Biotechnology Letters **24:** 1833–1837, 2002.

Microbial analysis of a phototrophic sludge producing hydrogen from acidified wastewater

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Received 9 July 2002; Revisions requested 31 July 2002; Revisions received 3 September 2002; Accepted 3 September 2002

Key words: hydrogen, microbial analysis, phylogenetic, *Rhodobacter capsulatus*, wastewater

Abstract

Based on DNA-cloning analysis, the microbial community of a phototrophic sludge producing H_2 from an acidified wastewater was composed of 81% of a species resembling *Rhodobacter capsulatus* (with 99.2% similarity) and two unidentified species of the *Bacillus*/*Clostridium* group. The sludge produced a biogas comprising $82 \pm 2\%$ H_2, $13 \pm 2\%$ CO_2, $4.5 \pm 0.5\%$ N_2, and $0.5 \pm 0.2\%$ H_2S.

Introduction

H_2 is a clean energy, producing water as the sole product upon combustion. Many have predicted that it may replace fossil fuel as the energy of the next generation. H_2 is also an important industrial commodity. It is traditionally produced by hydrocarbon reformation or electrolysis of water. However, it may also be produced biologically by either autotrophic or heterotrophic microorganisms. Autotrophs, such as green algae and cyanobacteria, produce H_2 by splitting water using light and CO_2 as energy and carbon sources. Heterotrophs, such as acidogenic bacteria and phototrophic bacteria, produce H_2 by fermentation. Acidogenic bacteria convert complex organic substrates into H_2 and fatty acids, whereas phototrophic bacteria convert simple fatty acids and alcohols into H_2 and CO_2 using light as energy source. It is thus possible to harvest H_2 from wastewater in a two-step process using these two distinct groups of heterotroph.

Most of H_2 production studies have been conducted using pure cultures. Mixed acidogenic cultures have only recently been used for H_2 production from wastewater (Fang *et al.* 2002). Pure phototrophic cultures have been studied for H_2 production from only a few wastewaters, including those from productions of starch, sugar, and tofu. However, it is impractical to treat a wastewater using pure cultures, and yet there is no information in literature on H_2 production from wastewater using mixed phototrophic culture. This study was thus conducted to investigate the phylogenetic diversity, using the 16S rDNA-based techniques, of a mixed phototrophic sludge which was developed to produce H_2 from acidified wastewater.

Materials and methods

Phototrophic production

A mixed phototrophic sludge culture was first enriched from the sediment of a local reservoir. The enrichment was carried out in an anaerobic batch reactor at 30 °C using a phototrophic Medium 27 (DSMZ, Germany) and a light intensity of 200 W/m^2 (370 to 1060 nm). After eight sequential batches, the enriched sludge was used to seed the 450 ml complete-mix cylindrical photo-reactor (ACE Glass Incorporated, USA). The reactor was illuminated with a tungsten lamp at the center with a light intensity of 90 to 150 W/m^2 (370–1060 nm), as measured by a radiometer (IL 1400 Radiometer, International Light Inc., USA). A synthetic wastewater comprising acetate (900 mg l^{-1}), butyrate (1000 mg l^{-1}) and ethanol (100 mg l^{-1}) was used

as feed. The compositions of these substrates were chosen to simulate the effluent from an acidogenic H_2-producing reactor (Fang *et al.* 2002). In addition, each liter of the wastewater was also dosed with the following nutrients (as mg): monosodium glutamate 200, yeast extract 200, KH_2PO_4 500, $MgSO_4 \cdot 7H_2O$ 400, $NiCl_2 \cdot 6H_2O$ 0.02, NaCl 400, $CaCl_2$ 50, Fe(III) citrate 5, 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, and vitamin B_{12}0.04. Ar was used to purge the wastewater to ensure the anaerobic condition, because dissolved N_2 would inhibit the H_2 production of nitrogenase. The photo-reactor was operated with a hydraulic retention of 25 h at pH 8 and 32 °C. The compositions of biogas and effluent were analyzed following procedures reported previously (Fang & Liu 2002).

