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농축 하수오니 유래 열처리 혐기세균 복합체를 이용한 두부제조 폐수로부터 수소 생산

오유관*, 김미선*†
*한국에너지기술연구원 바이오에너지연구센터

Hydrogen Production from Tofu Manufacturing Wastewater by Heat-treated Anaerobic Microflora from the Concentrated Sewage Sludge

YOUKWAN OH*, MISUN KIM*†
*Bioenergy Research Center, Korea Institute of Energy Research,
71-2, Jang-dong, Yuseong-gu, Daejeon 305-343, Korea*

ABSTRACT

합성 및 두부 제조 폐수로부터 혐기 세균 복합체를 이용하여 수소를 생산하였다. 수소생산 혐기 세균 복합체는 하수처리장 농축 소화조에서 발생하는 슬러지를 90°C에서 20분간 열처리하여 얻었다. 혐기 세균 복합체는 37°C 회분식 운전조건에서 1% (w/v) 포도당 함유 PYG (peptone-yeast extract-glucose) 배지로부터 1.15 L-H_2/g-균체건조량의 수소를 생산할 수 있었고, 이때 주요 유기산으로 15 mM acetate와 32 mM butyrate가 생성되었다. 같은 발효조건에서 1.4% 전분과 0.07% 환원당을 포함하는 두부 제조 폐수로부터 1.76 L H_2/L-두부제조폐수의 수소를 발생하였다. 이와 같은 결과로부터 포도당과 두부 제조 폐수로부터 혐기세균 복합체에 의한 수소생산 효율은 각각 1.9과 0.9 mol H_2/mol 포도당을 나타내었다. 반연속운전(HRT, 12 시간)시 합성폐수를 이용하여 60일 이상 안정적으로 수소를 생산할 수 있었고, 이 때 혐기 세균 복합체는 1.3-2.0 L H_2/L-배양액을 발생하였다. PCR-DGGE(polymer chain reaction-denaturing gradient gel electrophoresis) 분석결과, 반응기 내 세균 복합체의 주요 미생물은 Clostridium 종이었다. 본 연구는 적절한 열처리를 통해 혐기 소화조 슬러지로부터 고활성 수소생산 세균 복합체를 얻을 수 있으며, 이들 세균 복합체를 이용하여 합성 및 두부제조 폐수로부터 효율적인 수소생산이 가능하다는 것을 나타내고 있다.

KEY WORDS : Hydrogen production(수소생산), Sewage sludge(하수슬러지), Tofu wastewater(두부폐수), Heat-treatment(열처리), Anaerobic microflora(세균 복합체)

1. Introduction

Biological H_2 production process had been advanced as a potential technology for producing clean and renewable energy. Feasibility for creating renewable H_2 production systems has been demonstrated through the anaerobic dark

†Corresponding author : bmmskim@kier.re.kr

fermentation and/or photo-fermentation of organic wastes and wastewater by many researchers. Organic substances such as sugars, starch, and cellulose, which are present in the waste effluents, are decomposed to organic acids and simultaneously H_2 is evolved by anaerobic dark-fermentation. The biological generation of H_2 from anaerobic fermentation of organic substrates promises to be an economical and sustainable technology for H_2 production if conversion efficiencies can be increased[1]. There are many efforts to improve the productivity of H_2 and stability of fermentation system by lowering the H_2-consuming bacteria and many non-H_2 producing bacteria in the reactor. Culture conditions that can be used to limit growth of methanogens include low pH, short hydraulic retention time (HRT), and sludge retention time (SRT) in continuous cultures. Another method that can enhance biological H_2 production is a heat-shock treatment to remove non-spore forming bacteria, such as methanogens, that consume H_2[2-5].

Disposal of organic biomass resources such as sewage sludge, food wastes, and agricultural waste is one of the major environmental problems in Korea because the area for the landfill is limited and the average increase of organic waste increases approximately 8% annually. Therefore serious considerations must be given to disposal of the organic wastes and effluents and lessening the burden of organic substances in the environment. Biological H_2 production is more attractive when organic wastewater or other waste can be used as the raw material.

