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Bio-hydrogen production from a marine brown algae and its bacterial diversity

Jae-Hwa Lee†, Dong-Geun Lee, Jae-Il Park, and Ji-Youn Kim

Department of Pharmaceutical Engineering, College of Medical Bioscience,
Silla University, Busan 617-736, Korea
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Abstract−The aim of this study was to determine how bio-hydrogen production was related to the composition of the bacterial community in a dark fermentation fed with marine brown algae (*Laminaria japonica*). The bacterial diversity was ascertained by 16S rDNA PCR-sequencing. A total of 444 mL of bio-hydrogen was produced from 10 g/L of dry algae in a 100 mL of culture fluid for 62 h. The pH varied from 8.74 to 7.05. Active bio-hydrogen production was observed from 24 to 48 h, and maximum bio-hydrogen production was 106 mL over 1 L gas. The bacterial community of the activated sludge consisted of 6 phyla, where H_2 producing and consuming bacteria coexisted. The only detectable bacterial phylum after bio-hydrogen generation with heat-treated (65 °C, 20 min) seeding was Firmicutes. *Clostridium* and *Bacillus* species constituted 54% and 46%, respectively, of the bacterial mixture and the most abundant species was *Clostridium beijierinckii* (34%). These results may provide a better understanding of how different bio-hydrogen communities affect hydrogen production and aid in the optimization of bio-hydrogen production.

Key words: Bio-hydrogen, Bacterial Diversity, Marine Algae, 16S rDNA

INTRODUCTION

The currently available energy sources for clean energy are thermonuclear energy, nuclear breeders, solar energy, wind energy, hydropower, geothermal energy, ocean currents, tides and waves [1]. However, all other types of energy sources, except for fossil fuels, can only be used as fuel through indirect methods such as electricity [1,2].

H_2 is traditionally produced by thermo-chemical or radiolytic methods; however, traditional methods are not economically valuable because they can only be applied where electricity is inexpensive. Hence, bio-hydrogen has gained considerable traction as a potential sustainable alternative to the conventional methods for H_2 production. Bio-hydrogen provides a sound elasticity for a sustainable energy system in view of the present energy crisis and environmental problems [3].

In addition to other physical and chemical sources, much interest has been focused on bioenergy such as bio-diesel, bio-ethanol, fatty acid (m)ethyl esters, bio-methanol, acetic acid, bio-hydrogen, and methane [4-6]. Kalia and Puroit [7] reported that biological H_2 production appears to be the most promising since it is non-polluting and can be produced from water and biological wastes. Bio-hydrogen production can be achieved through (i) biophotolysis of water by green algae and blue-green algae (cyanobacteria), (ii) photodecomposition of organic compounds by photosynthetic bacteria, (iii) dark fermentation of organic compounds, and (iv) hybrid systems such as a combination of fermentative and photosynthetic bacteria [3]. However, it is not economically practical to grow photosynthetic bacteria in large photo-bioreactors since the price of synthetic culture media is considerably high [8,9] and large surface areas or photo-bioreactors are required for photosynthetic bacteria. Hence, production of H_2 by fermentation of cheap organic materials may be more practical than photo-biological conversion [9].

A diverse range of factors have been studied with respect to fermentative biological hydrogen production such as the operating conditions (pH, temperature, hydraulic retention time and reactor type), feeding substrates, nitrogen, phosphate, metal ion, and source of inoculation [3,10]. Dasa and Veziroglu [3] identified two major aspects that are indispensable to optimization of biohydrogen production, namely, a suitable renewable biomass/wastewater and ideal microbial consortia that can efficiently convert biomass to hydrogen.

Park et al. [11] reported hydrogen production from marine large algae by sewage sludge microflora. There are several distinct advantages to using seaweeds as energy crops, which include the following: (i) no need for a large area for culture, (ii) the crop yield is considerably higher than that obtained on land, (iii) seaweed contains almost no non-degradable lignins, and (iv) many valuable extracts, such as alginate, can be extracted from the waste, which is important to environmental protection [12-14]. Park et al. [11] compared the heating time required for the seeding of activated sludge in regards to the production of bio-hydrogen; however, they could not determine what microbiota were present and responsible for bio-hydrogen production.

