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Phototrophic hydrogen production from acetate and butyrate in wastewater

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

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

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Abstract

Phototrophic hydrogen production was conducted using individual substrates, acetate and butyrate, which are the main products of dark fermentation. Effects of initial pH (ranging 5.0–10.0) and individual substrate concentrations (acetate ranging from 800 to 4100 mg/l, and butyrate ranging from 1000 to 5100 mg/l) on phototrophic hydrogen production were evaluated. The maximum hydrogen yields were 2.5 mol-H_2/mol-acetate at an initial pH of 8.0 treating 800 mg/l of acetate, 3.7 mol-H_2/mol-butyrate at an initial pH of 9.0 treating 1000 mg/l of butyrate. Analyses of DGGE (denaturing gradient gel electrophoresis) profiles of 16S rDNA fragments and FISH (fluorescent in situ hybridization) images show that both phototrophic hydrogen-producing sludges comprised only one predominant species resembling *Rhodobacter capsulatus* with over 80% relative abundance.

Keywords: Acetate; Butyrate; Hydrogen; Phototrophic; *Rhodobacter capsulatus*

1. Introduction

Hydrogen is an environmentally friendly fuel; it produces only water upon combustion. Many believe that hydrogen will replace fossil fuel as the energy source of the next generation [1]. Hydrogen is conventionally produced by chemical or electrolytical means. But, it may also be produced biologically by either autotrophic or heterotrophic microorganisms. Autotrophs, such as green algae and cyanobacteria, produce hydrogen by splitting water using light and carbon dioxide, respectively, as energy and carbon sources [2]. Heterotrophs, such as dark-fermentative bacteria and phototrophic bacteria, produce hydrogen by fermentation [3].

It is thus possible to harvest hydrogen from wastewater in a two-step process using these two distinct groups of heterotroph: dark-fermentative bacteria first convert complex organic substrates in wastewater into hydrogen and fatty acids, while the phototrophic bacteria further converted fatty acids into hydrogen and carbon dioxide.

Most studies of biohydrogen production so far were conducted using pure cultures. In these studies, hydrogen was produced by pure cultures from simple acids, such as acetate [4], butyrate [5], malate [6–8] and lactate [9–11], as well as several wastewaters, including those from productions of starch [12], sugar [13], and tofu [14].

However, it is impractical to treat a wastewater using pure cultures. Yet there is very limited information available on the biohydrogen production from wastewater using mixed acidogenic cultures [15,16], and none available on using mixed phototrophic cultures. This study was thus conducted to investigate the feasibility of hydrogen production from acetate and butyrate, the two key products

* Corresponding author. Tel.: +852 2859 2668; fax: +852 2559 5337.
E-mail address: hrechef@hkucc.hku.hk (H.H.P. Fang).

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of dark fermentation in biohydrogen production [15], by a mixed phototrophic culture. The effects of initial pH, initial acetate and butyrate concentrations on hydrogen production were investigated in batch experiments. The microbial community of the phototrophic sludge was also analyzed using the 16S rDNA-based techniques and the FISH (fluorescent in situ hybridization) method.

2. Materials and methods

2.1. Seed sludge

A mixed phototrophic sludge was first enriched from the sediment of a local reservoir in a batch reactor using a common phototrophic Medium 27 (DSMZ, Germany) containing (in 1 l): 0.30 g yeast extract, 0.50 ml ethanol, 1.00 g Na_2-succinate, 0.50 g (NH_4)-acetate, 5.00 ml Fe(III) citrate solution (0.1% in H_2O), 0.50 g KH_2PO_4, 0.40 g $MgSO_4 \cdot 7H_2O$, 0.40 g NaCl, 0.40 g NH_4Cl, 0.05 g $CaCl_2 \cdot 2H_2O$, 0.40 ml Vitamin B_{12} solution (10 mg in 100 ml H_2O), and 1.00 ml trace element solution. The sludge was enriched at 30 °C under anaerobic conditions using tungsten lamps as a light source at a light intensity of 200 W/m^2 (370–1060 nm). After eight batches of enrichment, the sludge was used to seed the batch reactors.