In this study, we described the biological H_2 production from anaerobic dark-fermentation by sewage sludge microflora. Hydrogen-producing microflora was isolated from sewage sludge and heat-treated to inactivate H_2 consumers and harvest spore-forming anaerobic bacteria. Using the heat-treated microflora as an inoculum, the optimum H_2 production conditions, and long-term stability of H_2 production were examined.

2. Materials and Methods

2.1 Seed microorganisms

Sewage sludge was collected from the anaerobic digester of municipal wastewater treatment facility in the area of Daejeon, Korea. Twenty mL of the sewage sludge was heat-treated for 20 min at 90°C in a serum bottle and used as the seed microorganisms. The heat-treated sewage sludge was cultivated in PYG medium supplemented with 1% (w/v) glucose at 37°C in a 1.3 L stirred-tank type anaerobic reactor (0.6 L of working volume). The reactor was flushed with argon gas for the anaerobic culture condition. Initial pH was adjusted to 7.0 and maintained to 5.5 during fermentation.

2.2 Culture conditions and Tofu manufacturing wastewater

Heat-treated sewage sludge was cultured at 37°C in a synthetic or a Tofu manufacturing wastewater media. As a synthetic medium, PYG medium supplemented with 1% (w/v) glucose and the followings: 10 g of peptone, 5 g of yeast extract, 0.5 g of cystein·HCl, 4 g of $Na_2CO_3 \cdot 10H_2O$, 0.9 g of K_2HPO_4, 0.9 g of KH_2PO_4, 0.9 g of NaCl, 0.9 g of $(NH_4)_2SO_4$, 0.09 g of $MgSO_4$, 0.09 g of $CaCl_2$, 0.1 mg of *p*-aminobenzoic acid, and 0.01 mg of biotin. The bottles were sealed and flushed with argon gas

for 15 min to establish the anaerobic condition. Fresh PYG media was added every 10 h interval, and H_2 production was monitored. The initial pH was adjusted to 7.0. pH was allowed to drop during the batch fermentation, while maintained to 5.5 during semi-continuous operation. Tofu manufacturing wastewater was collected from the final stage of bean curd separation in a local company.

2.3 Analyses

The head-space of a reactor was monitored for the H_2 concentration using a gas chromatograph (Model 14-B, Shimadzu, Kyoto, Japan) equipped with a molecular sieve 5A column (2 mm × 3 m, Alltech, USA) and a thermal conductivity detector. The concentration of organic acids was measured using HPLC (Model SCL-10A VP, Shimadzu, Kyoto, Japan) fitted with an Aminex HPX-87H organic acid analysis column (Bio-Rad, Hercules, USA). Reducing sugar contents were measured by the dinitrosalicylic acid (DNS) method, and starch contents were determined by the DNS methods after acid hydrolysis using 5.5 N HCl.

In order to identify the H_2-producing microorganisms, DNAs from the sludge culture were extracted by using the Ultraclean DNA kit (Mo Bio Laboatory Inc., USA)[6]. The 16S rDNA fragments were amplified by PCR using the forward primer 968f with a GC clamp at the 5′ end to stabilize the melting behavior of the DNA fragments and the reverse primer 1492R. PCR was conducted using the protocol: that is, initial denaturation for 5 min at 95°C and 30 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 72°C, extension for 90 s at 72°C, followed by a final extension for 10 min at 72°C. DGGE was carried out using the Dcode Universal Mutation Detection System (Bio-Rad, USA) in accordance with the manufacturer's instructions. PCR products were electrophoresed for 6 h at 100 V and 60°C on polyacrylamide gel (6%) containing a linear gradient ranging from 40% to 60% denaturant. After electrophoresis, polyacrylamide gel was stained with EtBr for 30 min, and then visualized on UV transilluminator. DNA fragments from the bands were purified and then PCR-amplified with the forward primer 968f without a GC clamp and the reverse primer 1492R. After PCR amplification, PCR products were sequenced. Search of the GenBank database was conducted using the BLAST program.

