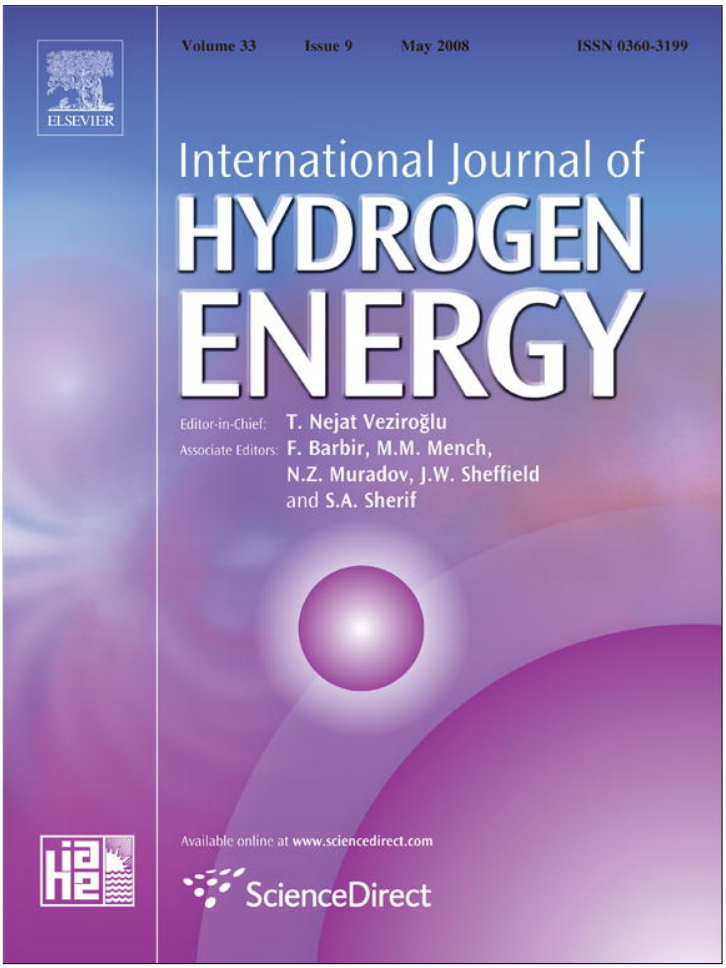

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Characteristics of a phototrophic sludge producing hydrogen from acetate and butyrate

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ABSTRACT

A mixed phototrophic sludge was enriched from the sediment of a local river for continuous hydrogen production from acetate and butyrate in a complete-mix reactor. At pH 7.0–7.5 and 30 °C, the optimal hydrogen production rate at 48 h of hydraulic retention time (HRT) for 150 days of steady-state operation averaged 8.0 ± 1.9 ml/l/h with 0.7 ± 0.1 g/l of biomass. The sludge yield averaged 0.32 ± 0.05 g-VSS/g-COD. Results of batch experiments showed an optimal pH of 8.5 and an optimal NH_4^+ concentration of 2 mM for hydrogen production. At 10 mM, NH_4^+ severely inhibited the hydrogen production. Three of the five OTUs classified from 26 clones developed from the seed sludge were phototrophs, based on phylogenetic analysis. Among them, OTU LA15, which is closely related to *Rhodobacter* sp., was most likely responsible to the hydrogen production.

1. Introduction

Hydrogen is an industrial chemical and an ideal fuel which produces only water upon combustion. Although it is traditionally produced by electro- or thermo-chemical process, hydrogen may also be produced biologically via either phototrophic or non-phototrophic (often referred as dark) fermentation. Dark fermentation converts less than 40% of chemical energy [1] in organic substrates into hydrogen, producing organic acids, mainly acetate and butyrate [2], plus small amount of alcohols as by-products. Phototrophic fermentation can, however, further convert these by-products of dark fermentation into hydrogen and it has thus attracted much research interests recently [3]. However, the technology development of phototrophic hydrogen production is still at the embryonic stage; studies were mostly conducted in batch reactors using single organic substrates. For the few studies using continuous reactor, tests were all conducted for pure cultures and for the limited duration of 10–20 days [4,5]. Furthermore, information on the effect of operational parameters for phototrophic hydrogen production, such as pH [6,7] and ammonium [8,9], were also limited to pure cultures. Similar information for mixed cultures using continuous reactors is still lacking.

This study was thus conducted to examine the characteristics of phototrophic hydrogen production from acetate and butyrate as a mixed substrate in a continuous reactor. Sludge in the continuous reactor under the steady-state condition was sampled for batch studies on the effects of pH and NH_4^+ concentrations. The phylogenetic diversity and the predominant phototrophic hydrogen-producing bacteria of the sludge were analyzed using the 16S rRNA gene-based techniques.

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2. Materials and methods

2.1. Experiment of hydrogen production in continuous reactor

A phototrophic hydrogen-producing seed sludge was first enriched from a sediment sample obtained from the North River (Guangdong, China). The enrichment was conducted in a 500 ml serum bottle at 30 °C under a light intensity of 200 W/m^2 using acetate and butyrate as carbon source. Light intensity was measured at the reactor surface using a radiometer (IL 1400 Radiometer, International Light Inc., USA). After six cycles of enrichment, the sludge showed a hydrogen production rate of 19.8 ml/l/h. The enriched sludge was then used to seed a 1-l complete-mix spherical glass photo-reactor for continuous hydrogen production experiments at 30 °C and pH 7.0–7.5 under a light intensity of 180 W/m^2. The feed solution was composed of 30 mM each of sodium acetate and sodium butyrate, plus the following nutrients (for each liter): 0.75 g K_2HPO_4, 0.85 g KH_2PO_4, 2.8 mg H_3BO_3, 0.75 mg $Na_2MoO_4 \cdot 2H_2O$, 0.24 mg $ZnSO_4 \cdot 7H_2O$, 2.1 mg $MnSO_4 \cdot 4H_2O$, 0.04 mg $Cu(NO_3)_2 \cdot 3H_2O$, 0.75 mg $CaCl_2 \cdot 2H_2O$, 2.0 mg EDTA, 0.2 g $MgSO_4 \cdot 7H_2O$, 11.8 mg $FeSO_4 \cdot 7H_2O$, 3.78 mg vitamin B1, 3.57 mg biotin, 5.25 mg *p*-aminobenzoic acid, 6.48 mg nicotinamide [10], plus 0.37 g glutamate, which was the sole nitrogen source. The feed solution had a chemical oxygen demand (COD) of 6300 mg/l and a C/N ratio of 80/1.