2.2. Operational conditions

Four series of batch experiments were conducted at 32 °C in 120 ml serum vials. Each batch experiment was conducted in duplicate. In series 1 and 2, the effects of initial pH (ranging 5.0–10.0 with step increments of 1.0) on hydrogen production from acetate (800 mg/l) and butyrate (1000 mg/l) were individually investigated. In series 3 and 4, the individual concentration effects of acetate (800–4000 mg/l) and butyrate (1000–5100 mg/l) on hydrogen production were investigated at respective initial pH of 7.0 and 8.0.

In the batch experiments, the hydrogen-producing medium contained the following nutrients (in mg/l): KH_2PO_4 500; $MgSO_4 \cdot 7H_2O$ 400; NaCl 400; $CaCl_2 \cdot 2H_2O$ 50; Fe(III) citrate 3.9; H_3BO_3 0.3; $Na_2MoO_4 \cdot 2H_2O$ 0.03; $ZnSO_4 \cdot 7H_2O$ 0.1; $CoCl_2 \cdot 6H_2O$ 0.2; $CuCl_2 \cdot 2H_2O$ 0.01; $MnCl_2 \cdot 4H_2O$ 0.03; $NiCl_2 \cdot 6H_2O$ 0.02; Vitamin B_{12} 0.04. The initial culture volume was kept at 100 ml. In all batches, the initial sludge concentration was kept at 400 mg/l of biomass, as measured by VSS. Argon was used to purge each tube to ensure anaerobic conditions. The tubes were submerged in a water-shaking bath illuminated with tungsten lamps at an average intensity of 200 W/m^2 (370–1060 nm).

2.3. Analyses of biogas, effluent and microbial population

The amount of biogas produced in each serum vial was measured using a glass syringe. The compositions of biogas and the effluent of the fermentation were analyzed following procedures reported previously [17]. The light intensity was measured by a radiometer (IL 1400 Radiometer, International Light Inc, USA).

In order to compare the microbial populations, genomic DNA were extracted from three phototrophic sludge samples, of which two were from the batch reactors of this study and the third one serving as a reference was obtained from a previous phototrophic hydrogen-producing study using a mixed substrate comprising acetate, butyrate and ethanol [18]. All extracted DNA were amplified with PCR primer set of *Eubacteria* domain-specific primer 968F-GC and universal primer 1392R using the GenAmp® PCR system 9700 (Perkin Elmer Ltd., Foster City, USA) [19]. The PCR amplified DNA fragments were analyzed by denaturing gradient gel electrophoresis (DGGE) following the previously established method using a denaturant gradient of 40–60% [19,20].

Microbial populations of the two sludges in this study were further compared using the method of fluorescence in situ hybridization (FISH). A Cy3-labeled EUB338 probe targeting most of the *Eubacteria* and an FAM-labeled Alf968 targeting most of the alpha-*Proteobacteria* were used [18,21]. The formamide concentration in the buffer was 20%. The hybridized images were obtained using a laser scanning confocal microscope (LSM 5 Pascal, Zeiss, Jena, Germany). The relative populations of *Eubacteria* and alpha-*Proteobacteria* were estimated from the respective fluorescent areas using the image analysis software MetaView (Universal Imaging).

2.4. Kinetic modeling

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

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

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 were determined by best fitting the hydrogen production data for Eq. (1) using the Matlab 6.0 with Optimization Toolbox 2.1 [23]. 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) was calculated by dividing P by the quantity of substrate (acetate or butyrate) in wastewater.

3. Results and discussions

3.1. Effect of initial pH

3.1.1. Acetate as substrate

Fig. 1 illustrates the effect of initial pH on (a) acetate degradation and (b) hydrogen production by phototrophic bacteria at an initial acetate concentration of 800 mg/l. Fig. 1a illustrates that the remaining acetate concentration after 280 h for the batch conducted at initial pH 5.0 was 610 mg/l, corresponding to only 23.8% of acetate degradation. The degradation efficiency rapidly increased to 94.0–99.6% for batches conducted at initial pH ranging from 6.0 to 8.0, and then decreased to 58.5% at pH 9.0 and 35.8% at pH 10.0. Fig. 1b illustrates the cumulative hydrogen production from acetate at various initial pH levels and the corresponding best-fit curves using Eq. (1). It illustrates that hydrogen was accumulated more for batches conducted at pH ranging from 6.0 to 8.0 than those at other pH levels.

