Bio-hydrogen production from wastewater

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Abstract The technically feasibility of converting organic pollutants in wastewater into hydrogen by a continuous two-step process was demonstrated. Two carbohydrates, i.e. glucose and sucrose, in wastewater were respectively acidified by dark fermentation at pH 5.5 with 6–6.6 hours of hydraulic retention in a 3-l fermentor, producing an effluent containing mostly acetate and butyrate, and a methane-free biogas comprising mostly hydrogen. The acidified effluent was then further treated by photo fermentation for hydrogen production. The overall yield based on the substrate consumed was 31–32%, i.e. 17–18% for dark fermentation and 14% for photo fermentation. It was found that under certain dark fermentation conditions, hydrogen-producing sludge was agglutinated into granules, resulting in a higher biomass density and increased volumetric hydrogen production efficiency. DNA-based analysis of microbial communities revealed that the respective predominant bacteria were *Clostridium* in dark fermentation and *Rhodobacter* in photo fermentation. Further investigations are warranted, particularly, in areas such as improving reactor design, treating protein and lipid rich wastewaters, and studying sludge granulation mechanisms and controlling factors.

Keywords Bio-hydrogen; *Clostridium*; fermentation; phototrophic; *Rhodobacter*; wastewater

Introduction

Anaerobic process has grown into a mature technology for wastewater treatment in the last two decades (Fang and Liu, 2001). In this process, organic pollutants are degraded through a series of chain reactions, each being carrying out by individual group of anaerobic microorganisms. Complex organic pollutants are first hydrolyzed, and fermented into fatty acids. Fatty acids are then converted to acetate and hydrogen, both of which are lastly converted into methane. As compared to the popular aerobic process, anaerobic process has at least two intrinsic advantages: low sludge yield and energy saving. It has been successfully applied to the treatment of high-strength industrial wastewaters, as well as to the treatment of municipal wastewater in tropical and subtropical countries (Fang and Liu, 2001). However, the final product of such conventional anaerobic processes, methane, has little commercial value except being used as fuel.

The alternative is to suppress methane production in the chain anaerobic reactions, and recover hydrogen as a key product. Hydrogen is a high-value industrial commodity with a wide range of applications. It is used for the syntheses of ammonia, alcohols and aldehydes, as well as hydrogenation of edible oil, petroleum, coal and shale oil (Hart, 1997). In addition, it is an ideal fuel, producing only water upon combustion. Many believe that hydrogen will replace fossil fuel as the next generation of energy supply (Rocha *et al.*, 2001). It may be directly converted into electricity by fuel cells or used in internal combustion engines (Hart, 1997). A hydrogen-based economy will have no risk of global warming, and will significantly improve the urban air quality.

Traditionally, hydrogen is produced by hydrocarbon reformation and water electrolysis (Hart, 1997). However, it is theoretically possible to recover hydrogen from wastewater during anaerobic degradation. Such a technology combining wastewater treatment with hydrogen production 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.

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Bio-hydrogen may be produced by both autotrophs and heterotrophs. Autotrophic microalgae produce hydrogen from water using carbon dioxide and photo energy. Heterotrophs convert organic compounds into hydrogen by either dark fermentation or photo fermentation. Environmental engineers are only interested in heterotrophs, which are capable of converting organic pollutants in wastewater into hydrogen. Among the dark-fermentative bacteria, two genera have been studied extensively for their hydrogen producing characteristics, i.e. *Clostridium* and *Enterobacter*. The former includes *C. butyricum* (Karube *et al.*, 1982), *C. pasteurianum* (Heyndrickx *et al.*, 1991) and *C. beijerinckii* (Taguchi *et al.*, 1993), whereas the latter includes *E. aerogenes* (Tanisho *et al.*, 1989) and *E. cloacae* (Kumar and Das, 2000). Among the phototrophic hydrogen-producing bacteria, several purple non-sulfur freshwater bacteria have been extensively studied, including *Rhodobactor sphaeroides* (Asada and Miyake, 1999), *Rhodobacter capsulatus* (Tsygankov *et al.*, 1998), *Rhodospirillum rubrum* (Zürrer and Bachofen, 1982), and *Rhodopseudomonas palustris* (Fedorov *et al.*, 1999). Phototrophic hydrogen production were also reported for non-sulfur marine bacteria, including *Rhodovulum* sp. (Yamada *et al.*, 1998), *Rhodobacter marinus* (Yamada *et al.*, 1996) *Rhodovulum* sp. NKBG16047 (Matsunaga *et al.*, 2000).