Community analyses are based on the identification of microbes. Traditional identification methods are based on culture, namely, the combination of isolating individual microbes and examining their physiological, biochemical, and morphological characteristics [15]. However, traditional methods have some limitations such as the inability to cultivate many environmental bacteria [16], failure of individual isolation by syntrophical growth of many microbes [17], and an uncertainty of several characteristics of microbes. To overcome the limitations of these culture methods, many molecular methods have been developed for the qualitative and quantitative analysis of microbial communities. All bacteria contain 16S rDNA; hence 16S rDNA-targeted methods have been extensively applied in the

†To whom correspondence should be addressed.
E-mail: jhalee@silla.ac.kr

study of wastewater [18] and tap water [16].

In this study, a microbial community that converts brown-algae into H_2 was analyzed using 16S rDNA analysis. *Laminaria japonica* was used as the substrate and the inoculating seed was activated sludge from a sewage treatment plant. Microbial diversity of this H_2 producing microbiota was analyzed by 16S rDNA based methods.

EXPERIMENTAL

1. Seeding Sludge and Feedstock

The methods used for seeding and feedstock were previously described by Park et al. [11]. Briefly, sludge was obtained from an anaerobic digester of a domestic sewage treatment plant at Su-young in Korea. Collected sludge was screened by a US standard No. 10-mesh (2.0 mm) sieve, and then heat treated (65 °C, 20 min). Brown algae, *Laminaria japonica,* were harvested from the Il-kwang coastal area in Korea and washed with fresh water to remove salt, sand and epiphytes. Washed seaweed samples were dried at room temperature. Dried samples were finely milled with a ball miller (DW-BM5, Dongwon Scientific Co., Korea), following thermal treatment in an autoclave (HB-506-6, Hanbaek Scientific Co., Korea) at 120 °C for 30 min. Materials were purchased from Sigma (St. Louis, MO, USA) and Aldrich (Milwaukee, WI, USA).

2. Operation Condition

Hydrogen production experiments were performed in 300 mL serum bottles with a working volume of 100 mL. Each bottle was filled with cooled seaweed (1 g), inoculum (1 mL), nutrient stock (1 mL), and phosphate buffer (0.07 M, pH 7.5). The nutrient stock solution was diluted to a liter can containing the following: 2.0 g of NH_4HCO_3, 1.0 g of KH_2PO_4, 0.01 g of $MgSO_4 \cdot 7H_2O$, 0.001 g of NaCl, 0.001 g of $Na_2MoO_4 \cdot 2H_2O$, 0.001 g of $CaCl_2 \cdot 2H_2O$, 0.0015 g of $MnSO_4 \cdot 7H_2O$ and 0.00278 g of $FeCl_2$. Bottles were wrapped in aluminum foil to eliminate substrate photolysis, and nitrogen gas was flushed to remove oxygen within the headspace for 10 min. The operational condition was 35 °C and 150 rpm without an inlet for gas. The gas accumulating in the head-space of the serum bottles was measured and sampled periodically for analysis of hydrogen content. Following gas measurements, samples were discarded to prevent possible errors, such as gas leakage, associated with the sampling procedure. Before each sampling event, a tedlar bag was used to equilibrate the pressure inside the bottle to the ambient pressure, and the volume in the tedlar bag was recorded and added to the total volume measured in the headspace. The hydrogen produced was calculated from the concentration of hydrogen in the headspace.

3. Analysis of Hydrogen

The composition of hydrogen and methane in the gaseous product was analyzed by using a high-density hydrogen and methane gas detector (Electrochemical Sensor, Model No. XP-3140, Comos Inc., Japan). The output signal displayed the % volumes of hydrogen and methane in the headspace of the fermentor, which were converted to mL/L. The system was calibrated once every two days by using the calibration cap provided with the instrument.

Standard methods were used to evaluate pH [19].