Table 1 summarizes the final pH and the three kinetic parameters in Eq. (1) for hydrogen production at various initial pH levels. Results show that the final pH for those batches conducted at the initial pH ranging from 6.0 to 8.0 was in the range of 6.8–8.2. For those conducted at the initial pH of 5.0, 9.0 and 10.0, the final pH was 5.3, 10.0 and 10.2, respectively, resulting in the lowering of hydrogen production and substrate degradation due to the inhibition of nitrogenase and other bacterial activities [24,25].

Table 1 also shows that the lag time was affected by the initial pH. At pH 5.0 and 6.0, the lag times were 74 and 41 h, respectively, much longer than the 19–28 h for those at the pH range of 7.0–10.0.

Based on the maximum production rate R_m and the hydrogen production potential P in Table 1, the maximum specific hydrogen production rate (ml/(g-VSS·d)) and hydrogen yield (mol-H_2/mol-acetate) were, respectively, calculated, as listed in Table 1. The maximum specific hydrogen production rate was sensitive to the initial pH. It increased from 18 ml/(g-VSS·d) at pH 5.0 to 474 ml/(g-VSS·d) at pH 7.0, and then decreased to 114 ml/(g-VSS·d) at pH 10.0. The hydrogen yield increased from 1.9 mol-H_2/mol-acetate at pH 6.0 to 2.5 mol-H_2/mol-acetate at pH 8.0 in spite of the fact that only 5.6% of acetate degradation efficiency increased. For the remaining pH of 5.0, 9.0 and 10.0, only a trace amount of hydrogen (0.2–0.5 mol-H_2/mol-acetate) was produced.

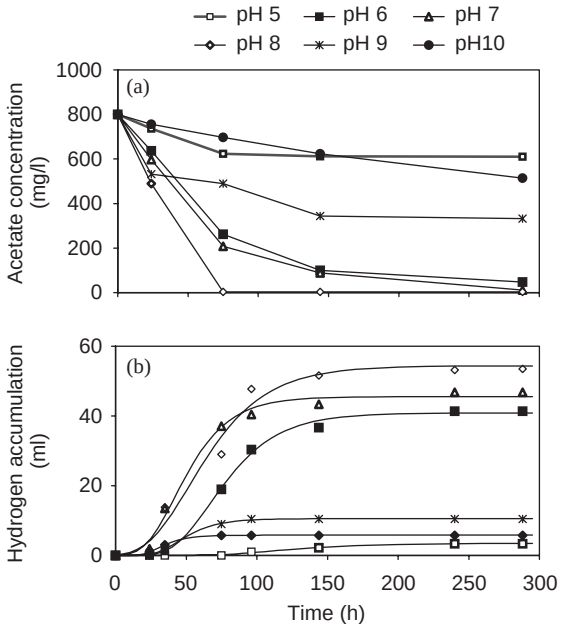

Fig. 1. Acetate degradation (a), and hydrogen accumulation (b) at various initial pH levels.

According to the stoichiometry of the following reaction, each mole of acetate can produce 4 moles of hydrogen:

$$CH_3COOH + 2H_2O \rightarrow 4H_2 + 2CO_2. \quad (2)$$

Thus, the maximum hydrogen conversion efficiency conducted at the initial pH 8.0 in this study was 62.5%. Such an efficiency was lower than the 72.8% by *Rhodopseudomonas* sp. [4], but much higher than the 7.6% by *R. sphaeroides* [5].

Overall, the favorable initial pH for hydrogen production from acetate by phototrophic bacteria was found in the

Table 1
Kinetic parameters and final pH for hydrogen production from 800 mg/l of acetate at various initial pH levels

Initial pH	Final pH	λ (h)	R_m (ml/h)	P (ml)	Specific H_2 production rate (ml/(g-VSS-d))	Hydrogen yield (mol/mol-acetate)
5.0	5.3	74	0.03	4	18	0.2
6.0	6.8	41	0.57	41	342	1.9
7.0	7.3	21	0.79	46	474	2.1
8.0	8.2	22	0.67	55	402	2.5
9.0	10.0	28	0.25	11	150	0.5
10.0	10.2	19	0.19	6	114	0.3

