for two pure cultures, i.e., 0.27 L/g for *E. cloacae* (Kumar and Das, 2000) and 0.29 L/g for *C. butyricum* (Kataoka et al., 1997).

Sucrose may be converted into either acetate or butyrate in the production of hydrogen. It produces eight moles of hydrogen when one mole of sucrose is converted to acetate, but only four moles of hydrogen when sucrose is converted to butyrate. Thus, the theoretical hydrogen yields for each gram of sucrose degraded are 0.57 L and 0.29 L for acetate and butyrate conversions, respectively. The hydrogen yield of 0.28 L/g found in this study and most of those listed in Table III were closer to the theoretical yield of 0.29 L/g for butyrate, which was accounted for 50% of organic products in the effluent. In a recent study of hydrogen production by starch acidification, Lay (2000) reported a hydrogen yield of 1.37 L/g by a mixed culture. Such a yield value was substantially higher than the theoretical yield even by assuming all the carbon in starch was converted to acetate. The reason for such an unusual yield was unclear.

The volumetric hydrogen production rate in this study was 13.0 L/(L · d), considerably higher than those reported by others, as a result of the high VSS content in the mixed liquor.

Microstructure of Hydrogen-Producing Granule

Previous studies have shown that methanogenic granules degrading carbohydrates were tightly packed with a smooth dense surface comprising a variety of fermentative acidogenic microorganisms (Fang et al., 1994, 1995b). The porous structure with multiple cracks on the surface of the HPG, as illustrated in Figure 2, is likely to facilitate the passage of nutrients and substrate as well as the release of hydrogen, which has a very limited solubility of 1.58 mg/L in water. Furthermore, the HPG in this study, unlike those of methanogenic UASB granules (Fang et al., 1994 and 1995a), did not exhibit a layered structure because of the simplicity of the acidification process.

Phylogenetic Analysis of the Microbial Species

Results in Table I and Figure 4 show that the two OTUs HPG-S-31 and HPG-S-15, comprising 46.2% of the clones, were most closely related to *Clostridium pasteurianum*. These two OTUs formed a group with *C. pasteurianum* with a node bootstrap value of 99% in the rDNA Cluster Ia of genus *Clostridium*. *C. pasteurianum* is a nonhomoacetogenic hydrogen-producing species present either singly or in pairs and excretes intracellular polysaccharide reserve (Hippe et al., 1989). Its growth might be stimulated by the fermentable carbohydrate, such as sucrose, producing acetate, butyrate, CO_2, and

Table IV. Comparison of EPS contents in hydrogen-producing and methanogenic granules.

Type of granule	Growth substrate	Total EPS (mg/g-VSS)	Protein / carbohydrates	Reference
Hydrogen producing	Sucrose	179	0.2	This study
Methanogenic	VFA	91	4.0	Veiga et al., 1997
Methanogenic	Hydrogen	37	1.6	Jia et al., 1996
Methanogenic	Glucose	23	2.4	Fang and Jia, 1996
Methanogenic	Paper wastewater	10	1.2	Morgan et al., 1990

H_2, as observed in this study. OTU HPG-S-13 comprising only two clones (3.8%) had a 92% similarity with the hydrogen-producing *Clostridium acidisoli*. It is likely a member of Cluster Ia of *Clostridium*, as illustrated in Figure 4.

The OTU HPG-S-3 comprising 10 clones (19.2%) was most closely related to *Clostridium tyrobutyricum* (98% similarity), another hydrogen-producing bacteria that uses only monosaccharides, not disaccharides like sucrose, as substrate. Thus, OTU HPG-S-3 probably used the hydrolyzed product of the sucrose as substrate. Figure 4 also shows that OTU HPG-S-3, *C. tyrobutyricum*, and *Clostridium kluyveri* formed a cluster with a node bootstrap value of 83% in the rDNA Cluster Ii of genus *Clostridium*.

OTU HPG-S-1 comprising seven clones (13.5%) was closely related (98% similarity) to *Sporolactobacillus racemicus* in the *Bacillus*/*Staphylococcus* group. This OTU likely belonged to the genus *Sporolactobacillus*, which are facultative anaerobes performing homolactic fermentation (Holt et al., 1994). It is unclear whether *Sporolactobacillus* produce hydrogen.

Because *Methanosaeta* was found abundant in most methanogenic granular sludges, many believed that the filamentous characteristics of *Methanosaeta* might be crucial to the formation of granules (Hulshoff Pol, 1989; Schmidt and Ahring, 1996). However, there was no filamentous bacteria found in the HPG based on microscopic observations. In an earlier study (Fang et al., in press) conducted at 37°C, pH 5.5, and 6 h HRT using glucose as sole substrate, it was found that the hydrogen-producing biomass remained in suspension without formation of granules. The suspended microbial community also comprised species in the rDNA Clusters IV, Ia, and Ii of *Clostridium*, same as those in the sucrose-degrading HPG of this study. However, the dominant populations differed in these two hydrogen-producing communities, probably because of the differences in temperature and substrate. It is unclear if there was any microbial species responsible for the formation of hydrogen-producing granules. Further study on this matter is warranted.

EPS in Hydrogen-Producing Granules

The agglutination of bacteria in natural ecosystems is related to their ability to produce EPS (Costerton et al., 1981). It is believed that EPS might also play an important role in the formation of methanogenic granular sludge (MacLeod et al., 1990). Figure 3b illustrates the presence of strands between adjacent cells. Although most of EPS had likely been removed by the supercritical CO_2 during sample preparation for SEM examination (Ravenscroft, 1991), these strands might represent residues of the dehydrated EPS.

Table IV summarizes the EPS contents in the HPG of this study and those reported for methanogenic granules for comparison. The total EPS content in methanogenic granules was substrate dependent, ranging 10–91 mg-EPS/g-VSS. This was substantially lower than the 179 mg-EPS/g-VSS for the HPG found in this study. Furthermore, the key EPS constituents were protein for methanogenic granules but carbohydrates for HPG.

CONCLUSIONS

Hydrogen-producing sludge agglutinated into granules in a well-mixed acidogenic reactor treating sucrose-containing synthetic wastewater at 26°C, pH 5.5, and 6 h hydraulic retention. At the volumetric loading rate of 48.6 g/(L · d), the reactor containing 20 g-VSS/L degraded over 97% of sucrose. It produced a methane-free biogas composed of 63% hydrogen. The volumetric production rate was 13.0 L-H_2/(L · d) and the yield was 0.28 L-H_2/g-sucrose. A total of 179.1 mg of EPS were extracted from each gram of granule. It contained 61.9% carbohydrate, 14.4% protein, 8.4% humic substance, 3.1% of uronic acid, and 0.15% DNA. Phylogenetic analysis of the microbial populations in the hydrogen-producing granules showed that 69.1% of the clones were affiliated with four *Clostridium* species in the family *Clostridiaceae*, and 13.5% with *S. racemicus* in the family *Streptococcaceae*.

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