The continuous reactor was operated in two stages. The first stage, which lasted 156 days, was conducted in an attempt to study the optimal operating condition at various hydraulic retention time (HRT), which increased stepwise initially from 72 to 144 h and then lowered to 48 h, similar to the HRT range of a previous study [5]. The second stage was conducted by keeping the HRT at 48 h for 150 days under steady-state condition as evidenced by the constant hydrogen production rate, biomass concentration, and effluent quality. Sludge at the second stage was collected for the subsequent batch experiments.

2.2. Batch experiments

Two series of batch experiments were conducted at 30 °C in 125 ml serum bottles under a uniform light intensity of 140 W/m^2, illuminating by a 200 W tungsten lamp. Each batch experiment was conducted in duplicate. Series 1 was conducted to investigate the effect of pH (ranging 6.0–9.0 with step increments of 0.5) on phototrophic hydrogen production. The pH remained unchanged throughout the batch experiments. Series 2 was conducted to investigate the effect of NH_4^+ concentration (ranging 0–15 mM). The media used in both series were same as the feed solution of the continuous experiment.

2.3. Chemical analysis

Production of biogas was measured by a syringe for the batch reactor and by water displacement for the continuous reactor. Compositions of biogas were analyzed by a gas chromatograph (Hewlett Packard 5890 II, USA) equipped with a thermal conductivity detector and a 30 m × 0.53 mm PLOT (Porous-Layer Open-Tubular) silica capillary column (Supelco Carboxen 1010) [11]. Concentrations of acetate and butyrate in the mixed liquor were measured by a high performance liquid chromatograph (SCL-10 AVP, Shimadzu, Kyoto, Japan) equipped with a 0.3 m × 6.5 mm column (OA-1000, Alltech, USA), and a SPD-10 (Shimadzu, Kyoto, Japan) UV-visible detector (210 nm), using 0.01 N sulfuric acid as the mobile phase with a flow rate of 0.80 ml/min. Other volatile fatty acids (VFA) in the mixed liquor, including propionate, *i*-butyrate, valerate, *i*-valerate and caproate, and alcohols, including methanol, ethanol, propanol and butanol, were analyzed by another gas chromatograph (6890 N, Agilent Technologies, USA) equipped with a hydrogen flame ionization detector and a 10 m × 0.53 mm fused-silica capillary column (HP-FFAP). COD and volatile suspended solids (VSS) were measured according to the Standard Methods [12].

2.4. Kinetic modeling

The cumulative hydrogen volume in the batch reactor followed the modified Gompertz equation [13]:

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

where H represents the cumulative hydrogen production (ml), λ the lag phase time (h), P the hydrogen production potential (ml), and R_m the maximum hydrogen production rate (ml/h). The values of P, R_m, and λ for each experiment were determined by best fitting the hydrogen production data for Eq. (1) using Microsoft's software Excel 2000.

2.5. DNA extraction and DGGE analysis of 16S rRNA gene

In order to compare the microbial diversity in hydrogen production, genomic DNA were extracted following established procedures [14] from various sludge samples, including (a) the seed sludge for the continuous reactor, (b) sludge samples collected from the continuous reactor at various stages of operation, and (c) sludge samples collected at the end of each series of batch experiments.

The 16S rRNA gene fragments of the genomic DNA extracted from the sludge samples were amplified by polymerase chain reaction (PCR) using the primer set of 341FGC (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG-GCCTACGGGAGGCAGCAG-3′) and 518R (5′-ATTACCGCGGC-TGCTGG-3′) by an automated thermal cycler (iCycler® Bio-Rad, Hercules, CA, USA) at the denaturing, annealing, and extension temperature of 95, 54, and 72 °C, respectively [15]. The PCR-amplified products were then screened by DGGE (denaturing gradient gel electrophoresis) using 8% gel with 40–60% denaturant gradients [14]. Electrophoresis was conducted in a 1 × TAE buffer solution at 200 V and 60 °C for 4 h, and the bands were stained with silver nitrate [15].

2.6. Cloning, sequencing, and phylogenetic analysis

The 16S rRNA gene of the seed sludge of the continuous reactor was further analyzed for its phylogeny. Firstly, the 16S

rRNA gene was amplified using the primer set of EUB8F (5′-AGAGTTTGATCMTGGCTCAG-3′) and universal primer 1392R (5′-ACGGGCGGTGTGTRC-3′) at the denaturing, annealing, and extension temperature of 95, 54, and 72 °C, respectively [14]. The PCR products were then cloned using the TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA) as described previously [14]. A total of 26 clones were selected for the plasmid recovery and sequenced using 1392R [16]. Five OTUs (operational taxonomy units) classified from these clones were then compared with the reference microorganisms available in the GenBank by BLAST search, and checked manually using the BioEdit [17]. A phylogenetic tree was then constructed using the neighbor-joining method by MEGA 2.1 [18]. Bootstrap re-sampling analysis for 1000 replicates was performed to estimate the confidence of tree topologies.

2.7. *Quantitative real-time PCR*

The 16S rRNA gene fragments of the five OTUs were aligned with those of their respective reference microorganisms retrieved from the GenBank using BioEdit [17]. A set of primer was then designed for extracting each target 16S rRNA gene fragment for qRT-PCR analysis. The concentration of each target 16S rRNA gene in a sample was quantified by the qRT-PCR conducted in 96-well, 0.2-ml thin-wall plates using 10 μl SYBR Green I Supermix (Bio-Rad, Hercules, CA, USA), plus 1 μl of each primer and 8 μl template. Each reaction was performed in a thermal cycler (iCycler IQ, Bio-Rad, Hercules, CA, USA) in triplicate. Cycling conditions began with an initial hold of 4 min at 95 °C, followed by 40 cycles consisting of 30 s each of denaturing at 95 °C, annealing at 54 °C and extension at 72 °C.

2.8. *Accession numbers*

The nucleotide sequences of OTUs reported in this paper have been assigned by the GenBank the following accession numbers: EU116430-EU116434.

