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ENVIRONMENTAL BIOTECHNOLOGY

Flow-FISH analysis and isolation of clostridial strains in an anaerobic semi-solid bio-hydrogen producing system by hydrogenase gene target

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Abstract By using hydrogenase gene-targeted polymerase chain reaction (PCR) and reverse transcriptase PCR (RT-PCR), the predominant clostridial hydrogenase that may have contributed to biohydrogen production in an anaerobic semi-solid fermentation system has been monitored. The results revealed that a *Clostridium pasteurianum*-like hydrogenase gene sequence can be detected by both PCR and RT-PCR and suggested that the bacterial strain possessing this specific hydrogenase gene was dominant in hydrogenase activity and population. Whereas another *Clostridium saccharobutylicum*-like hydrogenase gene can be detected only by RT-PCR and suggest that the bacterial strain possessing this specific hydrogenase gene may be less dominant in population. In this study, hydrogenase gene-targeted fluorescence in situ hybridization (FISH) and flow cytometry analysis confirmed that only 6.6% of the total eubacterial cells in a hydrogen-producing culture were detected to express the *C. saccharobutylicum*-like hydrogenase, whereas the eubacteria that expressed the *C. pasteurianum*-like hydrogenase was 25.6%. A clostridial strain M1 possessing the identical nucleotide sequences of the *C. saccharobutylicum*-like hydrogenase gene was then isolated and identified as *Clostridium butyricum* based on 16S rRNA sequence. Comparing to the original inoculum with mixed microflora, either using *C. butyricum* M1 as the only inoculum or co-culturing with a *Bacillus thermoamylovorans* isolate will guarantee an effective and even better production of hydrogen from brewery yeast waste.

Keywords *Clostridium* · Hydrogenase · Fluorescence in situ hybridization · Flow cytometry

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Introduction

Microbial production of hydrogen may solve not only the problems caused by organic waste but also create an alternative energy source in the near future. This is why fermentative biohydrogen production has been considered as an environmentally friendly process for recovering hydrogen from wastewater (Ueno et al. 1996) or solid waste (Lay 2000). As the general environmental engineering approaches for hydrogen production are performed in an open system, mixed microflora with species diversity usually makes the system difficult to operate in a stable manner. Isolation and characterization of the major hydrogen-producing contributors from each substrate-specific fermentation system should be helpful for operating the system and elevating hydrogen-producing efficiency. As traditional microbiological methods have a hard time distinguishing hydrogen-producing bacteria from other co-existing microorganisms in a fermentation system, limited information is available for biological processes with natural microflora, not to mention that the isolation and identification of major hydrogen-producing contributors in the system is difficult. Nowadays, methods for cellular characterization or 16S rRNA-based methods combining with other techniques, such as quinone profiles (Hiraishi

1988), denaturing gradient gel electrophoresis (DGGE) polyamine patterns (Auling et al. 1991), flow cytometry analysis (Amann et al. 1990), and immunofluorescence (Cloete and Steyn 1988; Howgrave-Graham and Steyn 1988; Palmer et al. 1993; Völsch et al. 1990) have been developed to analyze the composition of bacterial community in activated sludge (Nielsen et al. 1999), biofilms (Zhang and Fang 2000), ocean mats (Muyzer et al. 1993), and sediments (Fang et al. 2002; Devereux and Mundfrom 1994; Snaidr et al. 1997). However, these approaches could not reveal sufficient information on the contribution of specific microbes with particular metabolic activity.

Most hydrogen-evolving microorganisms synthesize [Fe]-hydrogenases, which contain iron-sulfur clusters and two Fe atoms at their active center, to catalyze the hydrogen production from protons and electrons (Cammack 1999). In a fermentative batch culture for hydrogen production, the cellular [Fe]-hydrogenase mRNA level at acidogenic stage is high, then the [Fe]-hydrogenase mRNA level decreases when the culture enters solventogenic stage (Gorwa et al. 1996). These variations always proportionally correlate with the hydrogenase protein level in cells and indicate that the expression of hydrogenase activity is regulated at the transcriptional level (Gorwa et al. 1996; Chang et al. 2006). In addition, most members of the genus *Clostridium* are known for evolving hydrogen during anaerobic fermentation (Esteso et al. 1996; Kataoka et al. 1997; Chen et al. 2005; Karube et al. 1982), their existence in many hydrogen-producing cultures had been reported (Fang et al. 2002; Lay 2001; Chang et al. 2006). For the above reasons, clostridial hydrogenase mRNA was considered as a key clue for monitoring and identifying the major clostridial strains that contributed to hydrogen production in a fermentation system. In a previous study, the predominant clostridial hydrogenases that may contribute biohydrogen production to an anaerobic semi-solid fermentation system have been detected by hydrogenase gene-targeted PCR and RT-PCR. A specific *Clostridium pasteurianum*-like hydrogenase (GenBank accession no. M81737) was detected from both DNA and RNA templates, whereas a specific *Clostridium saccharobutylicum*-like hydrogenase (GenBank accession no. U09760) was detected only from the RNA template. These reproducible results strongly suggested that the clostridia possessing these two specific hydrogenase genes were the major hydrogen producers in the system. The clostridial strain possessing the specific *C. pasteurianum*-like hydrogenase may be dominant at both RNA level and population level, whereas the clostridial strain possessing the specific *C. saccharobutylicum*-like hydrogenase may be less dominant in population (Chang et al. 2006). To verify this conclusion, hydrogenase gene-targeted fluorescence in situ hybridization (FISH) combining with flow cytometry analysis was used in this study to monitor the specific clostridial cells in our hydrogen-producing system. Based on the nucleotide sequences obtained from hydrogenase gene-targeted RT-PCR, the most likely candidates that contributed to hydrogen production were isolated and further characterized. These isolates were used to reconstruct a co-culture system for converting a complex substrate into biohydrogen.