DNA cloning, sequencing and phylogenetic analysis

Mixed liquor was sampled from the photo-reactor for DNA extraction, using the method described previously (Zhang & Fang 2001). DNA fragments of 1501 base pairs were amplified by polymerase chain reaction (PCR) from the extracted DNA using the *Eubacteria*-specific primer set of GM3F (5′-AGAGTTTGATCMTGGCTCAG) and GM4R (5′-GGTTACCTTGTTACGACTT). The amplified products were then cloned using the TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA) (Zhang & Fang 2001). A total of 48 colonies were selected for plasmid recovery. The inserted DNA fragments in all the plasmids were sequenced using an auto sequencer (ABI model 377A, Perkin-Elmer, Foster City, CA) and dRhodamine Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer, Foster City, CA) with the primer set of M13 Rev (5′-GTTTGATCCTGGCTCAG), M13 Univ (5′-CAGGAAACAGCTATGAC) and 1055R (5′-CACGAGCTGACGACAGCCAT) (Wu *et al.* 2001).

Each DNA sequence was manually edited with BioEdit and checked for possible chimeric artifacts using the CHECK-CHIMERA program. All DNA sequences were compared for similarity with those available in the GenBank by BLAST search. They were then aligned with the closest 16S rDNA sequences retrieved from the GenBank using BioEdit, followed by a manual check. Phylogenetic trees were constructed using the neighbor-joining method with MEGA 2.1 (Fang *et al.* 2002). 'Bootstrap' re-sampling analysis for 500 replicates was performed to estimate the confidence of tree topologies.

Fluorescence in situ *hybridization (FISH)*

Three oligonucleotide probes were selected for FISH study: (a) EUB338 targeting for most of the *Eubacteria*, (b) Alf968 for most of the alpha-*Proteobacteria*, and (c) SRB385 for sulfate-reducing bacteria (SRB). Oligonucleotide probes were labeled in the 5′-end with either Cy3 or 6-carboxy-fluorescein (FAM) by Integrated DNA Technologies (Coralville, IA).

Sludge from the photo-reactor was fixed with an aqueous solution containing 4% (v/v) paraformaldehyde, air dried on a glass slide, and dehydrated sequentially by ethanol solutions of 50%, 80% and 96% for 3 min each. The dehydrated sample was air dried again, and hybridized with 1 μl each of the oligonucleotide probe solution (100 ng μl^{-1}) and 10 μl hybridization formamide-containing buffer (0.9 M NaCl, 0.01% SDS, 20 mM Tris/HCl, pH 7.2). The formamide concentrations in the buffer were 20% for the oligonucleotide probes of EUB338/Alf968 and 30% for probes of EUB338/SRB385. The hybridization was carried out at 46 °C in a sealed moisture chamber for 90 min, followed by incubation in a washing buffer (0.07 M NaCl, 0.01% SDS, 20 mM Tris/HCl, 5 mM EDTA, pH 7.2) at 48 °C for 30 min. The slide was then rinsed briefly with distilled water and air dried before examination under a laser scanning confocal microscope (LSM 5 Pascal, Zeiss, Jena, Germany). The populations of *Eubacteria*, alpha-*Proteobacteria* and SRB in the mixed liquor were estimated from the areas hybridized with the respective fluorescent probes using the image analysis software MetaView (Universal Imaging).

Accession numbers

The three nucleotide sequence data reported in this paper have been assigned the following accession numbers for the GenBank, EMBL and DDBJ databases: AY128088, AY128089 and AY128090.

Results

H_2-producing characteristics of the sludge

Phototrophic reaction of converting a simulated acidified wastewater into H_2 was carried out for over 30 d. By day 20, the photo-reactor had reached steady state,

judging from the compositions of biogas and effluent, as well as the H_2 production rate. During days 20–30, the bioreactor degraded 85 ± 1% of acetate, 89 ± 1% butyrate and 96 ± 2% ethanol, producing a CH_4-free biogas, comprising 82 ± 2% H_2, 13 ± 2% CO_2, 4.5 ± 0.5% N_2, and 0.5 ± 0.2% H_2S. The daily H_2 production rate by each gram of sludge (as measured by volatile suspended solids) was 135 ml.

Based on stoichiometry, each mole of acetate, butyrate, and ethanol can produce 4, 10, and 6 moles of H_2. Accordingly, each liter of feed solution containing 900 mg acetate l^{-1}, 1000 mg butyrate l^{-1} and 100 mg ethanol l^{-1} could produce a total of 148 mmol H_2. In this study, the H_2 conversion efficiency of the mixed phototrophic sludge culture was only 12% of the theoretical value. For comparison, the phototrophic conversion efficiency of malate was 12% by *Rhodobacter sphaeroides* OU 001 (Eroglu *et al.* 1998), and that of lactate varied from 7% by *R. sphaeroides RV* (Fascetti & Todini 1995) to 24% by *Rhodobacter capsulatus* (Tsygankov *et al.* 1998).

