음식물 쓰레기로부터 분리한 *Clostridium* sp.의 고정화에 의한 생물학적 수소 생산

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Biological hydrogen production by immobilized cells of *Clostridium* sp. isolated from a food waste treatment process

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Introduction

Global climate change and environmental pollution due to the abuse and potential shortfall of fossil fuels make it important to search for alternative energy sources that are cost-effective, environmentally-friendly and renewable. Hydrogen (H_2) satisfies the above requirements because it has a higher energy yield (122kJ/g) by 2.75 times than hydrocarbon fuels and produces only water, not carbon combined end-product, when it is combusted as a fuel or converted to electricity. Among the hydrogen producing processes, biological methods can produce hydrogen from various renewable resources and are less energy intensive than chemical or electrochemical ones since they are carried out at ambient temperature and pressure. In Korea, food wastes have been regarded the main source of various environmental pollutions as they are the most abundant (11,464 t/d, 32.6% of municipal solid wastes) and the major recycling methods such as composting and feed stuffing are not appropriate to treat them due to the high salinity. Under this circumstance, employing food wastes as substrates for hydrogen production would be very attractive strategy because they are rich in carbohydrates and can provide carbon energy source for the mixed culture.

In this work, we isolated hydrogen-producing anaerobes (identified as genus *Clostridium*) from a food waste treatment process. The isolate was applied to an immobilized bioreactor packed by polyurethane foam. Cell immobilization is an effective method of improving the efficiency of substrate utilization and productivities of various fermentation processes. Effects of hydraulic retention time (HRT) on hydrogen production rate, composition of biogas, soluble metabolites, and hydrogen yield were investigated to identify for the proper conditions for hydrogen evolution with the isolate.

Materials and Methods

2-1. Isolation of microorganism from a food waste reactor

Sewage sludge was taken from the Pohang wastewater treatment plant, and used as microflora. The sludge was filtered through a No.10 mesh, and heated at 95 ^{o}C for 15 min to eliminate methanogens and make hydrogen producing microorganisms dominant. Food waste was taken from the cafeteria of the Pohang University of Science and Technology, and ground before refrigeration (79.5% VSS/TSS). It was mixed with deionized water in the ratio of 3 to 1, and used as medium. 1.5 L of the sludge was inoculated into 3.5 L of the medium in a 7 L jar reactor. The anaerobic condition was established by flushing the reactor with nitrogen gas. The medium was supplied to the reactor by a peristaltic pump at 3 d HRT. The reactor was run at 35 ^{o}C and stirred at 200 rpm. An automatic pH controller maintained the pH value at 5.2-5.5 with 5 N NaOH.

To isolate the microorganisms responsible for hydrogen production, serial dilutions of the broth from food waste reactor were plated and cultivated on both RCM (Reinforced Clostridial Medium, Merck) plates in the Gas-Pak anaerobic cultivation jar (BBL, UK) at 35 °C. The RCM was composed of the following materials (g/L): beef extract, 10.0; peptone, 5.0; yeast extract, 3.0; D(+) glucose 5.0; starch, 1.0; sodium chloride, 5.0; sodium acetate, 3.0; and L-cysteinium chloride, 0.5.

2-2. 16S rDNA sequencing and phylogenetic analysis

2-2-1. Determination of 16S rDNA sequence

After 48 h of incubation, the colonies were appeared on the plates and the 16S rRNA genes of the microorganisms were amplified by a following colony PCR method. In a 500 μL tube containing 100 μL of 5% Chelax 100, the cells were inoculated with the tip of a sterile platinum inoculating needle. After thirty-one colonies on each RCM were denatured for 5 min above 95 °C, the samples were centrifuged for 3 min at 12,000 rpm. The supernatant was used as a template for PCR. The PCR was performed using universal primers 27F and 1492R as follows: 94 °C for 5 min; 32 cycles consisting of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 1 min followed by 72 °C for 10 min. The amplicons were purified and sequenced. In addition, the isolates were checked for their ability to grow in RCM broth.

