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Characterization of Fe-hydrogenase genes diversity and hydrogen-producing population in an acidophilic sludge

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Abstract

According to the DNA sequences of six Fe-hydrogenase genes (FHG) of *Clostridium* species retrieved from the GenBank, a set of primers specific for Fe-hydrogenase genes were identified from their common conserved regions. The length of DNA fragments amplified using these two primers averaged 313 bps. This primer set was then used to investigate the FHG diversity in an acidophilic rice-degrading sludge by methods based on polymerase chain reaction (PCR). Eight new Fe-hydrogenase gene fragments were identified from the sludge, as a result. Similarity based on amino acids among the 14 hydrogenase genes (8 newly found plus 6 known ones) was 39–97%, which is comparable to the similarity of 41–82% among the 6 known hydrogenase genes alone. The low similarity indicates a great diversity on Fe-hydrogenase among the *Clostridium* species. The primer set was then used to monitor the change of hydrogen-producing microbial population in a batch reaction using the technique of quantitative real-time polymerase chain reaction (qRT-PCR) with SYBR Green I as the fluorescent reagent. Results showed that the hydrogen producers had an average generation time of 4.2 h, and a production rate of 7.0×10^{16} H_2-molecule $cell^{-1}$ h^{-1}.

Keywords: Acidophilic; Fe-hydrogenase gene; Hydrogen production; Primer; Quantitative real-time polymerase chain reaction

1. Introduction

The population diversity of a bacterial community is often analyzed using either the culture- or the 16S rDNA-based methods. However, both approaches are inadequate for the study of the population diversity of bacteria of certain functionality. Many bacteria in the community may be un-culturable and thus would be overlooked by the culture-based methods. On the other hand, the phylogenetic diversity resulting from the 16S rDNA analysis may not be relevant to the physiological diversity of the community. It is thus useful to characterize the bacterial population of specific functionality according to the functional gene, such as nitrification/denitrification (Purkhold et al., 2000; Okano et al., 2004), dis-

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similatory sulfate reduction (Wagner et al., 1998), methane production (Hales et al., 1996), and hydrogen production.

Hydrogenases are the enzymes that catalyze both hydrogen uptake and production. About 40 hydrogenase genes have been sequenced so far. All the known hydrogenases contain Fe, and some contain Ni and Se as well (Voordouw, 1992). Hydrogenases containing Ni and Se facilitate the uptake of hydrogen, whereas those containing Fe alone (Fe-hydrogenases) catalyze the production of hydrogen (Cammack, 1999). Fe-hydrogenase genes (FHGs) (Cammack, 1999) and hydrogen-producing microorganisms, especially clostridia (Adams and Stiefel, 1998; Nandi and Sengupta, 1998), have attracted much attention recently due to the increased interest of biological production of hydrogen. Many hydrogen-producing microorganisms belong to the genus *Clostridium*. The hydrogenase of several *Clostridium* species have been sequenced and characterized, including *C. pasteurianum* (Meyer and Gagnon, 1991), *C. acetobutylicum* (Santangelo et al., 1995; Gorwa et al., 1996), *C. perfringens* (Kaji et al., 1999) and *C. paraputrificum* (Morimoto et al., 2005). A sludge of mixed-culture has recently been used to produce hydrogen from wastewater (Fang et al., 2006). However, there is no information in literature so far about Fe-hydrogenase diversity of the mixed hydrogen-producing sludge.

In this study, a set of FHG-specific primers was identified from the DNA sequences of FHGs found in the GenBank. This primer set was then used to investigate the FHG diversity in an acidophilic rice-degrading sludge by polymerase chain reaction (PCR). The primer set was also used to monitor the change of hydrogen-producing microbial population in a batch reaction using the technique of quantitative real-time PCR (qRT-PCR).

2. Materials and methods

2.1. Primer design

The DNA sequences of FHGs of six clostridia available in the GenBank were retrieved and aligned using BioEdit (Hall, 1999). A set of two primers, designated as HydA-F and HydA-R, was consequently identified from the common conserved regions found in these FHGs.