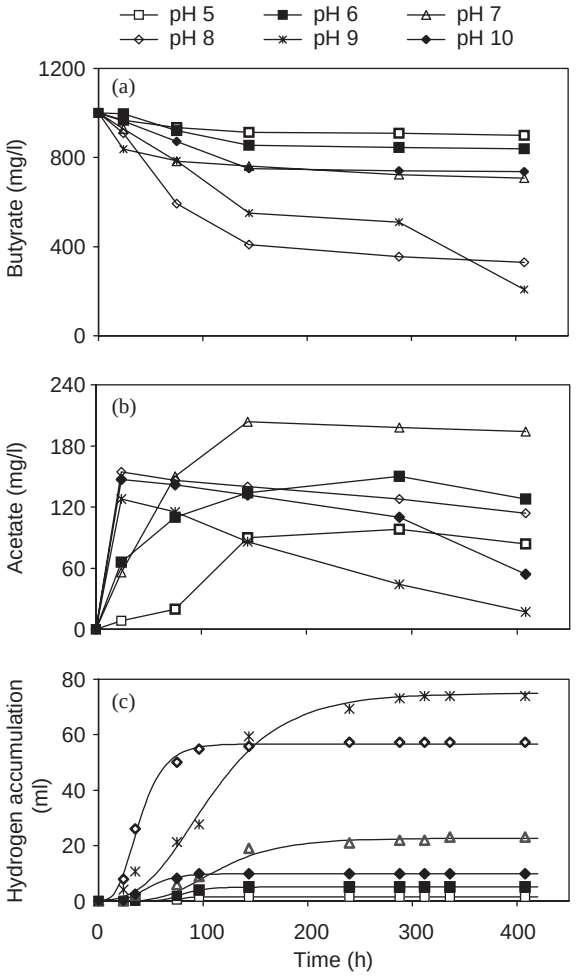

Fig. 2. Butyrate degradation (a), acetate accumulation (b), and hydrogen accumulation (c) at various initial pH levels.

range of 7.0–8.0, depending on hydrogen production rate or yield. For comparison, the optimal ranges for pure culture of *R. capsulatus* were, respectively, reported as pH 6.5–7.5 [24] and pH 8.5–9.0 [25].

3.1.2. Butyrate as substrate

Butyrate conversion to hydrogen produced acetate as a by-product, as shown in the following:

$$CH_3(CH_2)_2COOH + 2H_2O \rightarrow 2H_2 + 2CH_3COOH. \quad (3)$$

Fig. 2 illustrates the effects of initial pH on (a) butyrate degradation, (b) acetate accumulation, and (c) hydrogen production by phototrophic bacteria for batches degrading butyrate with an initial concentration of 1000 mg/l. Fig. 2a illustrates that for batches conducted at initial pH ranging from 5.0 to 7.0, the remaining butyrate concentration was 900–780 mg/l after 408 h, corresponding to 10–22% of butyrate degradation. The butyrate degradation efficiency increased to 70% and 79% for the batches operated at initial pH of 8.0 and 9.0, respectively, and decreased to 26% at pH 10.0.

Degradation of butyrate produced acetate, as illustrated in Fig. 2b. For the initial pH ranging from 5.0 to 7.0, the acetate concentration gradually increased during the initial 150 h, and then leveled off. However, for those conducted at initial pH ranging from 8.0 to 10.0, the acetate concentration reached maximum values during the first 25 h and then decreased.

The accumulation and degradation of acetate confirmed that hydrogen production from butyrate was a two-step process, producing acetate as an intermediate product. Fig. 2c illustrates the cumulative hydrogen production at various initial pH and the corresponding best-fit curves using Eq. (1). It shows that more hydrogen was accumulated at pH 8.0 and 9.0 than at other pH levels.