Materials and methods

Preparation of compost inoculum

The inoculum used in batch hydrogen-producing fermentation was prepared as previously described (Lay 2000). The compost that was prepared with rice straw and cattle dung was heated at 85°C for 3 h to remove non-endospore-forming bacteria. Ten grams of heated compost was mixed with 100 ml of distilled water and stirred for 3 h. Let the suspension stand still to settle down large particles, then the supernatant was used as inoculum. According to the observation of previous studies (Lay 2000, 2001), the supernatant contained hydrogen-producing clostridia.

Fermentative culture

Batch hydrogen-producing fermentation was carried out in a 100-ml serum bottle with a working volume of 80 ml. Each liter of growth medium contained the following: brewery yeast waste, 18.75 g; NH_4HCO_3, 80 g; KH_2PO_4, 40 g; NaCl, 0.4 g; $FeCl_2$, 0.11 g; $MgSO_4{\cdot}7H_2O$, 4 g; $Na_2MoO_4{\cdot}2H_2O$, 0.4 g; $CaCl_2{\cdot}2H_2O$, 0.4 g; and $MnSO_4{\cdot}7H_2O$, 0.6 g. This medium was prepared without sterilization. For general hydrogen production test, 64 ml of compost inoculum was inoculated to 16 ml of growth medium in a serum bottle. After flushing with N_2, the bottle was sealed with a rubber stopper and an aluminum crimp. For testing the ability of clostridial isolates in augmenting hydrogen production, the serum bottle containing 16 ml of growth medium and 64 ml of distilled water was flushed with N_2 and sealed first, then 1 ml of clostridial isolate culture (OD_{600}=2.0) in a peptone, yeast extract, glucose (PYG) medium (Chang et al. 2006) was inoculated by a syringe. Cultivation was grown at 40°C with agitation at 200 rpm.

Quantification of hydrogen production

The percentage of hydrogen in biogas was determined by a gas chromatograph (GC-8A, Shimadzu) equipped with a thermal conductivity detector and a stainless steel column (3.2 mm in inner diameter, 2 m in length) packed with Porapak Q (50/80 mesh). The operational temperatures of

the injection port, the oven, and the detector were 100, 60 and 100°C, respectively. Nitrogen was used as the carrier gas at a flow rate of 10 kPa.

Isolation of RNA and genomic DNA

Samples were harvested from the hydrogen-producing fermentation batch culture at middle exponential phase. After spinning at low speed (2,000×g, 5 min) to remove nondegraded solid material and impurities, the supernatant was centrifuged at 8000×*g* for 5 min to collect bacterial cells. The bacterial cells were washed once with 750 ml of STE buffer (10 mM Tris–HCl, pH 8.0, 1 mM ethylenediamine tetraacetic acid, 0.1 M NaCl) and suspended in 150 μl of STE buffer containing lysozyme (5 mg/ml), then the cell suspension was incubated at 37°C for 30 min. After homogenizing cell mixture with Mini-Beadbeater (Biospec Products, Bartlesville, Oklahoma), genomic DNA was purified by using the Ultraclean Soil DNA Isolation Kit (Mo Bio Laboratories, USA), then the Micro-Elute DNA Clean/Extraction Kit (GeneMark, Taiwan) was used to eliminate contaminants from DNA solution. The RNA template for RT-PCR was purified by using RNeasy Mini Kit (Qlagen, USA) and followed by the RQI DNase system (Promega, USA) to remove DNA contamination.

PCR and RT-PCR reaction and sequencing analysis

By using the Accessquick RT-PCR kit (Promega Biotech), RT-PCR amplification was performed with primer pairs Elf/Elr and L1f/Llr (Table 1), and the purified total RNA was used as template. After initial heating to 94°C for 3 min and holding at 48°C for 45 min, 35 cycles of 94°C (30 sec), 48°C (1 min), and 68°C (1 min) were performed for all the RT-PCR reactions. One RNA sample was pretreated with RNase A as negative control. The PCR products obtained were cloned to pGEM-T (Promega Biotech) and subjected to DNA sequencing. Nucleotide sequences were analyzed with the BLAST program (Basic Local Alignment Search Tool, http://www.ncbi.nlm.nih.gov/BLAST/) and phylogenetic analysis was performed by ClustalW algorithm (Feng-Doolittle and Thompson) for optimal alignment, and alignment analysis was performed by the global alignment algorithm (Wilbur and Lipman) for fast alignment with the DNAMAN Version 4.11 program (Lynnon Biosoft).

Specific clostridial hydrogenase gene probes

The clostridial hydrogenase gene-specific probes used in this study were designed by comparing the partial nucleotide sequences from nine clostridial hydrogenase genes (Fig. 1). These sequences include five clostridial hydrogenases gene found in Genebank database (accession numbers NP346675, AAA23248, NP563262, AAA85785, and AF148212), RT-PCR products RT-KE2 and RT-KE6 (accession numbers AAU88252 and AAT76847), and the hydrogenase genes (accession numbers AAU88256 and AAU88255) of two clostridial isolates (*Clostridium* Z5-1 and *Clostridium* Z2) previously obtained from our system (Chang et al. 2006). The nucleotide sequences of RT-KE2 and RT-KE6 represented the hydrogenase genes of two dominant hydrogen-producing clostridia in our fermentative system, and these two genes encode a specific *C. pasteurianum*-like hydrogenase and a specific *C. saccharobutylicum*-like hydrogenase, respectively (Chang et al. 2006). The non-conserved regions among these sequences were used to design specific probes for RT-KE2 and RT-KE6, and both probes (PRT-KE2 and PRT-KE6) were labeled with fluorescein isothiocyanate (FITC).