2.2. DNA extraction and PCR

An acidophilic hydrogen-producing sludge from a recent study (Fang et al., 2006) was used for the diversity study. The sludge was sampled at the end of a batch reaction, which was conducted at pH 4.5 and 37 °C treating a rice slurry containing 5.5 g-carbohydrate l^{-1} with 85 mg of inoculated sludge as measured by volatile suspended solids (VSS). The sludge had a maximum hydrogen production rate of 2100 ml g-VSS^{-1} d^{-1} and a hydrogen yield of 346 ml g-carbohydrate^{-1}, corresponding to 62.6% of theoretical yield by assuming maximum conversion of 553 ml g-carbohydrate^{-1}. The effluent was composed mostly of acetate (27.3%) and butyrate (67.1%).

The same sludge was also used to seed a new batch of hydrogen production under the same conditions for the population study. Genomic DNA was extracted from the sludge sampled throughout the batch which lasted 130 h. The FHG fragments were then amplified by PCR using the primer set of HydA-F and HydA-R. All PCR amplifications were conducted in a 30 μl buffer (Pharmacia Biotech Inc., Piscataway, NJ) containing 200 μmol each of the four deoxynucleotide triphosphates, 15 mmol $MgCl_2$, 0.1 μmol of individual primers and 1U of *Taq* polymerase (Pharmacia Biotech Inc. Piscataway, NJ). An automated thermal cycler (iCycler IQ, Bio-Rad, Hercules, CA, USA) was used for PCR amplification using the following program: an initial denaturation at 94 °C for 7 min; 35 cycles of denaturation (30 s at 92 °C), annealing (30 s at 52 °C) and extension (30 s at 72 °C); and a final extension at 72 °C for 10 min. The amplified PCR products were subsequently stored at 4 °C.

2.3. Cloning and sequencing

The PCR-amplified products in the diversity study were used to build a clone library with the TA Cloning Kit (Invitrogen, Carlsbad, CA) as described previously (Fang et al., 2006). A total of 28 clones were selected. The FHG fragments inserted in the plasmids of these clones by the whole-cell PCR using the primer set of M13F (5′-CAGGAAACAGCTATGAC-3′) and M13R (5′-GTTTGATCCTGGCTCAG-3′). The PCR

products were purified using a kit (Promega, Australia), and sequenced using primer M13R, an auto sequencer (ABI model 377A, Perkin–Elmer) and the dRhodamine Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin–Elmer).

2.4. Phylogenetic analysis

The sequences were then manually edited using BioEdit (Hall, 1999), and compared with the reference microorganisms available in the GenBank by BLAST search (Altschul et al., 1990). The DNA and the protein sequences of FHG fragments obtained from this study were aligned with their closest sequences retrieved from the GenBank using BioEdit for the construction of respective phylogenetic trees using the neighbor-joining method (Satio and Nei, 1987) with MEGA 2.1 (Kumar et al., 1993). Bootstrap re-sampling analysis (Felsenstein, 1985) for 500 replicates was performed to estimate the confidence of tree topologies.

2.5. Quantitative real-time PCR

In this study, the FHG fragment concentration in each sample was quantified using the qRT-PCR method 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 run in triplicate. qRT-PCR was performed in a thermal cycler (iCycler IQ, Bio-Rad., Hercules, CA, USA). Cycling conditions began with an initial hold of 10 min at 95 °C, followed by 50 cycles consisting of 30 s each of denaturation at 95 °C, annealing at 52 °C and extension at 72 °C.

A plasmid with the FHG fragment insert, referred as HydA plasmid, was extracted and dissolved in water. The molecular weight (MW) of pCR®2.1 plasmid (3929 bps) was 2.39×10^6 (Invitrogen, 1999) and the MW of the FHG fragment (313 base pairs) inserted in the pCR®2.1 plasmid was estimated as $660 \times 313 = 2.06 \times 10^5$. Thus, the estimated MW of HydA plasmid was 2.60×10^6.

DNA concentration in the HydA plasmid solution was measured as 1.27×10^3 ng μl^{-1} using a UV spectrometer (UV-160, Shimadzu, Kyoto, Japan), based on the assumption that each A_{260} unit corresponds to 50 ng μl^{-1} of double-stranded DNA (Sambrook et al., 1989). Thus, the molecular concentration of HydA plasmid was 2.96×10^{11} molecule μl^{-1}. As each plasmid molecule containing one copy of insert, the concentration of the inserted FHG in HydA plasmid solution was 2.96×10^{11} copy μl^{-1}. This solution was 10-fold serially diluted and used as the standards to establish the qRT-PCR calibration curve.