Table 2 summarizes the final pH and three kinetic parameters in Eq. (1) for pH ranging from 5.0 to 10.0. It shows that the final pH values in all batches were lower than the initial pH, except the one at initial pH 10.0. Such pH changes may be responsible for the incomplete degradation of butyrate for batches conducted at pH ranging from 5.0 to 7.0 and 10.0. Table 2 also shows that both maximum specific hydrogen production rate and yield were greatly affected by the initial pH. The maximum specific hydrogen production rate ranged from 42 to 768 ml/(g-VSS·d), the highest rate being at pH 8.0. The hydrogen yield from butyrate increased with pH from 0.1 mol/mol for the batch conducted at

Table 2
Kinetic parameters and final pH for hydrogen production from 1000 mg/l of butyrate at various initial pH levels

Initial pH	Final pH	λ (h)	R_m (ml/h)	P (ml)	Specific H_2 production rate (ml/(g-VSS·d))	Hydrogen yield (mol/mol-butyrate)
5.0	4.9	69	0.07	2	42	0.1
6.0	5.8	60	0.12	5	72	0.2
7.0	6.5	48	0.21	23	126	1.1
8.0	7.6	17	1.28	57	768	2.8
9.0	8.7	39	0.53	75	318	3.7
10.0	10.6	25	0.22	10	132	0.5

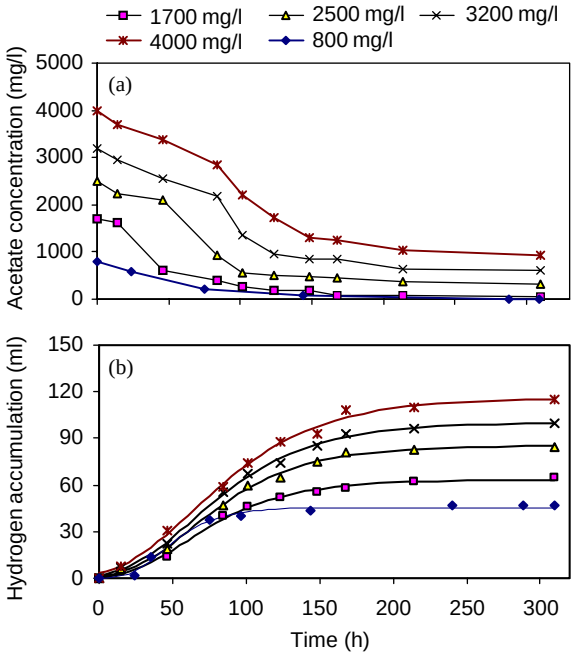

Fig. 3. Initial acetate degradation (a), and hydrogen accumulation (b) at various acetate concentrations.

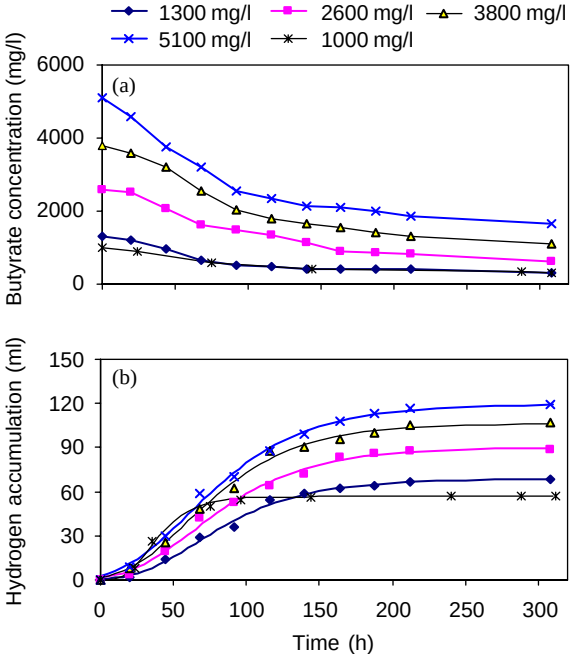

Fig. 4. Effects of butyrate concentrations on (a) butyrate degradation and (b) hydrogen accumulation.

pH 5.0 to 3.7 mol/mol for the one at pH 9.0, and then drastically decreased to 0.5 mol/mol at pH 10.0.

Based on reactions (2) and (3), each mole of butyrate can produce a maximum of 10 moles of hydrogen. Thus the maximum hydrogen conversion efficiency at pH 9.0 was 37.0%. Such a value was lower than the 62.5% of maximum conversion efficiency of acetate by the same sludge.

Overall, the pH favorable for hydrogen production from butyrate by phototrophic bacteria was in the range of 8.0–9.0.