In the bio-hydrogen production process, wastewater may be treated in two stages. Organic pollutants are first treated by dark fermentation to produce hydrogen and fatty acids; the acids are then further treated by photo fermentation for hydrogen production. The former reaction is mainly regulated by hydrogenase, whereas the latter by nitrogenase. Using glucose as the model organic pollutant, one mole of glucose can theoretically be converted into 12 moles of hydrogen. While the theoretical yields via dark-fermentation are only 4 moles of hydrogen with acetate as by-product. That means the maximum efficiency of producing hydrogen from glucose is only 33%, assuming acetate is the sole by-product. On the other hand, fatty acids, such as acetate and butyrate can be further converted to hydrogen via photo fermentation. However, these processes are thermodynamically unfavorable and thus require photo energy input.

A series of exploring experimental studies were conducted to demonstrate the feasibility of bio-hydrogen production from wastewater. Experimental conditions and results are summarized and discussed as follows.

Material and methods

Dark fermentation

Two dark fermentation experiments (Fang and Liu, 2002; Fang *et al.*, 2002a) were carried out in a 3 litre fermentor (Biostat B, B. Braun Biotech, Germany) using carbohydrates as substrate. In addition, each synthetic wastewater was composed of balanced nutrient. In the first experiment using glucose as substrate, fermentation was conducted at 36°C, pH 5.5 and 6.6 hours of hydraulic retention time (HRT). In the second experiment using sucrose as substrate, fermentation was conducted at 26°C, pH 5.5 and 6 hours of HRT. 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 litres.

Photo fermentation

Photo fermentation was conducted in a 450 ml complete-mix cylindrical photo-reactor (ACE Glass Incorporated, USA). The reactor was illuminated with a tungsten lamp at the center with a light intensity of 90–150 W/m^2 (370–1060 nm), as measured by a radiometer (IL 1400 Radiometer, International Light Inc, USA). A mixed phototrophic sludge enriched from the sediment of a local reservoir was used to seed the reactor. The wastewater was composed of acetate, butyrate and ethanol, plus balanced nutrient. The three

substrates and their concentrations were chosen to simulate the effluent of dark fermentation (Fang *et al.*, 2002a). Argon, instead of nitrogen, was used to purge the wastewater to ensure the anaerobic condition, because dissolved nitrogen would inhibit the hydrogen production of nitrogenase. The photo-reactor was operated at 32°C, pH 8.0, and 25 hours of HRT.

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, 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°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 (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 (Yu and Fang, 2000). Volatile suspended solids (VSS) were determined according to *Standard Methods* (APHA, 1992).

Phylogenetic analysis of hydrogen-producing sludges

Microbial communities of the dark and photo fermentation sludges were analyzed using the molecular techniques (Zhang and Fang, 2001; Fang *et al.*, 2002a, b). DNA in each sludge was extracted, and amplified by polymerase chain reactions (PCR) (Zhang and Fang, 2000). After screening by denaturing gradient gel electrophoresis, the PCR-amplified products were cloned, and then sequenced for phylogenetic analysis. Each DNA sequence, known as operational taxonomy unit (OTU), was edited with BioEdit (Hall, 1999) and checked for chimeric artifacts using the CHECK-CHIMERA program. All DNA sequences were compared for similarity with their close relative available in the GenBank by BLAST search. They were then aligned with the closest 16S rDNA sequences retrieved from the GenBank using BioEdit, followed by a manual check. Phylogenetic trees were constructed using the neighbor-joining method with MEGA 2.1 (Kumar *et al.*, 1993). Bootstrap resampling analysis for 500 replicates was performed to estimate the confidence of tree topologies.

Results and discussion

Dark fermentation

Both dark fermentation experiments were conducted at pH 5.5 and 6–6.6 hours of HRT. The pH was found as the optimal pH for hydrogen production (Fang and Liu, 2002). The optimal HRT for hydrogen production varied from 6 hours for degrading glucose (Lin and Chang, 1999), to 13.7 hours for sucrose (Liu and Fang 2001) and 17 hours for starch (Lay, 2000).