2.6. Accession number

The nucleotide sequence data reported in this paper have been assigned by the GenBank the following accession numbers: DQ342012–DQ342019.

3. Results and discussions

3.1. A new Fe-hydrogenase gene primer set

DNA sequences of FHG of six *Clostridium* species available in the GenBank, including *C. perfringens* (AB016775), *C. saccharobutylicum* P262 (U09760, formerly *C. acetobutylicum*), *C. paraputrificum* (AB159510), *C. pasteurianum* (M81737), *C. thermocellum* (AF148212), and *C. saccharoperbutylacetonicum* (AY827554), were retrieved. A set of two primers was consequently identified from the common reserved regions found in these FHGs. The following primer set was identified as: HydA-F (5′-TCACCACAACAAATATTTGGT-3′, $T_m = 52.8$ °C) and HydA-R (5′-GCTGCTTCCATAACTCC-3′, $T_m = 53.3$ °C). This primer set was then used for the diversity and population analysis on this study.

3.2. Diversity of FHGs in the acidophilic sludge

Based on 16S rDNA analysis in a previous study (Fang et al., 2006), clones developed from the extracted DNA of the acidophilic sludge were classified into nine operational taxonomy units (OTUs), all of which were affiliated with genus *Clostridium*. Phylogenetic analysis shows that eight of the nine OTUs (96.4% of population) form a distinct group with *Clostridium* sp. 44a-T5zd, but only remotely relate to any *Clostridium* species that have been characterized so far.

For comparison with the 16S rDNA analysis, the FHG fragments of the same sludge were cloned and sequenced in this study. A total of 28 clones were recovered. The FHG fragments of these clones, containing an

Table 1
Abundance of OTUs based on Fe-hydrogenase gene fragments

OTU	No. of clones	Abundance (%)
HydA-24	13	46
HydA-10	5	18
HydA-21	4	14
HydA-6	2	7.1
HydA-7	1	3.6
HydA-16	1	3.6
HydA-20	1	3.6
HydA-25	1	3.6
Total	28	100

average of 313 nucleotides, were sequenced. They were subsequently classified into eight OTUs, each of which was composed of clones with over 99% similarities. Table 1 shows that, among the eight OTUs, HydA-24, -10 and -21 were the most abundant, accounting for 46, 18 and 14%, respectively, of the population.

For further comparison, the amino acid sequences of these Fe-hydrogenases were deduced from the corresponding DNA sequences by BioEdit (Hall, 1999). Fig. 1 compares these amino acid sequences and those of six know hydrogenases of *Clostridium* species. Results show that the Fe-hydrogenase amino acid sequences obtained in this study have a conserved region at positions 36–41, which distinguishes them from the six known hydrogenases. This region may likely be the fingerprint specific to this group of acidophilic hydrogen-producers.

Table 2 shows the calculated similarities of amino acid sequences between the eight newly identified Fe-hydrogenase fragments and the six known *Clostridium* species. A similar table was also compiled (but not shown) for the similarity analysis of DNA sequences. Results show that OTUs found in this study were rather similar to each other, with 75–97% similarities based on amino acid sequences and 77–98% based on DNA sequences. The highest similarity (97.1%) was found between HydA-24 and -25 with a difference of only three amino acids out of a total of 104.

Table 2 also shows that the similarity based on amino acids among the 14 hydrogenase genes (8 found in this study plus 6 known ones) is 39–97%, which is comparable to the similarity of 41–82% among the 6 known hydrogenase genes alone. The OTUs obtained in this study have low similarities to *C. saccharoperbutylacetonicum* and *C. thermocellum*, i.e. 39–59% based on amino acid sequences and 54–64% based on DNA sequences. Instead, these OTUs were more closely related to *C. pasteurianum*, *C. paraputrificum*, *C. saccharobutylicum*, and *C. perfringens*, with similarities of 72–85% based on amino acid sequences and 72–83% based on DNA sequences. This indicates the great diversity on Fe-hydrogenase among *Clostridium* species.