Fluorescence in situ hybridization and flow cytometry analysis

Cell samples that were harvested from fermentative batch culture at actively hydrogen-producing stage were fixed with 4% paraformaldehyde and then preserved in 50% ethanol in phosphate buffer solution (PBS; 130 mM NaCl, 10 mM $NaHPO_4$, pH 7.2) at −20°C. The preserved cells were pelleted and then resuspended in 200 μl of hybridization buffer (0.9 M NaCl, 0.01% sodium dodecyl sulfate, 20 mM Tris–HCl buffer, pH 7.2) containing 5 ng/μl of FITC-labeled oligonucleotide probe (PRT-KE2, PRT-KE6, or EUB 338; Table 1). After incubating the cell suspension

Table 1 The primers and probes used for RT-PCR and PCR in this study

Primers/probes	Sequence	Specificity	Reference
E1f	GCTGATATGACAATAATGGAAGAA	hydrogenase gene	Chang et al. 2006
E1r	GCAGCTTCCATAACTCCACCGGTTGCACC	hydrogenase gene	Chang et al. 2006
L1f	AAATCACCACAACAAATATTTGGTGC	hydrogenase gene	Chang et al. 2006
L1r	ACATCCACCAGGGCAAGCCATTACTTC	hydrogenase gene	Chang et al. 2006
PRT-KE6	TAAGCCTTCCACTGTAGG	RT-KE6	AY652730
PRT-KE2	TAATCCTTCAACTGCTGG	RT-KE2	AY652729
EUB 338	GCTGCCTCCCGTAGGAGT	16S rRNA of eubacteria	Amann et al. 1990

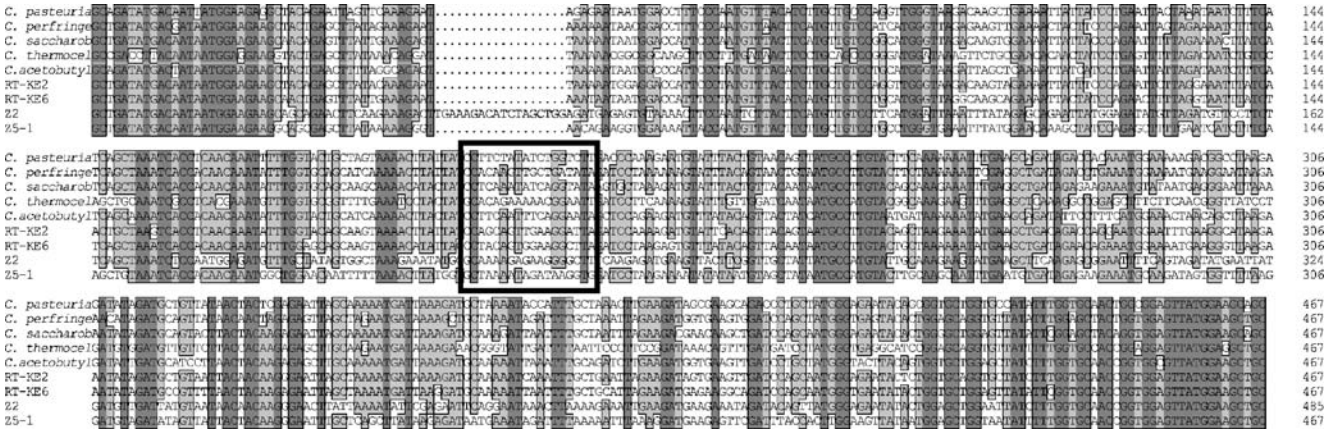

Fig. 1 Alignment of the partial nucleotide sequences of nine clostridial hydrogenase genes, which includes five clostridial hydrogenases found in Genebank database, RT-PCR products of RT-KE2 and RT-KE6, and clostridial isolates Z5-1 and Z2 from our system. (The probe design region was marked with *rough outline*)

at 50°C for at least 12 h, nonspecific binding was washed with 1×PBS washing buffer. Then, the cell suspension was passed through FACS Calibur flow cytometer (Becton Dickinson, NJ, USA), and collected data were analyzed with WinMDI2.8 (J. Trotter, 1993–1998, PUCL, USA).

Isolation of bacterial stains

One milliliter each of mixed microflora samples from fermentative batch culture was heated in 85°C water bath for 15 min to exclude non-spore-forming vegetative cells. Then, the cell samples were inoculated into 15-ml anaerobic pressure tubes containing 10 ml of PYG medium [each liter contained: peptone, 5 g; tryptone, 5 g; yeast extract, 10 g; K_2HPO_4, 2 g; cysteine HCl, 0.5 g; $FeSO_4$, 1.1 mg; resazurin, 10 mg; $CaCl_2$ (anhydrous), 0.2 g; $MgSO_4{\cdot}7H_2O$, 0.48 g; K_2HPO_4, 1.0 g; KH_2PO4, 1.0 g; $NaHCO_3$, 10 g; NaCl, 2 g; glucose, 10 g; glutathione, 0.25 g] and incubated at 40°C. Test tubes were sealed with rubber stoppers and screw caps and flushed with sterile nitrogen gas. After five times of transfer, enrichment cultures were streaked onto a solid PYG medium containing 1.5% agar and 0.25% phenylethyl alcohol and incubated in a GasPak jar (BBL GasPak 100TM Anaerobic System). Isolated colonies were transferred to and maintained in a liquid PYG medium for further analysis.