In the fermentor treating glucose at 36°C, steady state was reached after 30 days judging from composition and production rate of biogas, as well as the VFA and residual glucose concentrations in the effluent. During days 30–60, the fermentor degraded 99% of glucose, producing a methane-free biogas, comprising 64% hydrogen, 34% carbon dioxide and 2% nitrogen, and an effluent comprising mostly butyrate, acetate and ethanol (Fang *et al.*, 2002a). Degrading each gram of glucose produced 0.26 litre of hydrogen, which is comparable to the 0.25–0.29 litre values reported for the pure culture of Clostridium butyricum strain SC-E1 (Kataoka *et al.*, 1997). The specific hydrogen production rate was 4.6 l/(g-VSS·d), substantially higher than the 2.2 l/(g-VSS·d), reported for the conversion of starch at pH 5.2 at 17 hours of HRT (Lay, 2000).

In the second fermentor treating sucrose at 26°C, steady state was reached after 60 days. Under the steady state condition, the reactor degrading 97% of sucrose, producing an effluent composed mainly of butyrate and acetate, and a methane-free biogas comprising of 63% of hydrogen, plus carbon dioxide (35%) and nitrogen (2%). Under this condition, degrading each gram of sucrose produced 0.28 litre 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. In addition, it was interesting to note that the sludge in the fermentor developed into granules of an average size of 1.6 mm. The granules had a high density of biomass, and exhibited a satisfactory settling velocity of over 50 m/h. As a result, the reactor of granular sludge had a much higher volumetric hydrogen production than others. Further studies on the mechanism of sludge granulation and the controlling factors are warranted.

Table 1 summarizes the hydrogen production yields of the two hydrogen-producing dark fermentation sludges with those in literature, including those of batch processes, for comparison. Yields were defined as percentages of actual hydrogen production as compared to the theoretical values based on the amount of degraded substrates. Results show that the hydrogen yields of 17 and 18% found in the two continuous dark fermentation by mixed cultures were higher than most of previous studies, but slightly lower than the 21% reported by Ueno *et al.* (1996).

Nearly all the studies of hydrogen production by dark fermentation were conducted using carbohydrates as substrates. One study conducted by Cheng *et al.* (1999) used peptone as substrate found the hydrogen yield was only 0.015 l/g-peptone, substantially lower than those using carbohydrates.

Photo fermentation

Photo fermentation of a synthetic acidified wastewater into hydrogen reached steady state after 20 days. It degraded 85% of acetate, 89% butyrate and 96% ethanol in wastewater, producing a methane-free biogas, comprising 82% hydrogen, 13% carbon dioxide, 4.5% nitrogen, and 0.5% hydrogen sulfide. The hydrogen production rate was 135 l/(g-VSS·d), substantially lower than the rates in dark fermentation. Table 2 summarizes the hydrogen production yields of the mixed phototrophic sludge, and those of pure cultures reported in

Table 1 Hydrogen yields of dark fermentative culture

Microorganism	Substrate	Yield (%)	Reference
Continuous reactor			
Mixed culture	glucose	17	Fang and Liu, 2002
Mixed culture	glucose	14	Lin and Chang, 1999
Mixed culture	glucose	10	Nakamura *et al.*, 1993
Mixed culture	glucose	7	Mizuno *et al.*, 2000
Mixed culture	sucrose	18	Fang *et al.*, 2002a
Mixed culture	sugar wastewater	21	Ueno *et al.*, 1996
Clostridium butyricum	glucose	11–19	Kataoka *et al.*, 1997
Clostridium butyricum	alcohol wastewater	17	Suzuki *et al.*, 1978
Enterobacter aerogenes	glucose	9	Rachman *et al.*, 1998
Batch reactor			
Mixed culture	sucrose	3	Ginkel *et al.*, 2001
Mixed culture	cellulose	18	Ueno *et al.*, 1995
Mixed culture	cellulose	3	Lay, 2001
Clostridium beijerinckii	glucose	17	Taguchi *et al.*, 1992
Clostridium beijerinckii	starch	14	Taguchi *et al.*, 1992
Clostridium pasteuriannum	glucose	13	Brosseau and Zajic, 1982
Clostridium sp., strain No. 2	xylose	17	Taguchi *et al.*, 1995
Clostridium sp., strain No. 2	glucose	19	Taguchi *et al.*, 1995
Enterobacter cloacae	glucose	18	Kumar and Das, 2000