3.3. Phylogenetic analysis

Phylogenetic relationships of these newly identified FHG fragments and the known species are illustrated

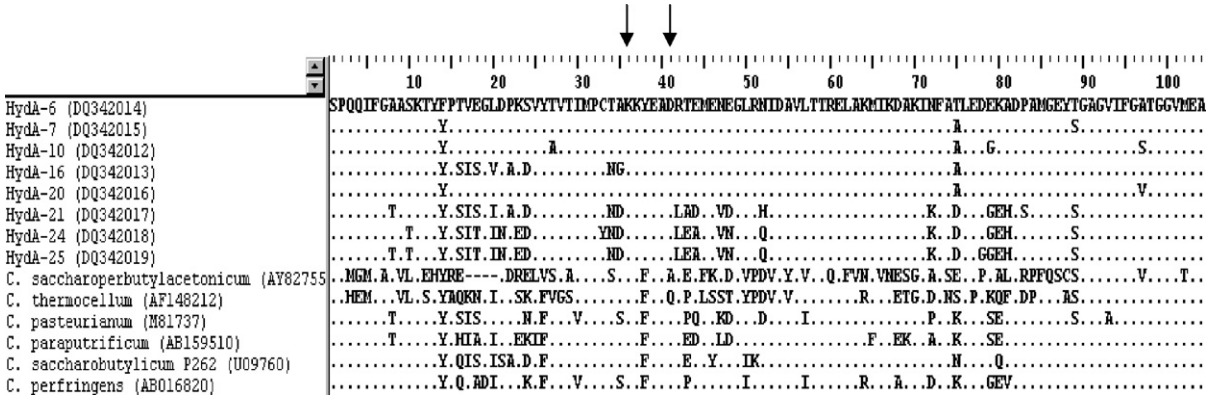

Fig. 1. Alignment of amino acid sequences of Fe-hydrogenases. The region between two arrows, positions 36–41, is the conserved fingerprint region specific to this group of acidophilic hydrogen-producers.

Table 2
Similarity comparison of amino acid sequences of Fe-hydrogenase gene fragments obtained in this study and those of known *Clostridium* species

Clostridium species and OTUs	*Clostridium* species						OTUs obtained in this study						
	1	2	3	4	5	6	HydA-6	HydA-10	HydA-7	HydA-20	HydA-16	HydA-21	HydA-24
2	0.51												
3	0.41	0.57											
4	0.44	0.59	0.82										
5	0.45	0.61	0.78	0.80									
6	0.42	0.62	0.82	0.80	0.82								
HydA-6	*0.43*	*0.57*	0.79	0.79	0.85	0.81							
HydA-10	*0.43*	*0.57*	0.78	0.78	0.83	0.81	**0.95**						
HydA-7	*0.45*	*0.59*	0.81	0.79	0.85	0.81	**0.97**	**0.96**					
HydA-20	*0.44*	*0.56*	0.78	0.78	0.84	0.80	**0.96**	**0.96**	**0.97**				
HydA-16	*0.43*	*0.55*	0.80	0.78	0.88	0.79	**0.90**	**0.89**	**0.91**	**0.90**			
HydA-21	*0.40*	*0.54*	0.81	0.78	0.78	0.74	**0.78**	**0.78**	**0.80**	**0.77**	**0.85**		
HydA-24	*0.39*	*0.54*	0.76	0.76	0.76	0.73	**0.77**	**0.77**	**0.79**	**0.76**	**0.81**	**0.88**	
HydA-25	*0.39*	*0.53*	0.77	0.77	0.75	0.72	**0.76**	**0.76**	**0.78**	**0.75**	**0.80**	**0.89**	**0.97**

Clostridium species: 1: *C. saccharoperbutylacetonicum*; 2: *C. thermocellum*; 3: *C. pasteurianum*; 4: *C. paraputrificum*; 5: *C. saccharobutylicum*; 6: *C. perfringens*.

in Fig. 2(a and b), respectively, according to their DNA and amino acid sequences. Some general features are found from Fig. 2(a and b). Eight OTUs may be classified into two groups, one comprises HydA-6, -7, -10, -16 and -20 and the other comprises HydA-21, -24 and -25, as evidenced by the high bootstrap values of 63–100%. These eight FHG fragments form a distinct branch in genus *Clostridium*, as previously shown from the phylogenetic analysis based on 16S rDNA (Fang et al., 2006). The FHGs were most closely related to *C. saccharobutylicum* (with 75–88% similarity based on amino acids), followed by *C. pasteurianum* (76–81%), *C. paraputrificum* (76–79%), and *C. perfringens* (72–81%). *C. saccharobutylicum* is a well-known hydrogen-producing, obligately anaerobic, spore-forming bacterium capable of fermenting various carbohydrates (Johnson et al., 1997). *C. pasteurianum* (Dabrock et al., 1992), *C. paraputrificum* (Evvyernie et al., 2000), and *C. perfringens* (Morimoto et al., 2005) are also known hydrogen producers.