3.2. Effects of substrate concentration

3.2.1. Acetate as substrate

Fig. 3 illustrates (a) acetate degradation and (b) hydrogen accumulation for batches conducted at initial pH 7.0 using acetate as a sole substrate at various concentrations (800–4000 mg/l). Fig. 3a illustrates that acetate was degraded rapidly in the initial 100 h and stopped after 300 h for all batches. The acetate degradation efficiency decreased with the increase of initial concentration. By 214 h, 99.4–96.3% of acetate was degraded for the initial concentrations ranging from 800 to 1700 mg/l but only 76.5% for 4000 mg/l.

Fig. 3b illustrates the cumulative hydrogen production from acetate at various initial concentrations and the corresponding best-fit curves using Eq. (1). It illustrates that the hydrogen production increased with the acetate concentrations from 800 to 4000 mg/l. A similar trend was also observed for hydrogen production from acetate (492–1804 mg/l) by *Rhodobacter sphaeroides* [4]. However, Kim et al. [26] reported that hydrogen production was

Table 3
Kinetic parameters and final pH for hydrogen production from acetate at various initial concentrations

Concentration (g/l)	λ (h)	R_m (ml/h)	P (ml)	Specific H_2 production rate (ml/(g-VSS.d))	Hydrogen yield (mol/mol-acetate)	Final pH
0.8	21	0.79	46	474	2.1	7.3
1.7	20	0.60	63	360	1.4	7.5
2.5	19	0.75	85	450	1.3	7.9
3.2	18	0.82	100	492	1.2	8.1
4.0	14	0.88	116	528	1.1	8.3

Table 4
Kinetic parameters and final pH for hydrogen production from butyrate at various initial concentrations

Concentration (g/l)	λ (h)	R_m (ml/h)	P (ml)	Specific H_2 production rate (ml/(g-VSS.d))	Hydrogen yield (mol/mol-butyrate)	Final pH
1.0	17	1.28	57	768	2.8	7.6
1.3	25	0.62	68	372	2.9	7.6
2.6	19	0.75	90	450	1.9	7.4
3.8	17	0.94	106	564	1.6	7.3
5.1	14	0.97	120	582	1.3	7.2

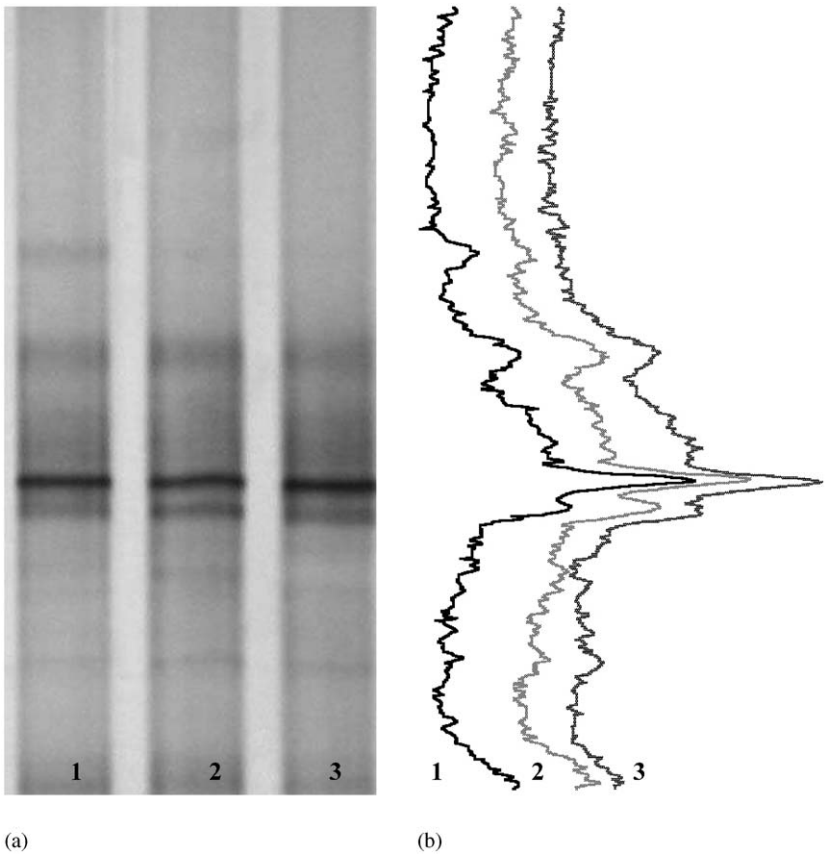

Fig. 5. The DGGE profiles with a denaturant gradient from 40% to 60%: (a) figureprints and (b) digitized profiles of three phototrophic hydrogen-producing communities degrading (1) acetate, (2) butyrate, and (3) a mixture of acetate, butyrate and ethanol.

independent of concentration (for acetate and lactate ranging from 820 to 4100 mg/l) by *Rhodopseudomonas palustris*.