3. Results and discussion

3.1. *Hydrogen production in continuous reactor*

The continuous complete-mix reactor was operated at 30 °C and a light intensity of 180 W/m^2 using acetate and butyrate as a mixed substrate. The pH of the mixed liquor remained at pH 7.0–7.5, same as in the feed solution. The biogas contained 80–90% of hydrogen, the remaining fraction being carbon dioxide. Fig. 1 illustrates the hydrogen production characteristics, including (a) volumetric hydrogen production rate (ml/l/h), (b) concentration of biomass as measured by VSS (g/l), and (c) substrate removal efficiency (%). The continuous reactor was operated in two stages. The first stage (days 1–156) was operated in an attempt to examine the effect of HRT, which was increased stepwise from 72 to 144 h and then lowered to 48 h. The attempt was later abandoned due to the difficulty to keep the reactor operated under steady-state condition for some HRTs. However, results of the first phase plotted in Fig. 2 clearly show a substantial decrease of volumetric hydrogen production rate for biomass concentrations in the reactor exceeded 0.8 g/l. Such a decrease may be due to the reduction of light penetration resulting from the increased biomass.

The second stage (days 157–306) was then conducted at a constant HRT of 48 h by keeping a steady biomass concentration at 0.7 ± 0.1 g-VSS/l. This operation under steady state condition lasted 150 days, which was considerably longer than any of the previous study using continuous reactor [4,5]. Results in Fig. 1 show that when operated under steady state at 30 °C, pH 7.0–7.5, a light intensity of 180 W/m^2, the reactor consistently removed $54.4 \pm 6.0\%$ acetate and $32.4 \pm 6.4\%$ butyrate, and produced hydrogen at a volumetric rate of 8.0 ± 1.9 ml/l/h and a specific rate of 11.5 ± 2.7 ml/g/h. In addition to the residual acetate and butyrate, the effluent contained 30–50 mg/l propionate, 50–60 mg/l isobutyrate, and 30–60 mg/l caproate. The residual COD in the mixed liquor plus the corresponding COD in hydrogen and increased biomass totaled 5480–6700 mg/l, accounting for 87–106% of the COD in the feed solution.

The specific hydrogen production rate of 11.5 ± 2.7 ml/g/h was considerably higher than the 5.2–5.6 ml/g/h reported for the degradation of mixed acids by another phototrophic sludge [19,20] and the 2.5–9.8 ml/g/h for the degradation of acetate by *Rhodopseudomonas palustris* [21,22], but lower than the 37.8 ml/g/h for the degradation of mixed acids by *Rhodobacter capsulatus* [5].

The volumetric hydrogen production rate of 8.0 ± 1.9 ml/l/h in this study was in the mid range of the reported 1.6–14.7 ml/l/h in other batch studies using acetate or butyrate as substrate for various pure cultures, including *R. palustris* [21], *Rhodobacter sphaeroides* [7], *R. capsulatus* [23], and mixed cultures of phototrophic bacteria [20,24,25]. It was however lower than the reported 12.5–21.0 ml/l/h from mixed organic acids by the pure culture of *R. capsulatus* [5] and 48 ml/l/h by *R. sphaeroides* [4] in continuous reactors.

The acetate removal efficiency of $54.4 \pm 6.0\%$ was higher than the $32.4 \pm 6.4\%$ for butyrate, indicating a higher degradation rate for acetate by this phototrophic sludge. This removal efficiency of acetate was also higher than the 17.3% for *R. palustris* in batch reactor [21] and comparable to the 66% for continuous hydrogen production by *R. capsulatus* [5]. But, the butyrate removal efficiency of $32.4 \pm 6.4\%$ was lower than the 67.6–76.4% by another phototrophic sludge in batch reactor [25].

The sludge yield under the steady-state condition averaged 0.32 ± 0.05 g-VSS/g-COD-removed. This yield was higher than the 0.17–0.20 g-VSS/g-COD-removed reported for the dark fermentative hydrogen production from glucose [26] or sucrose [27], and comparable to the 0.33 g-VSS/g-COD-removed for dark fermentation using sucrose [28].

3.2. *Effect of pH*

Sludge in the continuous reactor at 48 h of HRT was collected under steady-state condition for two subsequent series of batch experiments. Fig. 3 illustrates data of hydrogen production at pH ranging 6.0–9.0 and the corresponding plots of Eq. (1) using the best-fitted kinetic parameters summarized in Table 1. The COD balance in this series was 97–108%.