Table 2 Hydrogen yields of phototrophic sludge and bacteria

Microorganism	Substrate	Yield (%)	Reference
Continuous reactor			
Mixed culture (81% *Rhodobacter*)	acetate, butyrate & ethanol	14	Zhang *et al.*, 2002
Rhodobacter sphaeroides RV	lactate	7	Fascetti and Todini, 1995
Rhodobacter sphaeroides O.U.001	malate	12	Eroglu *et al.*, 1999
Rhodobacter capsulatus	lactate	28	Tsygankov *et al.*, 1998
Rhodospirillum rubrum	lactate	80	Zürrer and Bachofen, 1982
Rhodopseudomonas palustris	acetate, propionate, butyrate & ethanol	—	Otsuki *et al.*,1998
Batch reactor			
Rhodobacter sphaeroides O.U.001	malate	7	Sasikala *et al.*, 1991a
Rhodobacter sphaeroides O.U.001	lactate	75	Sasikala *et al.*, 1991b
Rhodobacter sphaeroides RV	lactate	56	El-Shishtawy *et al.*, 1998a
Rhodobacter capsulatus ST410	malate	73	Ooshima *et al.*, 1998
Rhodobacter sphaeroides RV	tofu wastewater	16	Zhu *et al.*, 1999
Rhodobacter sphaeroides O.U. 001	sugar refinery	—	Yetis *et al.*, 2000
Rhodobacter sp M-19	starch wastewater	60	Yokoi *et al.*, 2002
Rhodopseudomonas sp.	acetate	73	Barbosa *et al.*, 2001
Rhodovulum sp NKPB 160471	malate	—	Yamada *et al.*, 1998

literature for comparison. Results show that the hydrogen yield found in this preliminary study was 14%, which were lower than most of the reported yields of pure cultures but still higher than some others. Since there was no prior study of using mixed culture for phototrophic hydrogen production from wastewater, comparison with yields of pure culture could be misleading. Table 2 also shows that most of pure culture studies used lactate as substrate. The hydrogen conversion efficiency from lactate could be as high as 75% by Rhodobacter sphaeroides O.U.001 (Sasikala *et al.*, 1991b), and 80% Rhodospirillum rubrum (Zürrer and Bachofen, 1982). Beside these two bacteria, other pure cultures having been studied included Rhodospirillum, Rhodopseudomonas, and Rhodovulum. Of all pure cultures, Rhodobacter was the mostly studied for phototrophic hydrogen production.

Phylogenetic analysis

Figure 1 illustrates the phylogenetic tree constructed for the major populations in the three hydrogen-producing sludges and their respective close relatives, using the neighbor joining algorithm with 500 times of bootstrapping. For the glucose-degrading sludge, results show that most of the 16S rDNA clones were affiliated with two families in *Bacillus/Clostridium* group (low G+C gram positive bacteria), including 64.6% in Clostridiaceae and 3.1% in Streptococcaceae. The three predominant OTUs, HPB-G1-5, HPB-G1-11 and HPB-G1-15, representing 43.8% of the clones, were most closely related to *Clostridium cellulosi* and assigned into *Clostridium* Cluster IV. The HPB-G1-6 (12.5%) fell into the *Clostridium* Cluster Ia which contains a few hydrogen-producing bacteria, such as *C. acetobutylicum*, *C. butyricum* and *C. botulinum*. The HPB-G1-8 (8.3%) was most closely related to another hydrogen-producing bacterium, *C. tyrobutyricum* in the *Clostridium* Cluster Ii. Meanwhile, HPB-G1-4 (18.8%) was closely affiliated with species of *Klebsiella*, *Enterobacter* and *Citrobacter*, and could be assigned into the family Enterobacteriaceae.

For the sucrose-degrading sludge, 69.1% of populations were affiliated with *Clostridium*. The predominant HPG-S-31 and HPG-S-15, comprising 46.2% of the clones,