4. DNA Extraction, Amplification and Analysis of 16S rDNA

The 16S rDNA sequence analysis was performed to determine the composition of the bacterial community in the samples. Template genomic DNA was extracted and purified according to the manual provided with the bacterial genomic DNA prep kit (SolGent. SGD62-S120). Briefly, the sludge and fermented culture broth were centrifuged at 10,000 ×g for 10 min to harvest the bacterial community. Pellets were resuspended with 300 µl of the cell resuspension solution after the supernatant was discarded. Two µl lysozyme (100 mg/ml, Sigma) was added and incubated at 37 °C for 1 hour. After centrifugation (10,000 ×g, 1 min), the pellet was resuspended with 300 µl of cell lysis solution. Protein precipitation solution (100 µl) was added and vigorously vortexed for 20-30 seconds. The supernatant was transferred to a new 1.5 ml micro tube containing 300 µl of 100% isopropanol after centrifuge (10,000 ×g, 5 min). The solution was mixed by gently inverting 50 times followed by centrifugation (10,000 ×g, 1 min). After discarding the supernatant, 500 µl of the washing buffer was added and the tube containing the solution was inverted several times to wash the DNA pellet. After centrifugation (10,000 ×g, 1 min), the DNA pellet was air dried and 50 µl of elution buffer was added. This genomic DNA was used for PCR.

PCR amplification of the 16S rDNA sequences was carried out using the eubacterium specific primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GACGGGCGGTGTGTAC-3') [18]. After heat treatment at 95 °C for 15 min, 30 amplification cycles (95 °C for 20 sec, 50 °C for 40 sec, 72 °C for 1.5 min) were conducted following the final extension (72 °C, 5 min). In the case of bacteria, 1,500 bp DNA fragments were amplified. One µl of purified genomic DNA was added to 25 µl of the PCR reaction solution. Amplified DNA fragments were confirmed to be bacterial 16S rDNA with a length of 1,500 bp by agarose electrophoresis.

After purification of PCR products using the PCR purification kit (SolGent, SPP02), the T-Blunt cloning Kit (SolGent, SOT01-K020) was used to clone the 16S rDNA PCR products. Transformed *E. coli* was grown and selected from LB plates containing 100 µg/ml ampicillin and 50 µg/ml kanamycin after incubation at 37 °C for 16 hours. Colony PCR was done in 25 µl of the PCR reaction solution (SolGent). Primers were M13(-20)F/M13R T-Blunt vector primers(M13(-20) F: 5'-GTAAAACGACGGCCAGT-3', M13R: 5'-AGCGGATAACAATTTCACACAGGA-3'). After heat treatment at 95 °C for 2 min, 30 amplification cycles (95 °C for 20 sec, 58 °C for 40 sec, 72 °C for 1.5 min) were conducted following the final extension (72 °C, 5 min). Amplification of 1,600 bp DNA fragments was confirmed by agarose electrophoresis.

The purified PCR products, which were approximately 1,600 bp in length, were sequenced using M13(-20)F primer. Sequencing was performed with a Big Dye terminator (Cycle sequencing kit, Applied BioSystems, USA). Sequencing data was analyzed by NCBI blast software.

RESULTS AND DISCUSSION

1. Hydrogen Production

Fig. 1(a) shows the effect of heating the seed on hydrogen production. All heating treatments showed a similar production of hydrogen until 37 h. Hydrogen production from non-heated inoculums stopped at 37 h, while heated seeding conditions yielded hydrogen production up to 62 h. The total amount of generated hydrogen was 250 mL and 444 mL from non-heated seeding and heated seeding,