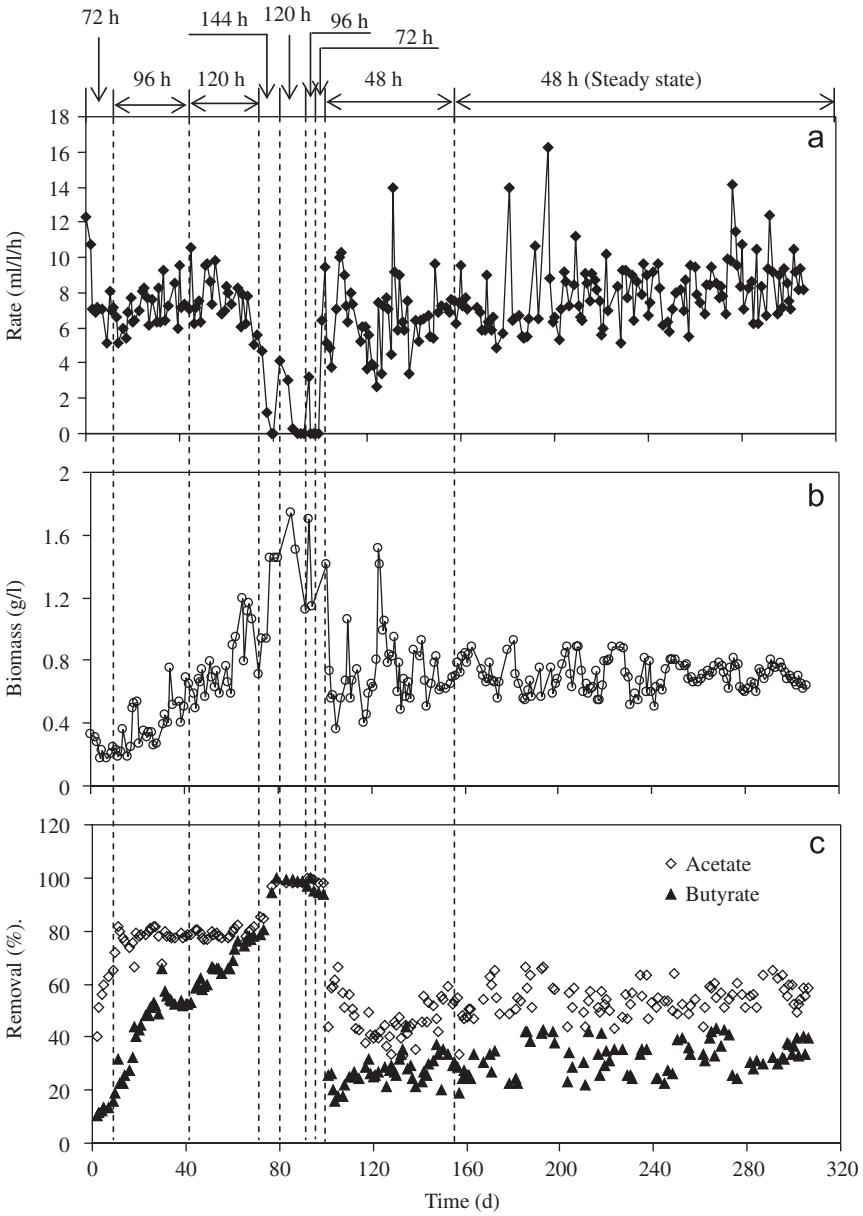

Fig. 1 – Hydrogen production in continuous reactor: (a) volumetric hydrogen production rate, (b) biomass concentration, and (c) substrate removal efficiency.

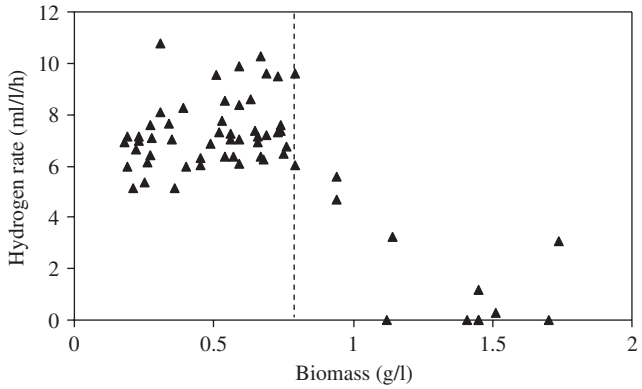

Fig. 2 – Volumetric hydrogen production rates at various biomass concentrations.

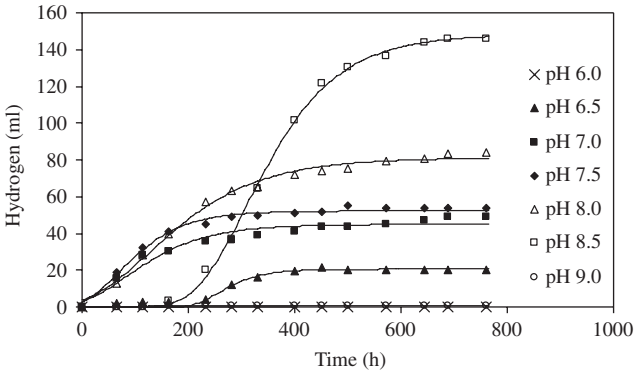

Fig. 3 – Hydrogen production at pH 6.0–9.0.

Table 1 – Kinetic parameters for hydrogen production at pH 6.0–9.0, and the corresponding biomass increase and substrate removal

pH	λ (h)	R_m (ml/h)	P (ml)	Maximum specific H_2 rate (ml/g/h)	Biomass increase[a] (g/l)	Removal efficiency (%)		
						Acetate	Butyrate	COD
6.0	–	0	0	0	0.07	0	7.4	2.6
6.5	210	0.16	20.5	24.6	0.34	10.5	15.3	14.1
7.0	0	0.20	44.9	30.8	0.58	20.3	13.0	16.2
7.5	0	0.29	52.2	44.6	0.45	11.6	13.5	14.2
8.0	0	0.26	81.0	40.0	0.41	28.0	38.4	16.2
8.5	185	0.57	148.2	87.7	0.52	29.4	35.1	26.9
9.0	88	0.01	0.8	1.5	0.16	0	0	4.4

[a] The initial biomass concentration was 0.065 g/l.

Results show that little hydrogen was produced at pH 6.0 and 9.0. The optimal pH for hydrogen production was pH 8.5 with a maximum amount of 148.2 ml hydrogen produced and a rate of 5.7 ml/l/h, but with a long lag phase of 185 h. At pH 7.0–8.0, the sludge produced considerably lower amounts of hydrogen (44.9–81.0 ml) and at lower rates (2.0–2.9 ml/l/h). The optimal pH of 8.5 found in this study was comparable to the reported pH 8.5–9.0 for *R. capsulatus* [29] and pH 8.0–9.0 for a mixed phototrophic sludge [25], but higher than the pH 7.0–8.0 reported by others [6,7,25,30]. The maximum volumetric hydrogen production rate of 5.7 ml/l/h at pH 8.5 in this study was comparable to the reported 5.3–6.7 ml/l/h using mixed phototrophic sludge [25], and higher than the 2.2–4.9 ml/l/h by *R. sphaeroides* [6,7].

Table 1 also compiles data of maximum specific hydrogen-producing rate, increased quantity of biomass and the removal efficiencies of acetate, butyrate and COD. Results show that the biomass had the maximum yield at pH 7.0, which was comparable to the reported pH 6.8–7.2 [6,7,30], even though a high pH 8.5–9.0 was reported for *R. capsulatus* [29]. The maximum specific hydrogen production rate of 87.7 ml/g/h at pH 8.5 in this study was considerably higher than the 3.5–16.8 ml/g/h reported in literature [6,7,25].