Sequencing and phylogenetic analysis

The DNA sequences of 48 colonies revealed that the phototrophic H_2-producing sludge was composed of only three populations. The predominant population, labeled as PSB-M-1 (Accession number AY128090), had 39 clones (81%), whereas the other two, labeled as PSB-M-2 (AY128089) and PSB-M-3 (AY128088), had 6 and 3 clones, respectively. Figure 1 illustrates the phylogenetic tree of the three species identified in this study and their respective close relatives based on full 16S rDNA sequence. By comparing the DNA sequences of these three populations with those available in the GenBank, the predominant PSB-M-1 was most like a member of genus *Rhodobacter*, resembling *R. capsulatus* with 99.2% DNA similarity. *Rhodobacter* is a phototrophic purple non-sulfur bacteria genus in the sub-division of alpha-*Proteobacteria. R. capsulatus* is previously known as *Rhodopseudomonas capsulata, Rhodonostoc capsulatum, Rhodopseudomonas capsulatus* and *Rhodonostoc capsulatum.* Its phototrophic H_2-producing characteristics had been well documented (Tsygankov *et al.* 1998).

PSB-M-1 was also closely related to three isolates of *Rhodobacter* (Accession numbers AB017796, AB017797, AB017798) with 99.4% similarity, and to *R. sphaeroides* with 95.8% similarity. PSB-M-2 and PSB-M-3 are likely members of the *Bacillus*/*Clostridium* group. PSB-M-2 was related closely

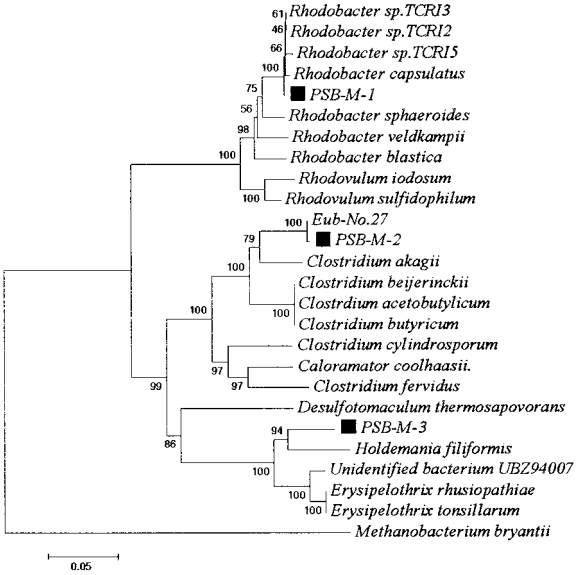

Fig. 1. The phylogenetic tree of the three species identified in this study and their respective close relatives based on full 16S rDNA sequence. The tree based on Jukes-Cantor distance was constructed using the neighbor-joining algorithm with 1000 bootstrappings. *Methanobacterium bryantii* was selected as the out-group species. The scale bar represents 0.05 substitution per nucleotide position. Numbers at the nodes are the bootstrap values.

to an uncultured bacterium Eub No. 27 with 99.2% similarity, followed by *Clostridium akagii* (93.5%) and *Clostridium butyricum* (92.3%). On the other hand, PSB-M-3 was most closely related to *Holdemania filiformis* with 92.3% similarity, distantly to an isolate of activated sludge (Accession number Z94007) with 91.7% similarity and to *Erysipelothrix tonsillarum* with 91.6%. Both *Holdemania* and *Erysipelothrix* are genera in the *Bacillus*/*Clostridium* group, which is also known as the low G+C subdivision.

Although 0.5% of H_2S was detected in the biogas, there was no SRB-related species detected in the mixed phototrophic H_2-producing sludge.

FISH

Out of the three species detected in the phototrophic H_2-producing sludge, only the *R. capsulatus* resembling PSB-M-1 was a member of alpha-*Proteobacteria.* Thus, the Alf968 probe specific for alpha-*Proteobacteria* was used for FISH analysis of PSB-M-1 in the mixed phototrophic H_2-producing sludge. A universal probe for *Eubacteria*, EUB336, was used for comparison. Figure 2 is the FISH image of the sludge. It illustrates that PSB-M-1 accounted