Results

Detecting the specific bacterial cells by FISH and flow cytometry

The clostridia possessing the *C. pasteurianum*-like or *C. saccharobutylicum*-like specific hydrogenase genes might be the major hydrogen producers in the system (Chang et al. 2006). To distinguish these bacterial cells, cell samples at the middle exponential phase of fermentative culture were subjected to fixation treatment, after FISH was preformed with probes PRT-KE6, PRT-KE2 (Table 1) under optimized conditions to detect these specific clostridial cells. Another same process was also carried out, except for using probe EUB338 to detect all eubacterial cells in the samples (data not shown). According to the Side Scatter (SSC) and Forward Scatter (FL1) statistics of the bacterial cells that were subjected to FISH treatment without any probe (negative control), the basal point of quarter line was set at X=560 and Y=336 (Fig. 2a). The results after detecting a cell sample with probe EUB338 and counting with flow cytometry revealed that of the 74.3% of the total counted signals, 10,000 cells represented eubacterial cells (Fig. 2b). On the other hand, 25.6% of the total counted signals represented the predominant hydrogen-evolving bacteria that expressed the specific *C. pasteurianum*-like hydrogenase and were detected by probe PRT-KE2 (Fig. 2c). Whereas only 6.6% of the total counted signals represented the predominant hydrogen-evolving bacteria that expressed the specific *C. saccharobutylicum*-like hydrogenase and were detected by probe PRT-KE6 (Fig. 2d). The mean fluorescent intensity of the counted particles in the cell samples without adding the probe was 25.75, and the mean values of cell sample hybridized with probes EUB338, PRT-KE2, and PRT-KE6 were 66.98, 30.01, and 28.91, respectively (Fig. 3). These results suggested that the hydrogen-producing clostridia represented a large portion of inhabitants in the sample. The mean value of fluorescent intensity obtained after probing with PRT-KE2 and PRT-KE6 was less than that of probing with EUB338, this may be due to the expression level of 16S rRNA genes, which is higher than that of hydrogenase gene.

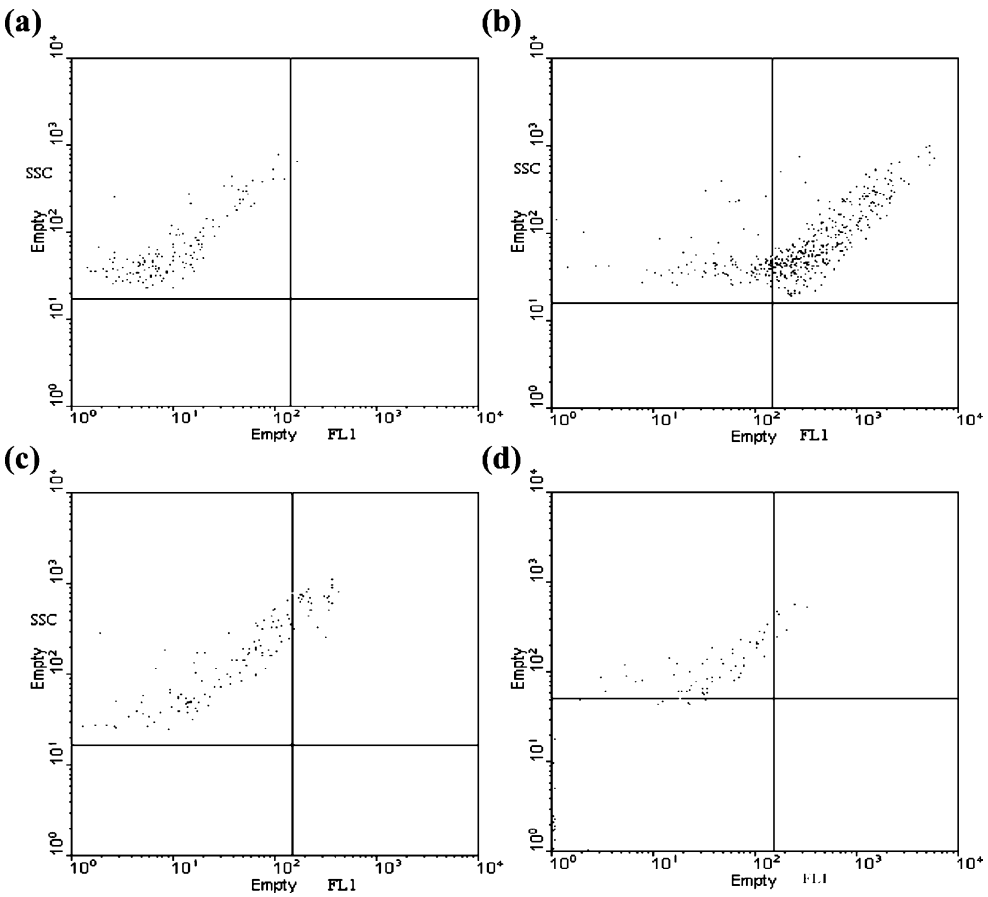

Fig. 2 Flow cytometric analysis of the bacterial sample from fermentative culture. The bacterial cells were subjected to FISH treatment with different probes. **a** non-probe negative control, **b** probe EUB338, **c** probe PRT-KE2, and **d** probe PRT-KE6. *SSC* is the cell complexity detected by the side-angle light scatter. *FL1* is the signal fluorescence intensity. The *basal point* of quarter line is at (*X*=560, *Y*=336)