3.3. *Effect of* NH_4^+ *concentration*

Fig. 4 shows data of hydrogen production at initial NH_4^+ concentrations up to 15 mM, and the corresponding curves of Eq. (1) using the best-fitted kinetic parameters summarized in Table 2. At the end of hydrogen production, there was no residual NH_4^+ found in the mixed liquor in batches with 0.5–5 mM of NH_4^+, and only trace NH_4^+ residues (0.02–0.06 mM) were found in batches dosed with 10–15 mM of NH_4^+. Results show that hydrogen production was stimulated by 0.5–5 mM of NH_4^+ with a rate ranging 4.8–5.3 ml/l/h, and it was suppressed by 10–15 mM of NH_4^+. At the optimal concentration of NH_4^+ of 2 mM, the amount of hydrogen produced and volumetric production rate were 214.4 ml and 5.2 ml/l/h, respectively. At 10–15 mM, NH_4^+ severely inhibited the hydrogen production activity. These findings were similar to those

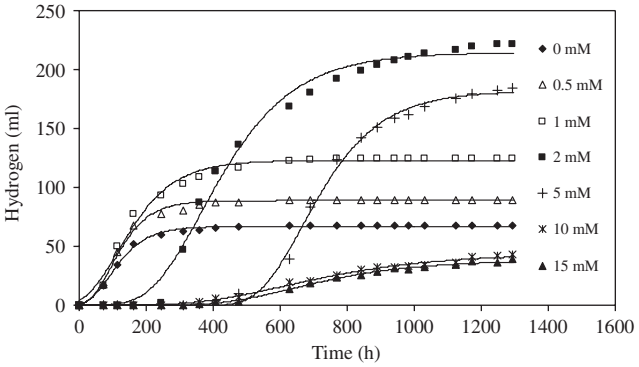

Fig. 4 – Hydrogen production at NH_4^+ concentrations ranging 0–15 mM.

of a former study of *Rhodopseudomonas* sp., in which hydrogen production was the highest at 1 mM of NH_4^+, but was reduced by 50% at 5 mM of NH_4^+ and ceased completely at 15 mM of NH_4^+ [31]. However, in a study of immobilized culture of *R. sphaeroides*, the highest hydrogen production was at 5 mM of NH_4^+, and there was no obvious inhibition by NH_4^+ at concentrations as high as 10 mM [32], suggesting the immobilized biomass had a higher degree of tolerance to NH_4^+.

Table 2 also compiles data of maximum specific hydrogen-producing rate, increased quantity of biomass and the removal efficiencies of acetate, butyrate and COD. The maximum specific hydrogen production rates of 69.6–76.8 ml/g/h at 0.5–5 mM of NH_4^+ were much higher than the 5.2–16.8 ml/g/h for mixed phototrophic sludge [20,25] and the 9.8–28.4 ml/g/h for *R. palustris* [21,33,34]. The biomass increase was 0.53–0.54 g/l for NH_4^+ concentrations at 0.0–0.5 mM, but was up to 0.77 g/l at 1 mM of NH_4^+ and 2.04 g/l at 15 mM of NH_4^+.

The acetate removal efficiency of 62.0% at 2 mM of NH_4^+ was slightly higher than the 54.4 ± 6.0% in the continuous reactor of this study and the 55% reported in batch reactor in another study [35]. The 55.9% degradation of butyrate at 2 mM of NH_4^+ was much higher than the 32.4 ± 6.4% in continuous reactor.

Table 2 – Kinetic parameters for hydrogen production at ammonium concentrations ranging from 0 to 15 mM, and the corresponding biomass increase and substrate removal

NH_4	λ (h)	R_m (ml/h)	P (ml)	Maximum specific H_2 rate (ml/g/h)	Biomass increase[a] (g/l)	Removal efficiency (%)		
						Acetate	Butyrate	COD
0	28	0.40	66.8	58.0	0.53	12.8	23.8	9.3
0.5	34	0.53	88.8	76.8	0.54	21.3	21.4	17.0
1	20	0.48	122.9	69.6	0.77	37.0	29.0	23.1
2	213	0.52	214.4	75.4	1.08	62.0	55.9	43.4
5	536	0.52	181.9	75.4	1.51	25.3	76.0	48.5
10	356	0.06	43.9	8.7	1.82	51.6	66.0	80.5
15	429	0.07	38.2	10.1	2.04	50.1	64.1	89.3

[a] The initial biomass concentration was 0.065 g/l.

Table 3 – Phylogenetic affiliation of operational taxonomic units

OTU	Phylogenetic relationship		No. of clones	Abundance (%)
	Closest species in GenBank	Similarity (%)		
LA15	*Rhodobacter* sp. TCRI 14	99.4	11	42.3
LA01	*Rubrivivax gelatinosus* AB03	99.9	10	38.5
LA17	*Rhodobacter sphaeroides*	99.6	2	7.7
LA09	*Pseudomonas* sp. 3A_7	92.0	2	7.7
LA05	*Rubrivivax gelatinosus*	91.6	1	3.8
Total			26	100.0

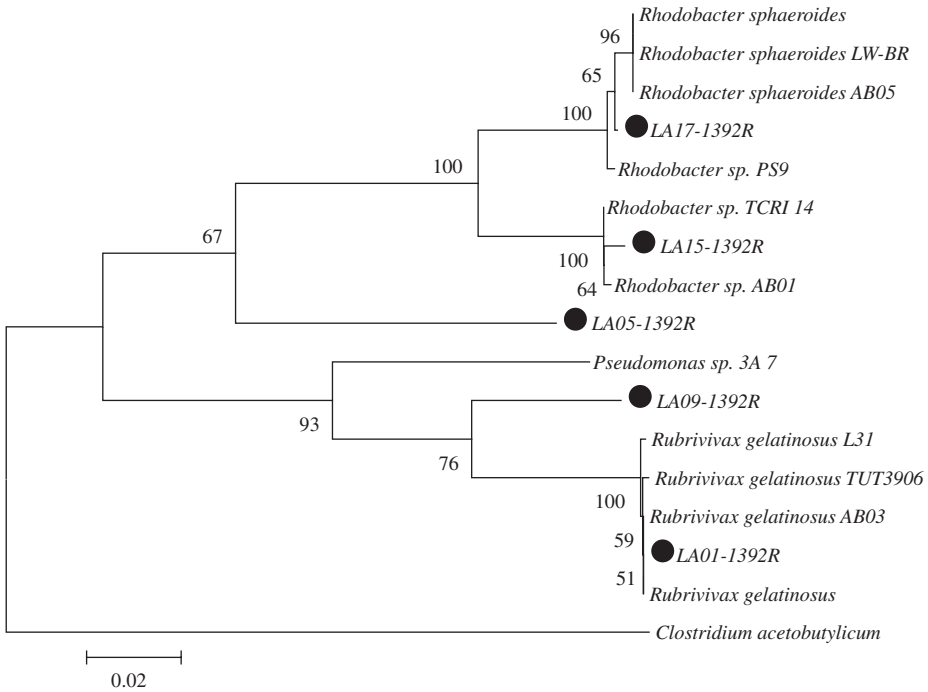

Fig. 5 – Phylogenetic tree of the OTUs in the phototrophic hydrogen-producing sludge and their close relatives based on partial 16S rRNA gene sequences. The tree based on Jukes–Cantor distance was constructed using neighbor-joining algorithm with 1000 bootstrappings. Clostridium acetobutylicum was selected as the outgroup species. The scale bar represents 0.02 substitution per nucleotide position (• OTUs).