2-2-2. Phylogenetic analysis

Phylogenetic analysis was performed using the neighbor-joining methods. Multiple sequence alignments, construction of a neighbor-joining phylogenetic tree and 1000-replicate bootstrap analysis for evaluation of the phylogenetic tree topology were carried out with CLUSTAL W and PHYLIP software packages.

2-3. Set-up and operations of fixed-bed bioreactors with polyurethane foam matrix

A cylindrical acryl column reactor (diameter 7.0 cm x height 65 cm) with a working volume of 2.5 L was used for the continuous culture. Fresh medium was supplied from the bottom by a peristaltic pump and evolved gas and effluent liquid were discharged from the top of the reactor. The effluent of the bioreactor was introduced into a gas-liquid separator. The gas phase was replaced with N_2 gas at the beginning of the culture, and cells were cultivated anaerobically at 37 ^{o}C without pH control. After 24 h of incubation in batch culture with recycling to increase the cell density in the fixed bed to a stable level, continuous cultivation was initiated by feeding sterilized medium at HRT of 24 h and decreased to 1 h with a peristaltic pump without recycling system under anaerobic conditions. During the cultivation, the pH and soluble metabolites (volatile fatty acids and alcohols) in the effluent was monitored as a function of time.

The fixed-bed column was packed with synthetic polymer, polyurethane foam (Golden Co., Ltd., Korea) with 96–97% high void fraction as the support for microbial growth. The density of the support was 22.6 g matrix/L reactor and 1 cm^3 cubes of polyurethane foam (800 ea.) were packed inside the cylindrical reactor following sterilization. The biomass content on the immobilized support was in range of ca. 0.41–1.23 kg VSS/kg matrix. The initial pH of the medium (RCM) was controlled at 6.7, which was found to be favorable for hydrogen production, by adding a buffer solution containing 2.3 g/L of potassium phosphate dibasic and 0.7 g/L of potassium phosphate monobasic [1].

2-4. Analytical methods

The percentage of hydrogen gas was analyzed by a gas chromatography (Model 6890N, Agilent Inc.) equipped with PDD Detector (Pulsed Discharge Detector) and CARBOXEN^{TM} 1010 Plot Fused Silica Capillary column. The temperatures of oven, injector and detector were 120, 150 and 250 °C, respectively. Helium gas was used as the carrier gas at a flow rate of 20 mL/min with a split ratio of 100:1. The concentration of effluent metabolites such as volatile fatty acids (VFAs), alcohols and glucose was measured by using high-performance liquid chromatography (HPLC 1100 series, Agilent Inc.). RID (Refractive Index Detector) and Aminex HPX-87H (Bio-Rad Laboratories) were used as a detector and an organic acid analysis column, respectively. The column was operated at 40 °C with 8 mN H_2SO_4 as the carrier liquid at a flow rate of 0.6 mL/min.

Results and Discussion

3-1. Characterization of hydrogen producing isolates

Four strains capable of producing hydrogen were isolated from a food waste treatment reactor under anaerobic conditions. Microscopic examination showed that the isolates were in rod-shape (not shown in figure). The nearly full-length sequences of 16S rRNA genes (1,406 bp) were determined for the four isolates. The determined sequences were compared with database sequences by the BLAST search and a phylogenetic tree was constructed to show the relationship between microorganisms deduced from the sequence determined and reference strains (Fig. 1). Identified JO1, JO6 and JO7 were related to hydrogen producing bacteria such as *C. tyrobutyricum*, *C. leptum*, *C. acetobutylicum*, and *C. sporosphaeroides*. Among them, *Clostridium* sp. with the highest similarity (100%) to *C. tyrobutyricum* ATCC 25755 was selected for further hydrogen fermentation studies.