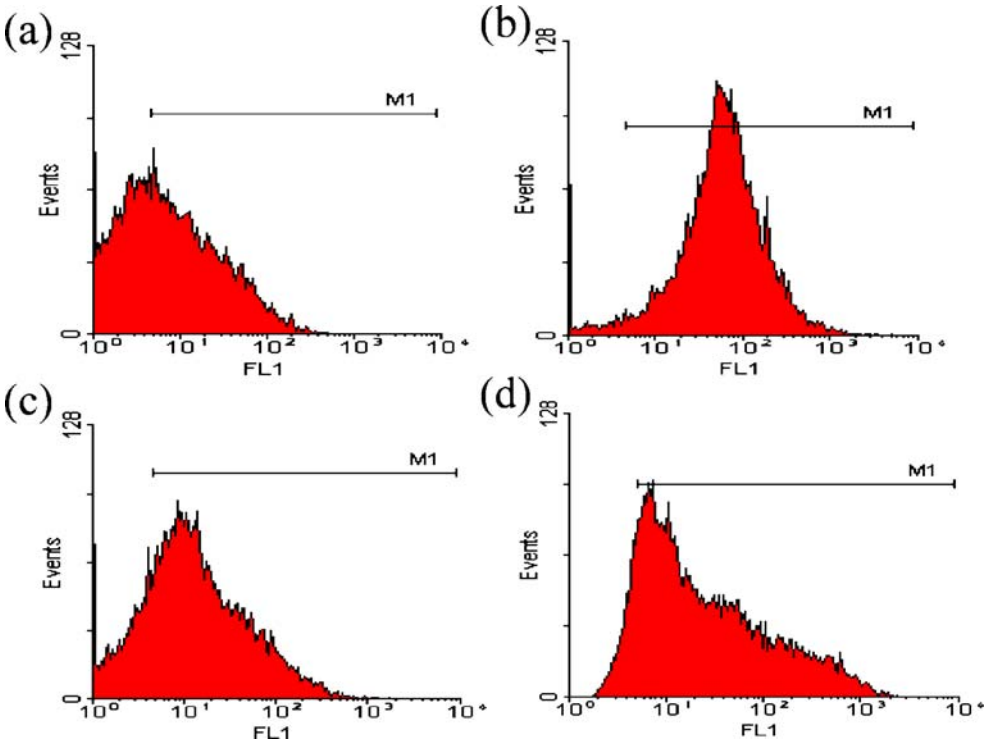

Fig. 3 The mean fluorescent intensity analysis of the bacterial sample from fermentative culture, the bacterial cells were subjected to FISH treatment with different probes by flow cytometry. **a** non-probe negative control, **b** probe EUB338, **c** probe PRT-KE2, and **d** probe PRT-KE6. *SSC* is the cell complexity detected by the side-angle light scatter. *FL1* is the signal fluorescence intensity. The mean fluorescent intensity is counting with the *M1 bar region*

Identification and characterization of the clostridial isolates

To isolate the predominant hydrogen-producing clostridia in the fermentative culture, bacterial cells collecting at optimal hydrogen-producing phase was heated at 80°C to kill non-spore forming bacteria, and then anaerobically cultivated on PYG agar containing 0.25% phenylethyl alcohol to selectively inhibit Gram-negative bacteria. The growing colonies were checked for hydrogen production, and their hydrogenase genes and 16S rRNA genes were sequenced. Finally, five clostridial isolates designated as Z2, Z4-4, Z5-1, M1, and W8, were isolated from the culture and showed close relationship in phylogenetic analysis (Fig. 4), but only the hydrogenase nucleotide sequence of isolate M1 showed 100% identity to that of the specific *C. saccharobutylicum*-like hydrogenase gene (RT-KE6 and RT-KL4, GenBank accession no. AAT76847; Fig. 5). Meanwhile, the similarity in hydrogenase nucleotide sequence among these isolates is only 75–85%. Owing to the 16S rRNA nucleotide sequence of isolate M1 (GenBank accession no. DQ462577) that showed 99% identity with that of *Clostridium butyricum*, isolate M1 was designated as *C. butyricum* strain M1.

The strain M1 is a strict anaerobe, when incubated aerobically, no growth could be found on a solid or liquid PYG medium. After growing anaerobically on PYG agar for 1 week, M1 formed small (1 to 2 mm in diameter), white, and lens-shaped colonies with a smooth edge. When M1 was grown anaerobically in 80 ml of PYG broth at 40°C, it had a doubling time of 1.98 h and could produce 12 ml (0.11 mol H_2/mol glucose) of hydrogen in 30 h.