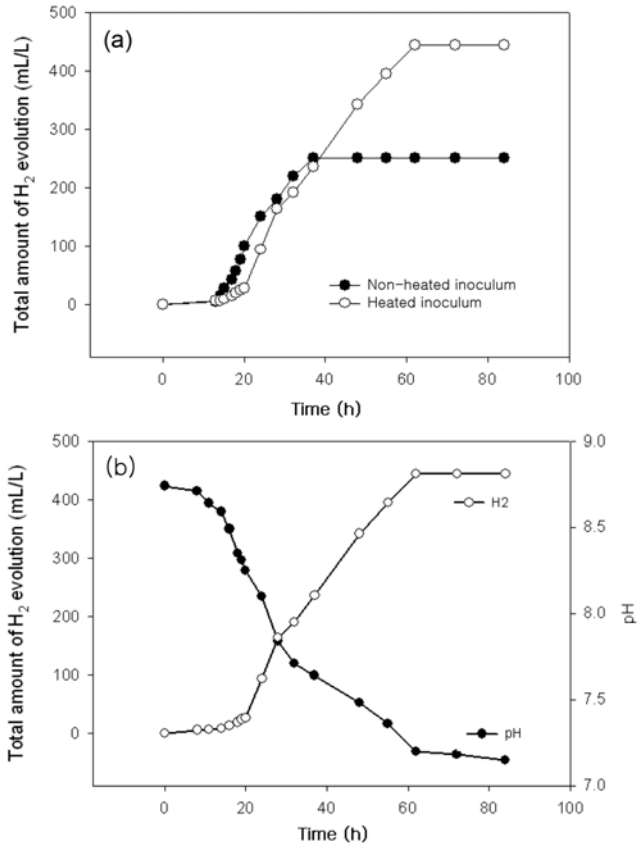

Fig. 1. Hydrogen production with or without heat-treatment of the seed (a) and change of hydrogen production and pH (b) for the heat-treated seed in a 300 mL serum bottle mixed at 150 rpm and 35 °C. The seed was heat treated (65 °C, 20 min) activated sludge obtained from a sewage treatment plant and the substrate was marine brown algae, *Laminaria japonica*. The concentration of the seed and substrate was 1%. Values shown are averages of bottles run in duplicate.

respectively, from 10 g/L of dry algae (*Laminaria japonica*) in a 100 mL of culture fluid for 62 h. Heat-treated inoculum produced 77.6% more hydrogen compared to the non-heated inoculum.

Fig. 1(b) represents the variation of pH and production of hydrogen as a function of time under the same condition used in Fig. 1(a). The total amount of H_2 produced was 444 mL and a total of 2,045 mL of CH_4 was generated from 10 g/L of dry algae (*Laminaria japonica*) in a 100 mL of culture fluid for 62 h. Park et al. [11] reported that the amount of H_2 produced by fermentation was 1,075 mL/L. In the study by Park et al. [11], a gas collector system was used as the test fermentor, while we did not use a gas collector system. As a result of this change, it is plausible that inhibition of H_2 production and/or consumption occurred. Bio-hydrogen production was detected after 8 h (5.9 mL/L). Park et al. [11] did not detect bio-hydrogen at 6 h, which may have been due to the adaptation and concentration of the hydrogen producing bacteria. Active production of bio-hydrogen and a decrease of pH was observed after 24 h (Fig. 1(b)). Bio-hydrogen production increased more than 10-fold from 24 to 48 h compared to the initial 24 hour-period. Maximum hydrogen production, 106 mL-H_2/L-total gas, was observed after 48 h in this study, while Park et al. [11] detected maximal hydrogen production at 39 h in a 5 L fermentor. The bio-hydrogen production rate fluctuated from 24 to 48 h and declined after 48 hours. Very little production was detected after 72 hours. A decrease in pH was also observed as a function of time. Yang et al. [20] reported that the pH of the fermentation solution dropped significantly over the fermentation time, which likely resulted from the production of volatile fatty acids and CO_2 during the production of bio-hydrogen. Nath et al. [21] reported that microorganisms predominantly produce acetic and butyric acids together with hydrogen gas from carbohydrates. Hence, the decrease in pH was most likely dependent on microbial activity. Wang et al. [10] postulated that pH was an important factor in bio-hydrogen production, since it may influence hydrogenase activity and the metabolic pathway of hydrogen-producing bacteria. It has been demonstrated that increasing pH over an appropriate range could increase the ability of hydrogen-producing bacteria to generate hydrogen during fermentative hydrogen production. Using cheese processing wastewater as a substrate, Yang et al. [20] reported a decrease in biogas production when the pH was decreased to 4.0; however, production was recovered after the pH was increased. In this study, the pH changed rather dramatically (8.1 to 7.4) between 24 and 62 h and active bio-hydrogen production was observed during this period. Park et al. [11] selected pH 7.5 phosphate buffer as the optimal buffering conditions after testing other buffers at different pH values. The pH was 7.48 after 48 h where maximum hydrogen production was observed. However, the pH varied from 8.70 to 7.04 during the experiment and changed from 8.25 to 7.20 during the 20 h to 62 h time period (Fig. 1(b)). These results suggest that a more controlled pH may facilitate an increase in bio-hydrogen production. H_2 can be converted to CH_4 by methanogenic bacteria. Production of methane gas was not observed at pH 5.5, which may be due to the repression of methanogenic activity under acidic conditions such as when using probiotic wastewater [22] and glucose [23] as the substrate. However, Yang et al. [20] found that CH_4 was still produced even though the pH dropped below 5.0, which should inhibit methanogenesis. These reports indicate that more studies will be needed to further understand the effect of pH on bio-hydrogen production. Hence, we cannot conclusively say that the decline in bio-hydrogen production originated from a decrease in pH and/or substrate as shown in Fig. 1. The effect of SCOD, namely the substrate, is under investigation using a fed-batch test.