Table 3 summarizes the three kinetic parameters for acetate concentration ranging from 800 to 4000 mg/l. Results show that the lag time (ranging 14–21 h) was not greatly affected by the initial acetate concentration. Table 3 also shows that the hydrogen yield from acetate decreased from 2.1 to 1.1 mol/mol with the increase of acetate concentration from 800 to 4000 mg/l. The decrease of hydrogen yield was possibly due to nutrient deficiency with the increase of acetate concentration. The maximum specific hydrogen production rate fluctuated between 360 and 528 ml/(g-VSS·d) for acetate concentration ranging from 800 to 4000 mg/l.

Table 3 also lists the final pH at various acetate concentrations. It shows that the final pH increased with the acetate concentration from pH 7.3 at 800 mg/l to pH 8.3 at 4000 mg/l.

3.2.2. Butyrate as substrate

Fig. 4 illustrates (a) butyrate degradation and (b) hydrogen accumulation for batches conducted at initial pH 8.0 using butyrate as a sole substrate at various concentrations. Fig. 4a illustrates that the butyrate degradation rate decreased with the increase of initial butyrate concentrations. For butyrate concentration of 1000–5100 mg/l, the degradation rate was 67.6–76.4%, lower than the 76.5–99.4% observed for the degradation of acetate.

Fig. 4b illustrates the hydrogen production at various butyrate concentrations and the corresponding best-fit curves using Eq. (1). It shows that hydrogen production increased with butyrate concentration, as expected.

Table 4 summarizes the final pH and the three kinetic parameters. The final pH decreased with the increase of the initial butyrate concentration from pH 7.6 at 1000 mg/l to pH 7.2 at 5100 mg/l. Such a decrease may be responsible for the incomplete degradation of butyrate. Based on the values of R_m and P, the maximum specific hydrogen production rate and hydrogen yield were calculated, as listed in Table 4. Table 4 shows that the hydrogen yield from butyrate increased from 2.8 mol/mol at 1000 mg/l of butyrate to 2.9 mol/mol at 1300 mg/l, and then decreased to 1.3 mol/mol when the butyrate concentration further increased to 5100 mg/l. The maximum specific hydrogen production rate increased with the butyrate concentrations from 372 ml/(g-VSS·d) at 1300 m/l to 582 ml/(g-VSS·d) at 5100 mg/l. However, an unexpectedly high rate of 768 ml/(g-VSS·d) was observed at 1000 mg/l butyrate. The reason for such a high rate was unclear.

3.3. Microbial characteristics of sludge

The DGGE images in Fig. 5a illustrate that the three phototrophic hydrogen-producing sludges were composed of similar population with only one identical predominant species. The digitized DGGE profiles [19] of the three sludge, illustrated in Fig. 5b, show that the relative abundances of such a predominant population were 81%, 84% and 78%, respectively, in sludges degrading acetate, butyrate and the mixed substrates. The DNA sequence of such predominant population in the reference sludge was identified in a previous study [18], and was assigned an accession number of AY128090 by the GenBank. This species was found to be closely related to *Rhodobacter* sp. TCRI2 (with 99.4% similarity), *Rhodobacter capsulatus* (99.2%) and *Rhodobacter sphaeroides* (95.8%), all of which belong to the family of *Rhodobacteraceae* of alpha-*Proteobacteria*. The relative abundance of this *R. capsulatus*-like species was also confirmed by the FISH analysis. The FISH images in Fig. 6 illustrate that alpha-*Proteobacteria* accounted for