To compare with the hydrogen productivity of original fermentative batch culture that inoculated with the microflora suspension prepared from a compost, 1 ml (OD_{600}=

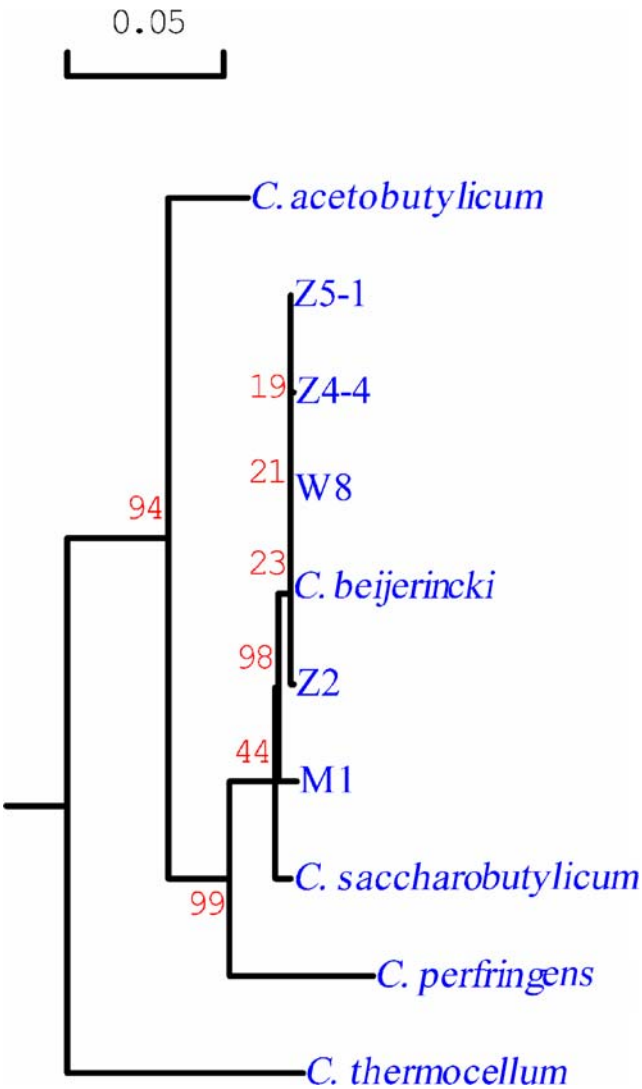

Fig. 4 Phylogenetic analysis of 16S rRNA sequences of the isolated strains and five published species of clostridial strains

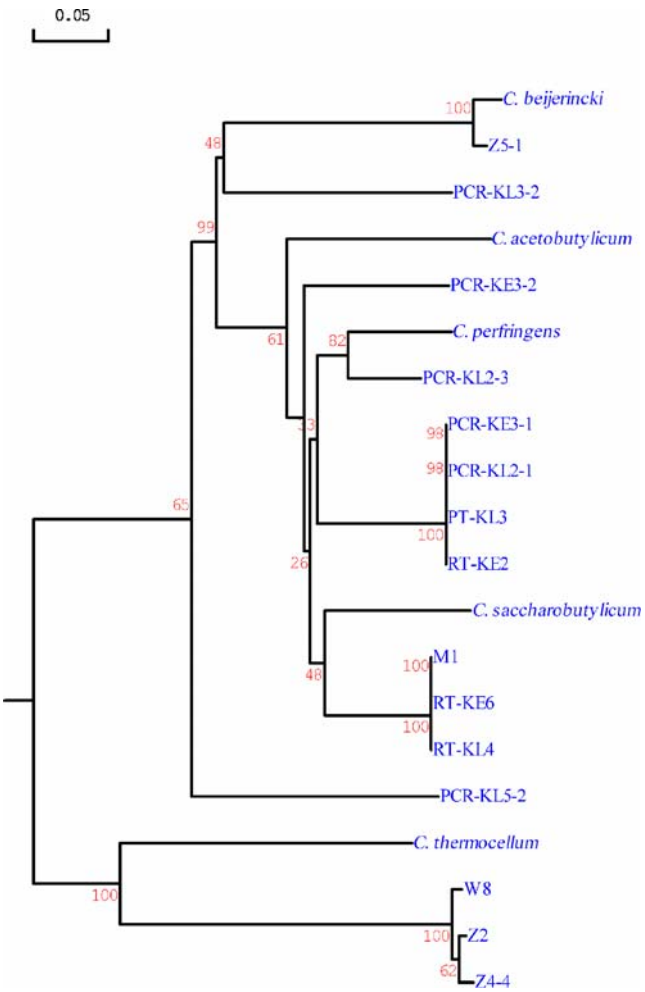

Fig. 5 Phylogenetic analysis of all the hydrogenase nucleotide sequences from RT-PCR, PCR, *C. butyricum* strains M1 (accession no. DQ462578), other isolated strains (M1, Z2, Z4-4, Z5-1, and W8), and five clostridial hydrogenases (accession numbers NP346675, AAA23248, NP563262, AAA85785, and AF148212) of GenBank. The bootstrap values were shown between the branch nodes

2.0) of *C. butyricum* M1 culture on PYG broth was inoculated into 80 ml of brewery yeast waste medium with anaerobic pre-treatment by N_2 flushing and sterilization, the same amount of medium but without inoculation was used as control. After 40 h of growing period, the batch culture with *C. butyricum* M1 produced twofold more concentration (40%) and producing rate (0.6 ml H_2/h) of hydrogen than the culture inoculated with the microflora suspension (Fig. 6b and c). The evolution of hydrogen by *C. butyricum* M1 continued for more than 100 h, then the accumulation rate slowed down (Fig. 6c) and almost reached its maximal production of about 50 ml after 180 h (Fig. 6a). This result showed that inoculation of *C. butyricum* M1 could shorten the production process and still produce as much hydrogen as the original inoculum of the mixed microflora.