3.4. qRT-PCR calibration curve and detection limit

The technique of quantitative real-time PCR has emerged recently, owing to the advanced development of fluorogenic chemistry, as an effective means for the detection and quantification of microorganisms at very low concentrations (Zhang and Fang, 2006). Comparing with the conventional hybridization- and PCR-based techniques, qRT-PCR not only has better sensitivity and reproducibility, but also is quicker to perform and has a minimum risk of amplicon carryover contamination.

In this study, a calibration curve was established for the HydA plasmid concentration (in Log_{10}) against the number of cycles needed to reach the detection threshold (C_t value). Fig. 3 illustrates a linear relationship over five orders of concentration, from 2.2×10^3 to 2.2×10^8 HydA-copy μl^{-1}. The slope of -3.38 (with a R^2 of 0.998) corresponds to a PCR efficiency of 97.7% (Devers et al., 2004).

The validity using Fig. 3 for the quantification of genomic DNA of the hydrogen-producing sludge was subsequently confirmed by an additional set of tests. The quantification of serially diluted solution of genomic DNA sample from the hydrogen-producing sludge was conducted to confirm the PCR efficiency

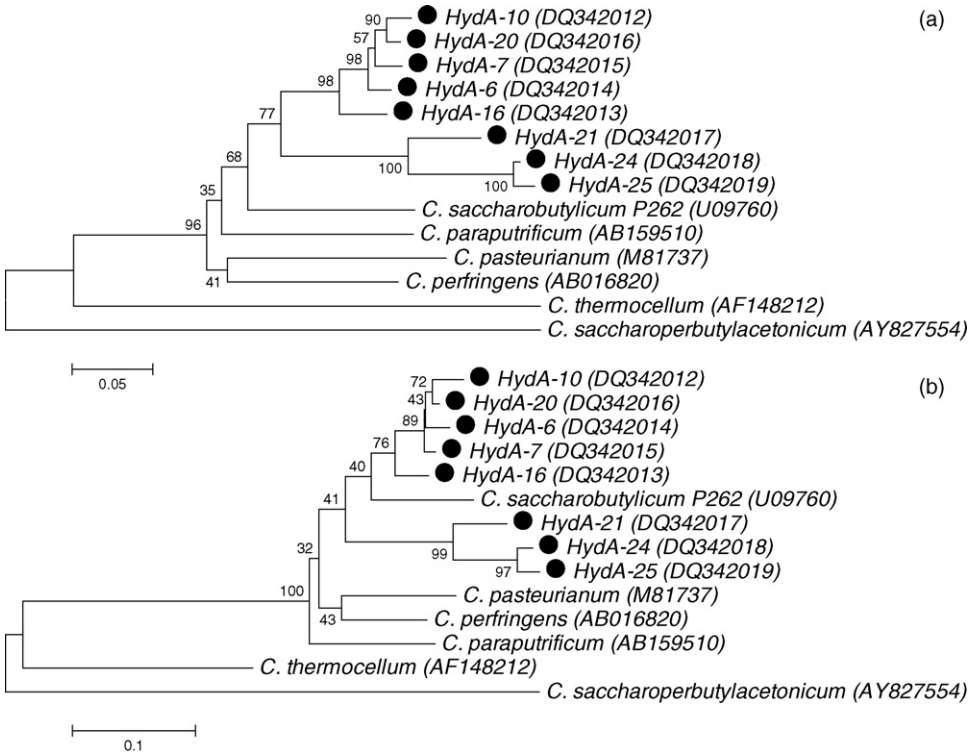

Fig. 2. Phylogenetic trees of Fe-hydrogenase based on (a) DNA sequences; and (b) amino acid sequences.

using the primer set. A plot similar to Fig. 3 also shows a linear relationship between the concentration of the serially diluted solutions and the corresponding C_t values. The PCR efficiency was 95.4%, as estimated from the slope of −3.44 (with a R^2 of 1.000).