2. Bacterial Community

Microbial communities were analyzed before the sludge was heat-treated and after 62 h, where bio-hydrogen production was almost complete. Since heat-treated sludge was shown to increase hydrogen production, we quantified the bacterial composition of the fresh-activated sludge and the heat-treated sludge after bio-hydrogen production was almost complete. Table 1 represents the composition of the microbial community within the activated sludge before heat treatment. The phylogenetic assignment of the 50 clones based on bacterial divisions was conducted by using the EMBL/GenBank database in the BLAST network service; phylogenetic affiliations are in Table 1. Uncultured and unclassified bacterial sequences were 26% and 12%, respectively. Uncultured bacteria were bacteria that could not be cultured by the present methods. Unclassified bacteria were culturable, but their phylogenetic positions could not be determined. Diverse bacterial sequences were detected, except for uncul-

Table 1. Composition of bacterial groups in the activated sludge used for bio-hydrogen production before heat-treatment based on culture-independent 16S rDNA analyses

Phylum	Closest species in GenBank	# of clones	Abundance (%)
Firmicutes		7	14
	Paenibacllus sp.	1	2
	Ruminococcus metamorphum	1	2
	Clostridiaceae bacterium WN101	1	2
	Pelotomaculum sp.	1	2
	Uncluted Clostridia	1	2
	Bacterium TC8	1	2
	Uncultured *Symbiobacterium* sp.	1	2
Proteobacteria		8	16
	Aquaspirillum metamorphum	1	2
	Pelobacter propionicus	1	2
	Uncultured Syntrophaceae	4	8
	Uncultured Rhodocyclaceae	1	2
	Curvibacter delicans	1	2
Verrucomicrobia		8	16
	Uncultured Verrucomicrobia	7	14
	Bacterium Ellin5102	1	2
Spirochaetes		3	6
	Uncultured Spirochetales	3	6
Bacteroidetes		2	4
	Uncultured Bacteroidetes	1	2
	Porphyromonadaceae bacterium	1	2
Chloroflexi		3	6
	Uncultured Chloroflexi	1	2
	Caldilinea aerophila	1	2
	Dehalococoides sp.	1	2
Unclassidied		6	12
	Candidate division OP1	2	4
	Cloacamonas acidaminovorans	3	6
	Bacterium rM4	1	2
Uncultured		13	26
	Uncultured bacterium	13	26
Total		50	100

tured bacterial sequences, and the predominant phyla were determined to be Proteobacteria (16%), Verrucomicrobia (16%), and Firmicutes (14%). Hydrogen is a key intermediate in the anaerobic digestion of organic matter and could be produced from wastewater [24] by suppressing the activity of hydrogenotrophic methanogens. However, little information is available on the hydrogen-producing microbial community. Bio-hydrogen production was observed regardless of heat treatment of the activated sludge; however, the volume of bio-hydrogen produced was small in the non-heat treated sludge [11]. Table 1 strongly implies the co-existence of hydrogen producer bacteria and degrading methanogens, which may result in the reduction of bio-hydrogen. Genes for bio-hydrogen production have been found in (*Candidatus*) *Cloacamonas acidaminovorans* [25], which indicates that they can produce bio-hydrogen at the appropriate conditions. Two uncultured Syntrophaceae- and a single *Dehalococcoides*-like sequences and one uncultured bacterium-like sequence were best matched with a sequence that originated from methanogenic communities [26-28].