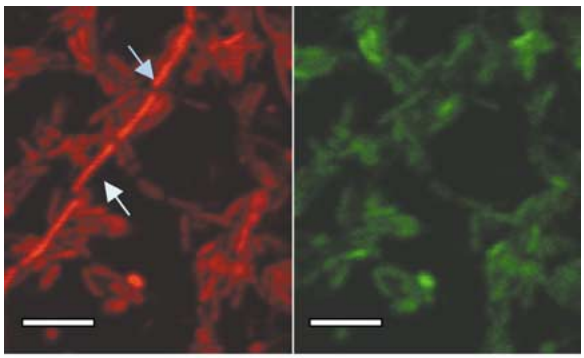

Fig. 2. FISH images of the phototrophic H_2-producing sludge: (a) cells hybridized with a *Eubacteria*-specific probe (EUB338) labeled with Cy3, and (b) cells hybridized with an alpha-*Proteobacteria* probe (Alf968) labeled with FAM. The arrows indicate the cells other than alpha-*Proteobacteria* (bar = 4 μm).

for 88 $\pm$ 6% of the total bacterial population, judging by the fraction of FISH image area emitted fluorescence of Alf968. Furthermore, the sludge hybridized with SRB338 did not show any fluorescent image, indicating the absence of SRB in the phototrophic H_2-producing sludge.

Discussion

Phototrophic H_2-producing bacteria

Microorganisms, such as algae and cyanobacteria, are capable of producing H_2. But, only phototrophic bacteria are heterotrophic and thus can be used for wastewater treatment. In this study, only one population resembling *R. capsulatus* was found in the mixed culture sludge even though many other phototrophic bacteria are capable of producing H_2, such as *Rhodospirillum* (Zürrer & Bachofen 1979), *Rhodovulum* (Yamada *et al.* 1998) and *Rhodopseudomonas* (Otsuki *et al.* 1998).

The predominance of a population resembling *R. capsulatus* in this H_2-producing sludge could be due to several reasons. Firstly, the original sediment sample from which the sludge was enriched might be rich in such a population and low in biodiversity. Secondly, unlike other phototrophic bacteria, *R. capsulatus* is capable of utilizing H_2 as an electron donor in the absence of others (Imhoff 1988) by a reversible, membrane-bound hydrogenase. Thus, as the organic substrates became depleted near the end of each batch enrichment process, *R. capsulatus* might out-compete other phototrophic H_2 producers. Thirdly, *R. capsulatus* often becomes predominant in an unselective media (Imhoff 1988), as during the enrichment process.

Other populations in the phototrophic H_2-producing community

In addition to *R. capsulatus*, two species in the *Bacillus*/*Clostridium* group were also found in the phototrophic H_2-producing sludge: a *Clostridium* and an unknown species. Many species in *Bacillus*/*Clostridium* group, especially *Clostridium*, are acidogenic H_2-producing microorganisms, including *C. acetobutylicum*, *C. butylicum*, *C. butyricum*, *C. kluyveri* and *C. pasteurianum* (Fang *et al.* 2002). However, it is uncertain whether the two unidentified *Bacillus*/*Clostridium* species found in this study were capable of producing H_2.

The presence of sulfate-reducing bacteria (SRB) could suppress the phototrophic H_2-production. Furthermore, the presence of sulfide even at low concentrations could inhibit the bioactivity of most purple nonsulfur bacteria; for example, the growth of *R. capsulatus* was not affected at 0.8 mM sulfide but was completely inhibited at 2 mM (Imhoff 1988). In this study, the H_2S concentration in biogas was 0.5%, equal to 0.08 μM in the mixed liquor. Results of this study show that H_2S at such a concentration does not inhibit the growth of *R. capsulatus* nor its H_2 production. Since the SRB were absent in the sludge based on results of cloning and FISH analysis, the H_2S detected in the biogas is likely to be produced from the yeast extract by the *Clostridium.*

Although N_2 can inhibit the H_2-producing capacity of nitrogenase, results of this study showed that N_2 at 4.5 $\pm$ 0.5% did not affect the H_2-producing activity of nitrogenase. Similar results were reported by Lichtl *et al.* (1997) that N_2 at 5.1% (v/v) did not inhibit the H_2 production by the nitrogenase of *Nostoc flagelliforme.*

Quantification of the microbial populations

In this study, the relative abundance of major species in the mixed H_2-producing culture was quantified by the abundance of clones and the fluorescent areas in the FISH image. The relative concentration of the predominant bacteria, *R. capsulatus*, was 81% by clone abundance and 88 $\pm$ 6% by FISH image. These results were consistent to each other, despite the potential bias in DNA extraction and PCR amplification.

Acknowledgement

The authors wish to thank the Hong Kong University Development Fund for the financial support of this study.

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