3.4. Phylogenetic analysis

Based on the cloning–sequencing analysis, five OTUs were classified from the 26 clones developed from the enriched sludge which was used to seed the phototrophic hydrogen-producing reactor. Table 3 shows the number of clones and relative abundance of each OTU, as well as the closest species found in the GenBank and the corresponding similarity by BLAST analysis. The two most predominant OTUs in the seed sludge were LA15 (42.3%) and LA01 (38.5%).

The phylogenetic tree in Fig. 5 shows that three of the five OTUs, i.e. LA01, LA15, and LA17 (in total accounting for 88.5% of the population), are affiliated with phototrophic purple non-sulfur bacteria, such as *R. gelatinosus*, which is classified as *incertae sedis* in the order *Burkholderiales* of beta-*Proteobacteria* [36], and two others in the family of *Rhodobacteraceae* of alpha-*Proteobacteria* [25]. LA15 was closely related (similarities 99.4% and 98.8%, respectively) to *Rhodobacter* sp. TCRI14 (accession number AB017799) and *Rhodobacter* sp. AB01 (accession number EF655911), whereas LA17 was closely related (99.0%) to *R. sphaeroides* AB05 (accession number EF655913), and LA01 was closely related (99.9% and 99.4%, respectively) to *R. gelatinosus* AB03 (accession number EF655912) and *R. gelatinosus* L31 (accession number EF094990).

Fig. 6 compares the DGGE profiles of the initial microbial population in the continuous reactor and those under the steady-state condition at 48 h of HRT. It shows that the band representing LA15 was most dominant throughout, whereas the two bands representing LA01 and LA17 vanished gradually. The predominance of LA15 was also observed in the DGGE profiles of samples collected at various pH and ammonium (NH_4^+) concentrations. This suggests that the *Rhodobacter* sp. was most likely responsible to the hydrogen production in this study.

3.5. Quantification of LA01, LA15, and LA17

DNA sequences of the five OTUs and *Pseudomonas* sp. 3A_7 (AY689026), *Rhodobacter* sp. TCRI 14 (AB017799), two *R. sphaeroides* strains (AB196355, DQ915852), as well as two *R. gelatinosus*

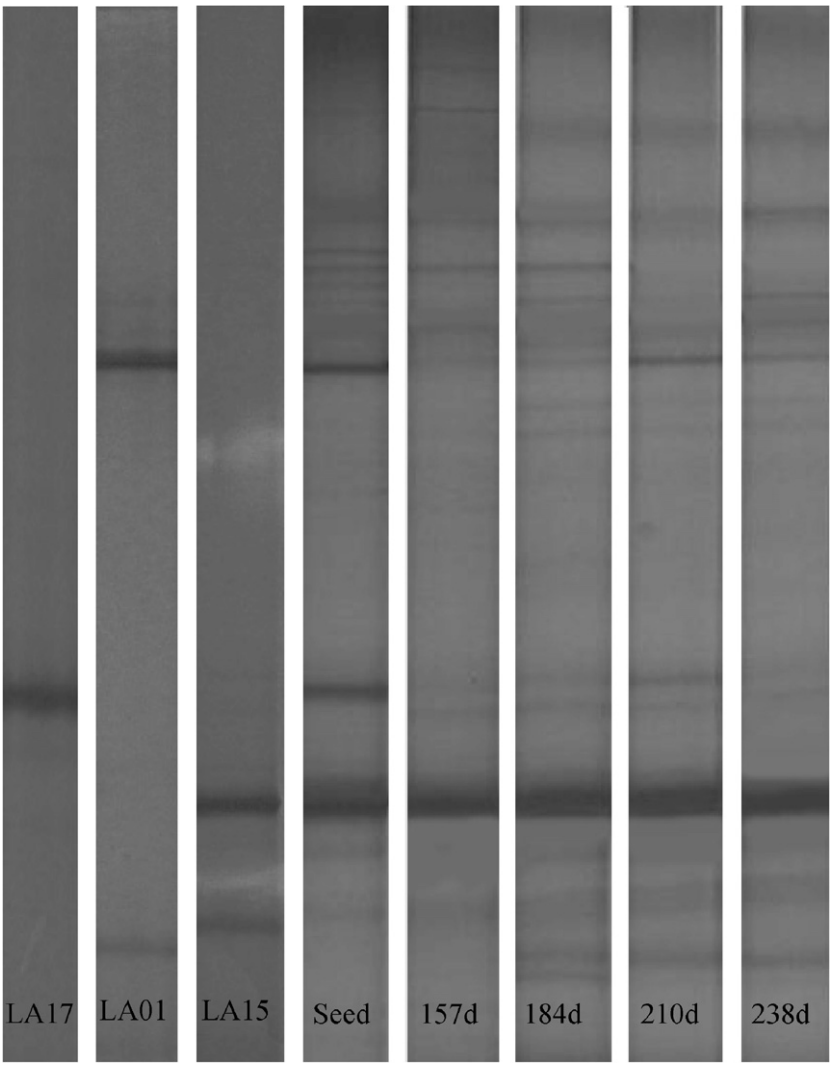

Fig. 6 – DGGE profiles of phototrophic hydrogen-producing sludge samples under steady-state condition with a HRT of 48 h.

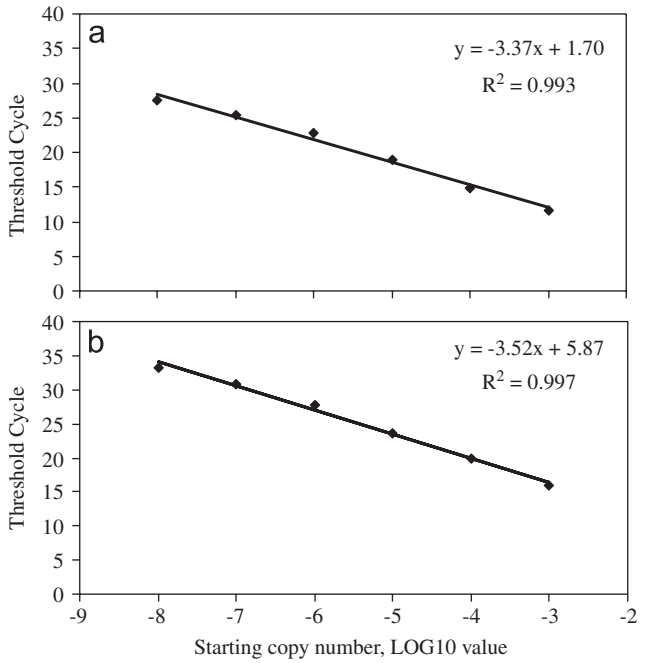

Fig. 7 – Standard curves of (a) 16S rRNA gene fragment of OTU LA15, and (b) the genomic DNA of the hydrogen-producing sludge.