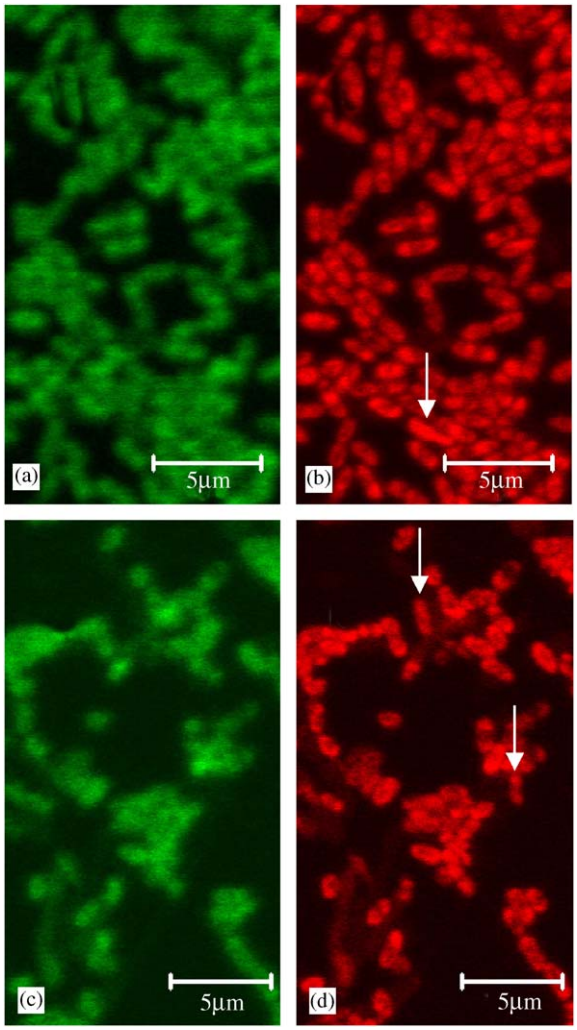

Fig. 6. FISH images of the phototrophic H_2-producing sludges: (a) acetate-degrading sludge hybridized with alpha-*Proteobacteria* probe (Alf968) labeled with FAM, (b) acetate-degrading sludge hybridized with a *Eubacteria*-specific probe (EUB338) labeled with Cy3, (c) butyrate-degrading sludge with probe Alf968 labeled with FAM, (d) butyrate-degrading probe hybridized with probe EUB338 labeled with Cy3. The arrows indicate the cells other than alpha-*Proteobacteria*. (bar = 5 μm).

88 ± 6% of microbial population in the acetate-degrading sludge and 85 ± 5% in the butyrate-degrading sludge.

A great number of phototrophic purple bacteria can produce hydrogen using simple substrates, such as lactate, acetate, butyrate and malate. Most of these phototrophic hydrogen-producing bacteria phylogenetically belong to four distinct groups of alpha-*Proteobacteria*: *Rhodospirillum* [27], *Rhodopseudomonas* [4], *Rhodobacter* [28] and *Rhodovulum* [29], including *R. sphaeroides* [14], *Rhodospirillum rubrum* [9], *Rhodovulum* sp. [29],

Rhodopseudomonas palustris [30], *Rhodopseudomonas* sp. [31], *Rhodobacter marinus* [32], *R. capsulatus* [28]. However, it appears that only one *R. capsulatus*-like species was predominant in the three hydrogen-producing sludges. *R. capsulatus* has been isolated from various environments, such as soil, cow pasture, duck farm, lake, stagnant cistern, and brackish area of Balic Sea [33]. It could grow rapidly using acetate and butyrate as substrates, as observed in this study. The lack of microbial diversity of these sludges could have resulted from the origin of the seed sludge, as well as the medium and the procedures used in the enrichment process. It should be noted that, although *R. capsulatus* can produce hydrogen, it can also consume hydrogen upon the depletion of organic substrate [34]; the process is catalyzed by a reversible, membrane-bound hydrogenase [35].

4. Conclusions

Phototrophic hydrogen production was investigated using acetate and butyrate, which are the main products of dark fermentation, as individual substrates. The maximum hydrogen yields were 2.5 mol-H_2/mol-acetate at initial pH of 8.0 treating 800 mg/l of acetate, 3.7 mol-H_2/mol-butyrate at initial pH of 9.0 treating 1000 mg/l of butyrate. Analyses of DGGE profiles of 16S rDNA fragments and FISH images show that both phototrophic hydrogen-producing sludges comprised only one predominant species resembling *Rhodobacter capsulatus* with over 80% relative abundance.

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