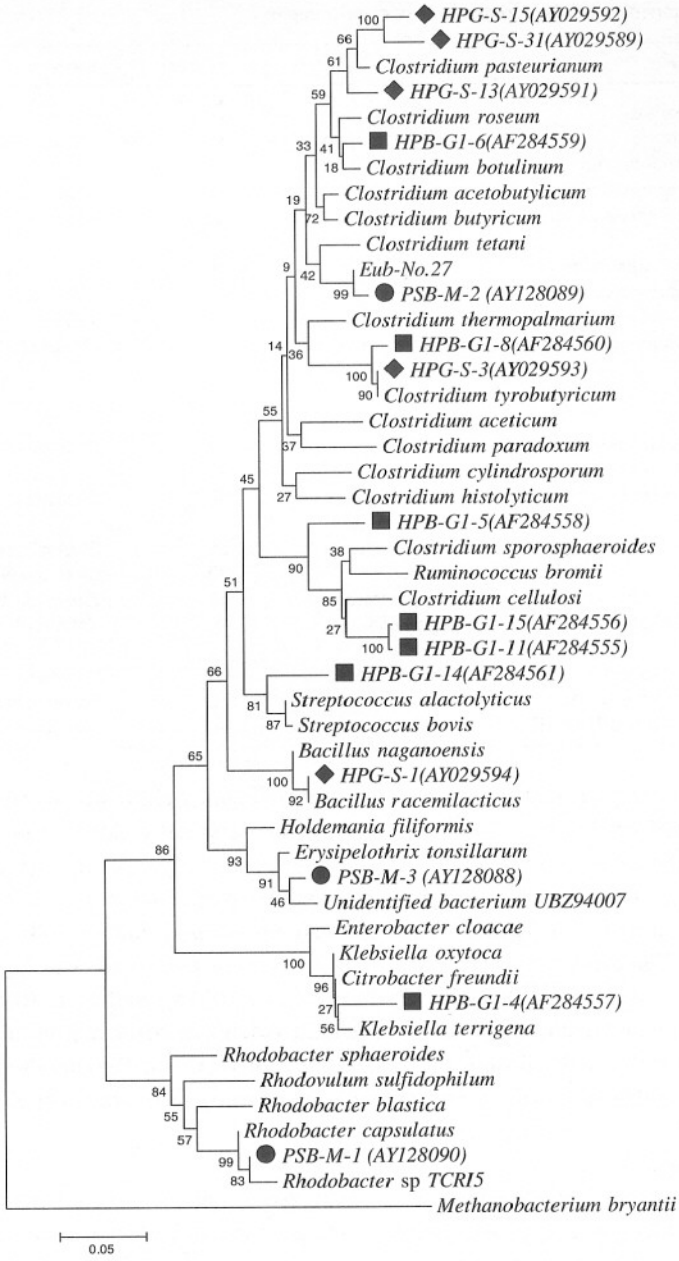

Figure 1 Phylogenetic tree of the major populations (accession numbers in parenthesis) in the three hydrogen-producing sludges based on partial 16S rDNA sequences. *Methanobacterium bryantii* was selected as the out-group species. The scale bar represents 0.05 substitution per nucleotide position. Numbers at the nodes are the bootstrap values. ◆ OTUs of glucose-degrading hydrogen-producing sludge; ■ OTUs of sucrose-degrading hydrogen-producing sludge; ● OTUs of phototrophic hydrogen-producing sludge.

were most closely related to *C. pasteurianum* and could be assigned into the *Clostridium* Cluster Ia together with HPG-S-13 (3.8%). The HPG-S-3 (19.2%) was most closely related to *C. tyrobutyricum* in the *Clostridium* Cluster Ii. HPG-S-1 (13.5%) was closely related to *Sporolactobacillus racemicus* in the Sporolactobacillaceae family.

For the phototrophic sludge treating acidified wastewater, the *R. capsulatus* resembling PSB-M-1 was predominant (81.3% of the clones). Two other minor population were

members of the *Bacillus/Clostridium* group. PSB-M-2 (12.5%), which was closely related to an uncultured bacterium Eub No.27 (99.2%), was likely a member of the *Clostridium* genus. PSB-M-3 (6.3%) was most closely related to an unidentified bacterium (Accession number: Z94007) isolated from activated sludge with 91.7% similarity and *Erysipelothrix tonsillarum* (91.6%).

Future developments

Although the technical feasibility of production of bio-hydrogen from wastewater has been demonstrated, the technology of bio-hydrogen production from wastewater is still at its infancy stage. Considerable developments are needed in order to apply this technology to full scale operations. Further studies in the following areas are warranted: (a) dark fermentation of substrates other than carbohydrates, such as proteins and lipids, (b) improvement of reactor design to increase sludge retention in fermentors, (c) improvement of photo efficiency for photo reactors, (d) improvement of process resistance against nitrogen and ammonium, etc.

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