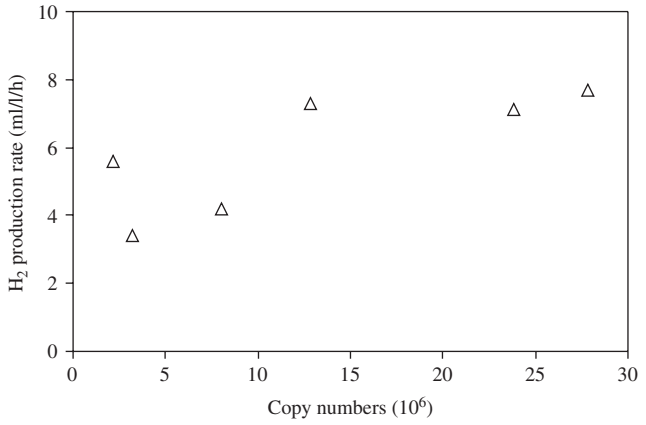

Fig. 8 – Correlation between hydrogen production and copy numbers of LA15 in sludge samples.

strains (AB016167, AB250625) available in the GenBank, were compared. Three forward primers, targeting OTUs LA01, LA15, and LA17, respectively, were identified from the common reserved regions: RG-F (5′-GGATCCTGCAGAGATGTGGGAG-3′, $T_m = 58\,^{\circ}C$), TCRI-F (5′-AGCGCAACCCACACTTCC-3′, $T_m = 57\,^{\circ}C$), and RS-F (5′- CCACGTCCTTAGTTGCCAG-3′, $T_m = 60\,^{\circ}C$). These forward primers and universal reverse primer 1392R (5′-ACGGGCGGTGTGTRC-3′) were then used for the analysis of relative population concentration in this study.

Fig. 7 shows the qRT-PCR standard curves [37] of (a) 16S rRNA gene fragment of OTU LA15, and (b) the genomic DNA of the hydrogen-producing sludge. Fig. 7(a) shows a slope of −3.37 ($R^2 = 0.993$) for the LA15 plasmid, corresponding to a 98.0% PCR efficiency [37]. Fig. 7(b) shows that the corresponding curve for the genomic DNA sample from the hydrogen-producing sludge had a slope of −3.52 ($R^2 = 0.997$), corresponding to a 92.3% PCR efficiency. The similarity of the two PCR efficiencies confirms the validity of using qRT-PCR for the microbial quantification of the sludge samples [37,38]. Similar standard curves were also obtained for the quantification of LA01 and LA17 plasmids with respective PCR efficiencies 82.7% ($R^2 = 0.989$) and 95.9% ($R^2 = 0.967$), the corresponding PCR efficiencies of genomic DNA sample being 84.2% ($R^2 = 1.000$) and 93.4% ($R^2 = 0.998$), respectively.

Six sludge samples were taken from the continuous reactor at various HRT for genomic DNA extraction. Using the three primer sets specifically designed to target LA01, LA15, and LA17, the amounts of these OTU 16S rRNA gene (expressed as copy numbers) in each sludge sample were quantified by qRT-PCR using the established standard curves. Fig. 8 illustrates that the hydrogen production rate in the continuous reactor generally increased with the amount of LA15 in the reactor. Analysis was also conducted for LA01 and LA17, but could not find any similar correlations between hydrogen production and the amounts of these two OTUs. This further confirms the DGGE finding that LA15 was the species most likely responsible for the hydrogen production in the continuous reactor.

4. Conclusions

Photo-fermentative production of hydrogen reduced drastically for biomass concentrations exceeding 0.8 g/l at pH 7.0–7.5 and 30 °C. In a complete-mix continuous reactor, the optimal hydrogen production rate at 48 h of HRT for 150 days of steady-state operation averaged 8.0 ± 1.9 ml/l/h with 0.7 ± 0.1 g/l of biomass. The sludge yield averaged 0.32 ± 0.05 g-VSS/g-COD. Results of batch experiments showed an optimal pH of 8.5 and an optimal NH_4^+ concentration of 2 mM for hydrogen production. At 10 mM, NH_4^+ severely inhibited the hydrogen production. Three of the five OTUs classified from 26 clones developed from the seed sludge were phototrophs, based on phylogenetic analysis. Among them, OTU LA15, which is closely related to *Rhodobacter* sp., was most likely responsible to the hydrogen production.

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REFERENCES

[1] Li CL, Fang HHP. Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. Crit Rev Environ Sci Technol 2007;37:1–39.

[2] Li CL, Zhang T, Fang HHP. Fermentative hydrogen production in two upflow reactors. In: Proceedings of the 7th Mainland-Taiwan Environmental Protection Academic Conference, Taizhong, Taiwan, 2005. p. 359–362.