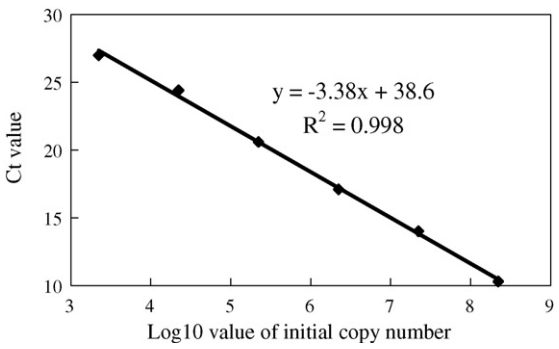

Fig. 3. Calibration curve of C_t vs. Log_{10} value of Fe-hydrogenase gene copy number.

3.5. Quantification of FHGs in hydrogen-producing sludge

Six sludge samples were taken in a batch experiment for genomic DNA extraction at 0, 24, 48, 72, 96 and 130 h. The hydrogenase primer set of HydA-F and HydA-R was then used to amplify the FHG fragments from these extracts. The image of the agarose gel stained ethidium bromide in Fig. 4 shows that the length of these PCR products was about 300 bps, comparable to the 313 bps average found in the FHGs of the six known *Clostridium* species. This further confirms the validity of the primer set of HydA-F and HydA-R. The specificity of the PCR amplification was also confirmed by the melting curve analysis, in which only a single peak was shown.

Using the calibration curve in Fig. 3, the FHG concentrations in the hydrogen-producing sludge samples at various time intervals were determined. The cell number of hydrogen-producers in each sample

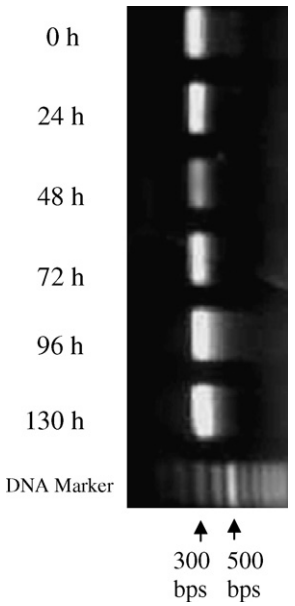

Fig. 4. Images of FHG fragments on agarose gel obtained by using the primer set of HydA-F/HydA-R.

was estimated by assuming that each cell contained one copy of FHG. The result was then used to estimate the generation time of hydrogen-producing cells, and the rate of hydrogen production. Fig. 5(a) illustrates the growth curve of hydrogen-producers over a 130 h period at pH 4.5. It shows that the hydrogen-producing bacteria population increased exponentially during the first 72 h and then leveled off as substrate became depleted. Fig. 5(b) illustrates the hydrogen accumulation over the same time period. The hydrogen production increased substantially after 38 h of lag time and then gradually leveled off after 90 h. During the exponential growth period, the average generation time for hydrogen-producing bacteria is estimated as 4.2 h, similar to the 4.5 h of *C. cellulolyticum* (Gelhaye et al., 1993) but longer than the 0.58 h of *C. botulinum* (Prescott et al., 2002). The maximum specific hydrogen production rate was 7.0×10^{16} H_2-molecule cell^{-1} h^{-1}.

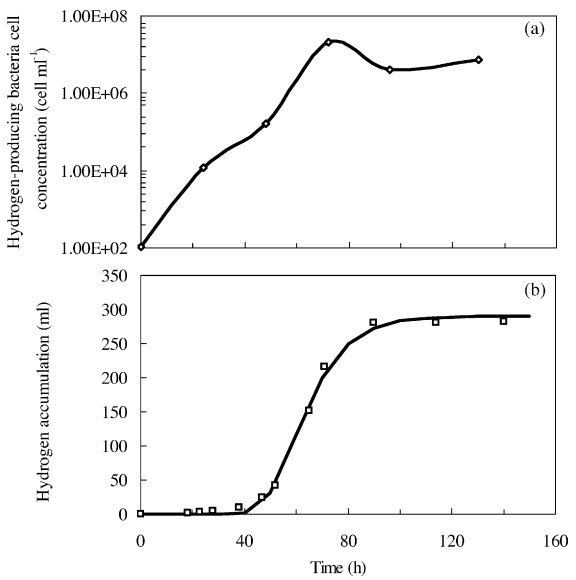

Fig. 5. (a) Concentration increase of hydrogen-producers; and (b) the corresponding hydrogen accumulation in a batch experiment.

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