Table 2 describes the composition of the bacterial community in the bio-hydrogen fermentor, which was fed brown-algae, after the bio-hydrogen production rate decreased. Only one phylum, Firmicutes, was detected. The lack of diversity in the microbial population was probably due to the heat treatment (65 °C, 20 min) of the seeding sludge [11]. According to a review by Kraemer and Bagley [29], heat treatment has been a commonly used method for killing methanogens (hydrogen-consuming microorganisms), leaving only sporogenic bacteria such as *Clostridium*, *Bacillus* and thermo-anaerobacterium. Only members of *Clostridium* and *Bacillus* were detected, where 54% of the 50 sequences were members of the *Clostridium* genus and the remaining 46% were members of the *Bacillus* genus. The most abundant known species were *Clostridium beijierinckii* (34%) and *Bacillus subtilis* (12%); however, a significant amount of an unknown *Bacillus* sp. was also present (16%). The *Clostridium* genus represents strict anaerobes that have a Gram positive cell wall

Table 2. Composition of bacterial groups after bio-hydrogen production with the heat-treated seed based on culture-independent 16S rDNA analyses

Class (Phylum)	Closest species in GenBank	# of clones	Abundance (%)
Clostridia		27	54
(Firmicutes)	*Clostridium beijierinckii*	17	34
	Clostridium acetobutyricum	4	8
	Clostridium butyricum	1	2
	Clostridium sp.	5	10
Bacillales		23	46
(Firmicutes)	*Bacillus subtilis*	6	12
	Bacillus megaterium	4	8
	Bacillus flexus	3	6
	Bacillus pulmilus	2	4
	Bacillus sp.	8	16
Total		50	100

structure, are unable to carry out dissimilatory sulfate reduction, and are capable of forming spores [30]. This genus also includes many species that have diverse metabolic activities and morphological properties [31]. Many Clostridia members are capable of producing bio-hydrogen, including *C. beijierinckii* [32], *C. acetobutylicum* [33], *C. butylicum* [34], *C. kluyveri* [35], and *C. pasteurianum* [36]. A totally of 44% of the 16S rDNA sequences were closest to bio-hydrogen producing *Clostridium* species such as *C. beijierinckii*, *C. acetobutylicum*, and *C. butylicum* (Table 2). The *Bacillus* genus represents strict or facultative aerobes that have a Gram positive cell wall structure and are capable of forming spores. In addition, this genus includes many species that have diverse metabolic activities and could be useful in the treatment of wastewater [18]. Many *Bacillus* species are able to produce bio-hydrogen. Porwal et al. [37] isolated and tested the capability of *B. subtilis*, *B. thuringiensis*, *B. cereus*, *B. licheniformis*, *B. pumilus*, *B. sphaericus*, and *Bacillus* sp. to produce bio-hydrogen. *B. subtilis*, *B. pulmilus*, *B. megaterium* and *B. flexus* were found in the bio-hydrogen producing fermentor (Table 2). *B. megaterium* was reported to produce bio-hydrogen [38] and *B. flexus* [39] was found to aid in bio-hydrogen production. A total of 30% of the 16S rDNA sequences were closest to bio-hydrogen producing *Bacillus* species such as *B. subtilis*, *B. pulmilus*, *B. megaterium* and *B. flexus* (Table 2). Kalia and Puroit [7] found that the *Bacillus* genus had many features appropriate for H_2 production: (i) they can survive under harsh conditions, hence could compete with other microbes (ii) they have large and versatile enzymatic activities such as lipase, amylase, protease, urease, cellulose, and lignin degrading laccase etc., hence a diverse range of different bio-wastes could be used as a substrate for bio-hydrogen production, (iii) they do not require light for H_2 production, (iv) *Bacillus* spores are being used as probiotics in humans and animals; thus, they pose no environmental health hazard, and (v) the *Bacillus* and *Clostridium* genus have different dehydrogenases, meaning that the Bacillus and Clostridial H_2 production systems operate under different metabolic controls. This may permit the co-existence of these two genus and may stimulate H_2 production at different conditions. In addition, Oh et al. [40] stated that less oxygen-sensitive facultative anaerobes are sometimes able to recover H_2 production activity after unintended oxygen damage by the rapid consumption of oxygen. Oh et al. [40] insisted that facultative anaerobes are better microbes than strict anaerobes for achieving fermentative hydrogen production. Nandi and Sengupta [41] also reported that the most common hydrogen-producing and facultative anaerobes are members of Class Enterobacteriaceae, Lactobacillaceae and Bacillaceae.

The combined results of this study may provide a better understanding of how the composition of different bio-hydrogen communities affects hydrogen production and aids in the optimization of bio-hydrogen production based on consideration of the composition of the bacterial community. Furthermore, isolation and study of each bacterium may result in more detailed understanding.

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