[3] Lee CM, Chen PC, Wang CC, Tung YC. Photohydrogen production using purple nonsulfur bacteria with hydrogen fermentation reactor effluent. Int J Hydrogen Energy 2002;27:1309–13.
[4] Fascetti E, D'Addario E, Todini O, Robertiello A. Photosynthetic hydrogen evolution with volatile organic acids derived from the fermentation of source selected municipal solid wastes. Int J Hydrogen Energy 1998;23(9):753–60.
[5] Shi XY, Yu HQ. Continuous production of hydrogen from mixed volatile fatty acids with *Rhodopseudomonas capsulata*. Int J Hydrogen Energy 2006;31(12):1641–7.
[6] Margaritis A, Vogrinetz J. The effect of glucose concentration and pH on hydrogen production by *Rhodopseudomonas sphaeroides* VM 81. Int J Hydrogen Energy 1983;8(4):281–4.
[7] Kim MS, Ahn JH, Yoon YS. Photo-biological hydrogen production by the uptake hydrogenase and PHB synthase deficient mutant of *Rhodobacter sphaeroides*. In: Miyake et al. (Eds.), Biohydrogen III, 2004. p. 45–55.
[8] Kelley BC, Meyer CM, Gandy C, Vignais PM. Hydrogen recycling by *Rhodopseudomonas capsulata*. FEBS Lett 1977;81:281–5.
[9] Zumft WG, Castillo F. Regulatory properties of the nitrogenase of *Rhodopseudomonas palustris*. Arch Microbiol 1978;117:53–60.
[10] Miyake J, Mao XY, Kawamura S. Hydrogen photoproduction from glucose by a co-culture of a photosynthetic bacteria and *Clostridium butyricum*. J Ferment Technol 1984;2(6):531–5.
[11] Fang HHP, Liu H, Zhang T. Bio-hydrogen production from wastewater. Water Sci Technol 2004;4(1):77–85.
[12] APHA, AWA, WPCF. Standard methods for the examination of water and wastewater, 21st ed. Washington, DC: American Public Health Association; 2005.
[13] Lay JJ, Li YY, Noike T. A mathematical model for methane production from landfill bioreactor. J Environ Eng 1998;124:730–6.
[14] Zhang T, Fang HHP. Phylogenetic diversity of a SRB-rich marine biofilm. Appl Microbiol Biotechnol 2001;57(3):437–40.
[15] Zhang T, Fang HHP. Digitization of DGGE (denaturing gradient gel electrophoresis) profile and cluster analysis of microbial community. Biotechnol Lett 2000;22:399–405.
[16] Fang HHP, Zhang T, Liu H. Microbial diversity of a mesophilic hydrogen-producing sludge. Appl Microbiol Biotechnol 2002;58(1):112–8.
[17] Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 1999;41:95–8.
[18] Kumar S, Tomura K, Nei M. MEGA: molecular evolution genetics analysis, version 1.0. Philadelphia: Pennsylvania State University; 1993.
[19] Zhang T, Liu H, Fang HHP. Microbial analysis of a phototrophic sludge producing hydrogen from acidified wastewater. Biotechnol Lett 2002;24:1833–7.
[20] Takabatake H, Suzuki K, Ko IB, Noike T. Characteristics of anaerobic ammonia removal by a mixed culture of hydrogen producing photosynthetic bacteria. Bioresour Technol 2004;95:151–8.
[21] Oh YK, Seol EH, Kim MS, Park S. Photoproduction of hydrogen from acetate by a chemoheterotrophic bacterium *Rhodopseudomonas palustris* P4. Int J Hydrogen Energy 2004;29:1115–21.
[22] Chen CY, Lee CM, Chang JS. Hydrogen production by indigenous photosynthetic bacterium *Rhodopseudomonas palustris* WP3-5 using optical fiber-illuminating photobioreactors. Biochem Eng J 2006;32:33–42.
[23] Shi XY, Yu HQ. Response surface analysis on the effect of cell concentration and light intensity on hydrogen production by *Rhodopseudomonas capsulata*. Process Biochem 2005;40:2475–81.
[24] Ike A, Murakawa T, Kawaguchi H, Hirata K, Miyamoto K. Photoproduction of hydrogen from raw starch using a halophilic bacterial community. J Biosci Bioeng 1999;88(1):72–7.
[25] Fang HHP, Liu H, Zhang T. Phototrophic hydrogen production from acetate and butyrate in wastewater. Int J Hydrogen Energy 2005;30:785–93.
[26] Fang HHP, Liu H. Effect of pH on hydrogen production from glucose by a mixed culture. Bioresour Technol 2002;82(2):87–93.
[27] Kim SH, Han SK, Shin HS. Effect of substrate concentration on hydrogen production and 16S rDNA-based analysis of the microbial community in a continuous fermenter. Process Biochem 2006;41(1):199–207.
[28] Yu HQ, Mu Y. Biological hydrogen production in a UASB reactor with granules. Ï: reactor performance in 3-year operation. Biotechnol Bioeng 2006;94(5):988–95.
[29] Tsygankov AA, Laurinavichene TV. Effects of illumination and pH on the growth rate and nitrogenase activity of *Rhodobacter capsulatus* grown with and without Molybdenum. Microbiology 1996;65:436–41.
[30] Sasikala C, Ramana CV, Rao PR. Regulation of simultaneous hydrogen production during growth by pH and glutamate in *Rhodobacter sphaeroides* O.U.001. Int J Hydrogen Energy 1995;20(2):123–6.
[31] Hoekema S, Bijmans M, Janssen M, Tramper J, Wijffels RH. A pneumatically agitated flat-panel photobioreactor with gas re-circulation: anaerobic photoheterotrophic cultivation of a purple non-sulfur bacterium. Int J Hydrogen Energy 2002;27:1331–8.
[32] Zhu HG, Suzuki T, Tsygankov AA, Asada Y, Miyake J. Hydrogen production from tofu wastewater by *Rhodobacter sphaeroides* immobilized in agar gels. Int J Hydrogen Energy 1999;24:305–10.
[33] Barbosa MJ, Rocha JMS, Tramper J, Wijffels RH. Acetate as a carbon source for hydrogen production by photosynthetic bacteria. J Biotechnol 2001;85:25–33.
[34] Chen CY, Lu WB, Wu JF, Chang JS. Enhancing phototrophic hydrogen production of *Rhodobacter sphaeroides* via statistical experimental design. Int J Hydrogen Energy 2007;32(8): 940–9.
[35] Kim MS, Baek JS, Lee JK. Comparison of H_2 accumulation by *Rhodobacter sphaeroides* KD 131 and its uptake hydrogenase and PHB synthase deficient mutant. Int J Hydrogen Energy 2006;31(1):121–7.
[36] Garrity GM, Brenner DJ, Krieg NR, Staley JT. Bergey's manual of systematic bacteriology. Volume Two: the proteobacteria. USA: Springer; 2005.
[37] Zhang T, Fang HHP. Application of real-time polymerase chain reaction for quantification of microorganisms in environmental sample. Appl Microbiol Biotechnol 2006;70:281–9.
[38] Devers M, Soulas G, Martin-Laurent F. Real-time PCR reverse transcription PCR analysis of expression of atrazine catabolism genes in two bacterial strains isolated from soil. J Microbiol Methods 2004;56:3–15.