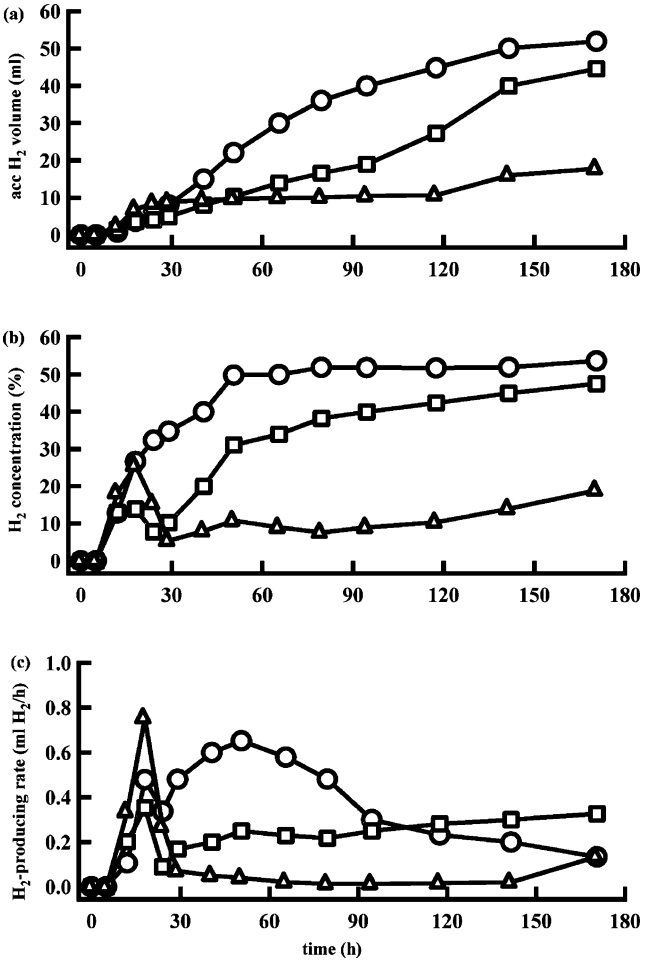

Fig. 6 The hydrogen productivity of the batch fermentative culture using brewery yeast waste as substrate. (*circle*) Inoculated with *C. butyricum* M1; (*square*) inoculated with a microflora of compost that was made from wasted rice straw and cattle dung; (*triangle*) non-inoculated control. **a** Accumulated volume of hydrogen, **b** hydrogen concentration, and **c** hydrogen-producing rate

Isolation and identification of a *Bacillus thermoamylovorans* strain

Except for the hydrogen producer, *Clostridium*, genus such as *Klebsiella* and *Bacillus* were also predominant population in our system, and they may also contribute to the biohydrogen production (Chang et al. 2006). To clarify their possible role, bacterial cells collecting at optimal hydrogen producing phase were enriched and then aerobically cultivated on PYG agar. The growing colonies were checked, 16S rRNA genes were sequenced, and as one of the isolate showed 99% identity to that of *B. thermoamylovorans*, the isolate was then designated as *B. thermoamylovorans* strain I (GenBank accession no. DQ860186). By culturing test on a skin milk agar plate and starch agar plate, the *B. thermoamylovorans* isolate could show the degrading activity of protease and α-amylase with clear zones (data not shown). The strain was also examined for hydrogen production, and the ability was not identified (data not shown).

Co-culture of the *C. butyricum* M1 and *B. thermoamylovorans* I for hydrogen production

To reconstruct the effective bacterial community for hydrogen production, 1 ml (OD_{600}=2.0) of both strain cultures on PYG broth was inoculated into 80 ml of sterilized brewery yeast waste medium without anaerobic pre-treatment by N_2 flushing. After 96 h of growing period, the co-cultured batch produced more than threefold hydro-

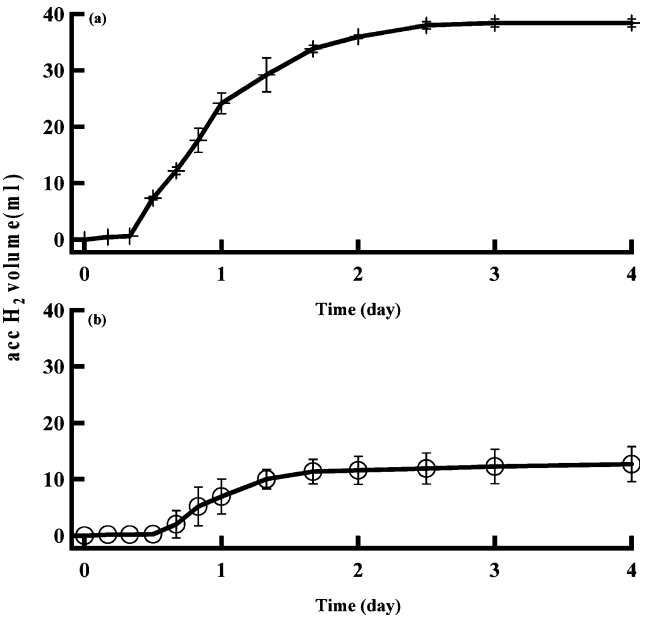

Fig. 7 The accumulated volume of hydrogen production profile of the batch fermentative culture using brewery yeast waste as substrate. **a** Co-culture with *C. butyricum* M1 and *B. thermoamylovorans*. **b** Inoculated with *C. butyricum* M1 only

gen gas (about 35 ml; Fig. 7a) than that of *C. butyricum* M1 (only about 12 ml; Fig. 7b). As the *B. thermoamylovorans* I could not perform hydrogen production, the result suggested that the *Bacillus* may function as an oxygen consumer and help to create an anaerobic condition for *C. butyricum* M1 to work.