3-2. Effect of HRT on the production and yield of hydrogen

Figure 2 shows that the environmental parameter of HRT has important influence on hydrogen production by *C. tyrobutyricum* JM1. At all HRTs, the biogas primarily consisted of CO_2 and H_2, while CH_4 was undetected. As HRT decreased from 24 to 2 h, the hydrogen production rate was increasing with the glucose conversion of 97–100%. The maximum hydrogen production rate at steady state conditions was obtained as 7.2 L/L/d at HRT 2 h as well as the maximum hydrogen content of 50%. The major metabolic product was butyrate as a concentration of ca. 4,000 mg/L. However, the rate was suddenly decreased at HRT 1h with the rapid accumulation of lactate and acetate. In addition, substrate utilization efficiency was at the range of 40–45%. It is reported that lactate is fermented in the presence of acetate to butyrate, CO_2, and H_2 by *Clostridium tyrobutyricum* [2]. Butyrate concentration was dominantly constant from HRT 24 to 2 h, but was replaced by lactate and acetate with decrease of hydrogen evolution at HRT 1 h. The phenomenon results in no reaction

time for the conversion of lactate to butyrate and H_2 in response to higher organic loading rate for lower HRT. It was supported by pH decrease from 5.5 (HRT 2 h) to 4.6 (HRT 1h) due to the no reduction of protons to the formation of H_2. Therefore, observation of predominant lactate production might be considered as a signal of inefficient hydrogen evolution by *C. tyrobutyricum*. The operation at HRT 1 h appeared to be unstable for hydrogen production. In addition, the maximum hydrogen yield was 223 mL/g hexose on the basis of glucose concentration of 5 g/L. The value was the higher than those of other researches [3].

Conclusion

Hydrogen producing *Clostridium* sp. showing the highest 16S rRNA gene sequence similarity (100%) to *Clostridium tyrobutyricum* ATCC 25755 were successfully isolated from a food waste treatment process. The immobilized system was an effective and stable approach for continuous hydrogen production for efficient utilization of carbon substrates with good hydrogen-producing performance.

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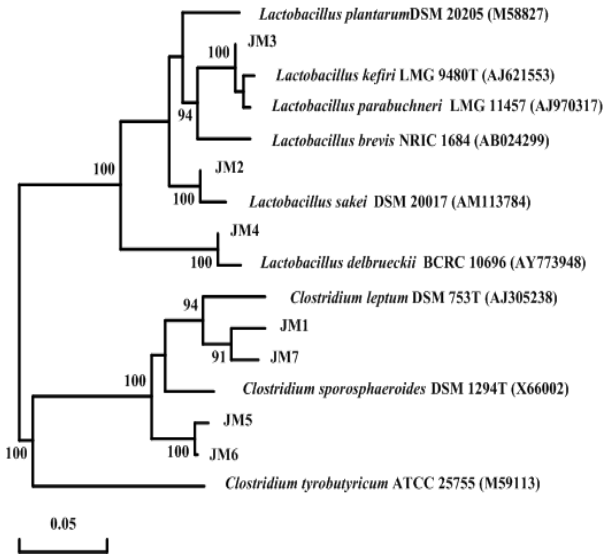

Fig. 1. Neighbor-joining showing phylogenentic positions of three hydrogen producing strains and Clostridium species based on 16S rRNA sequence comparisions.

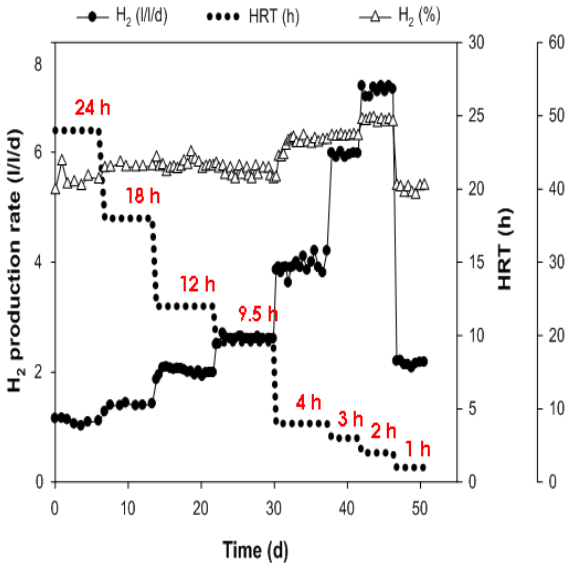

Fig. 2. HRT-dependent profiles of H_2 production rate and component of biogas in the fixed-bed reactor packed with polyurethane foam.