Discussion

By monitoring the activity expressed by functional gene, we developed a strategy for detecting and isolating the major players with specific metabolic ability such as hydrogen production in a mixed microflora system. The mRNA expressed by functional genes may represent the specific markers of real microbial activity. Thus, mRNA-targeted molecular analysis may offer us further information about how the microbial species contribute to the particular metabolic activity in a mixed microflora system. In a previous study, in addition to DGGE analysis of the microflora in a fermentation culture, hydrogenase-targeted RT-PCR provided us further information of bacterial strains responsible for anaerobic hydrogen production (Chang et al. 2006). It was surprising that only the nucleotide sequences of two different hydrogenase genes could be detected with high reproducibility. One belongs to the *C. pasteurianum*-like hydrogenase gene (GenBank accession no. M81737) and could be obtained from PCR products of both DNA and RNA templates, whereas the other belongs to *C. saccharobutylicum*-like hydrogenase gene (GenBank accession no. U09760) and could be obtained only from the RNA template. These reproducible results strongly suggested that the clostridial strains possessing these two different hydrogenase genes were the candidates of major hydrogen producers in the culture. The bacterial strain possessing the specific *C. pasteurianum*-like hydrogenase may be dominant at both RNA level and bacterial population level, whereas the bacterial strain possessing the specific *C. saccharobutylicum*-like hydrogenase may be less dominant in bacterial population (Chang et al. 2006). In this study, the result of FISH-flow cytometry analysis indicated that the signals detected by PRT-KE2 showed more population than probe PRT-KE6 and supported the speculation that bacterial strains expressing specific *C. pasteurianum*-like hydrogenase were dominant at both RNA level and bacterial population level. Our results also demonstrate the value of the functional gene mRNA-targeted molecular approaches for knowing the dynamics of the fermentative culture. However, it was noticed that the mean value of bacterial self-fluorescent intensity was close to that of the cell sample hybridized by either probe PRT-KE2 or PRT-KE6 (Fig. 8), and the overlapped region of the mean fluorescent intensity of each sample may affect the

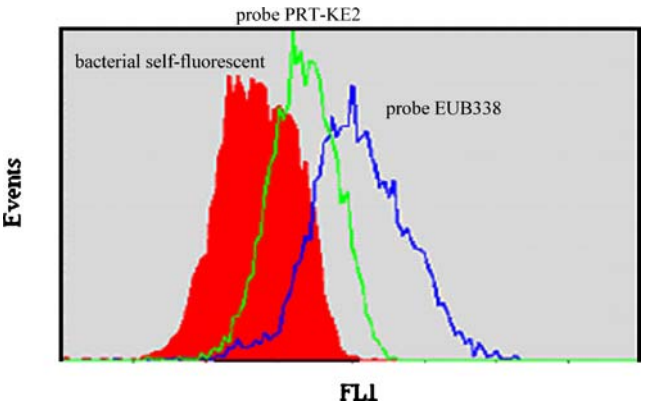

Fig. 8 The mean fluorescent intensity comparison between different probes (bacterial self fluorescent, probe PRT-KE2, and probe EUB338) of the bacterial sample from fermentative culture by FISH-flow cytometry

counting of cell number. The difference of the mean value of cell sample hybridized with probes EUB338, PRT-KE2, and PRT-KE6 may be due to the difference in transcriptional quantity of 16S rRNA gene and hydrogenase gene in a cell. The 16S ribosome RNA not only existed with this higher copies but also displayed with higher mean fluorescent intensity than the hydrogenase gene mRNA in a cell. To overcome the problem, a molecular biotechnique such as hydrogenase mRNA-targeted in-situ RT-PCR should be developed to amplify the fluorescent intensity.

Although the bacterial strain possessing the specific *C. pasteurianum*-like hydrogenase has not been isolated from the culture yet, another potential major player, *C. butyricum* M1, that could be detected by hydrogenase gene-targeted RT-PCR and FISH-flow cytometry was isolated and characterized. The result from inoculation of the isolate, *C. butyricum* M1, supports the idea that isolation and augmentation of the major hydrogen-producing microbes from the specific fermentation system should be helpful to operate the system and elevate the hydrogen-producing efficiency. The previous result of 16S RNA gene-targeted PCR-DGGE analysis also confirmed the existence of dominant clostridial species in our hydrogen-producing culture, this result also supported that the heating treatment of cell culture before inoculation was an effective method for enriching endospore-forming clostridial species (Chang et al. 2006). However, some aerobic *Bacillus* species and non-endospore-forming species of Enterobacteriaceae family such as *Klebsiella pneumoniae* and *Salmonella enterica* were also found, they might come from unsterilized brewery yeast waste (Chang et al. 2006). As some aerobic *Bacillus* species and facultative anaerobic members of Enterobacteriaceae are also known as hydrogen producers and anaerobic environment creators, their contributions in our hydrogen-producing system need to be elucidated. In fact, the result from co-culture of *C.*

butyricum M1 and *B. thermoamylovorans* I suggested that *Bacillus* species were good partners for creating an anaerobic environment for hydrogen producing clostridia, and the original mixed microflora system can be simplified to a bi-member fermentation system. Most importantly, these isolates are preservable for running the system and are helpful to operate the system stably. This may greatly contribute to construct an industrial plant for converting a complex substrate such as brewery yeast waste into bioenergy.

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