3. Results and Discussion

3.1 Heat-treatment of sewage sludge

The sewage sludge was heat-treated for 20 min at 90°C in a serum bottle to harvest anaerobic spore-forming H_2-producing microflora and inhibit the bioactivity of non-spore-forming and H_2-consuming bacteria. When 10% of fresh sewage sludge without heat-treatment was used as the seed culture during the anaerobic fermentation in PYG synthetic medium, H_2 was produced for 2 days and did not further evolved H_2 when fresh media was replaced every 8 - 10 h (Table 1). Hydrogen production continued over 60 days when the heat-treated sewage sludge was used at the same culture conditions. However, H_2 was not produced during the anaerobic fermentation with the repeated heat-treatment of sewage sludge, indicating the inactivation of microflora responsible for H_2 production with the severe heat-treatment. Heat-treatment of fresh or

enriched sewage sludge at 90℃ for 20 min in our experiment was enough to inactivate the H_2-consuming bacteria and methanogens to operate the anaerobic fermentation for producing H_2 over 2 months. It has been well-known that successful biological H_2 production by anaerobic dark fermentation requires inhibition of H_2-using microorganisms, such as homoacetogens and methanogens. Inhibition is commonly accompanied by heat-treatment of the inoculum to kill all microorganisms except for spore-forming fermentation bacteria. Other methods that have been used include the operation of reactors at high dilution rates, low pH, N_2-sparging, etc. The experimental conditions for the heat-treatment to inactivate the H_2-using bacteria or to improve the H_2 production vary on the different studies. Noike et al. observed that H_2 production rate was increased by elevating the reactor to 60℃ to inhibit the lactic acid bacteria which are major competitors to H_2-forming bacteria[7]. The dried sludges are baked at 100 - 104℃ for 2 h to kill the H_2-using bacteria and used for the H_2 production by many researchers[4,8,9].

Table 1 Cell growth and H_2 production by the microbial consortium of heat-treated sewage sludge

Time (day)	Heat treatment (90℃, 20 min)			
	-	+	+ +	+ + +
1	○	○	△	×
2	○	○	×	×
3	×	○	×	×
4	×	○	×	×
60	×	○	×	×

-, No heat-treatment of sludge; +, 14 hr fermentation in PYG media using inoculum after heat-treatment of sludge once; + +, 14 hr fermentation in PYG media using inoculum after heat-treatment of +; + + +, 14 hr fermentation in PYG media using inoculum after heat treatment of + +; Hydrogen productivity was shown as good, low, or none as ○ △ or ×, respectively.

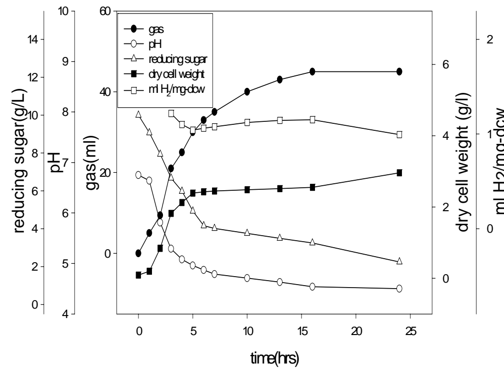


Fig. 1 Growth curve of the mixed culture, Hasu C-1, isolated from the sewage sludge. Symbol: ●, gas; ○, pH; △, reducing sugar; ■, dry cell weight; and □, H_2

3.2 Cell growth and H_2 production of 'Hasu C-1'

Heat-treated, H_2-producing microflora was named as 'Hasu C-1'. Hydrogen production and cell growth for 24 h(Fig. 1) and 25 days(Fig. 2) of the anaerobic fermentation were monitored. Hasu C-1 grew fast to reach 2.5 g dcw/L in a CSTR using PYG media containing 1% (w/v)

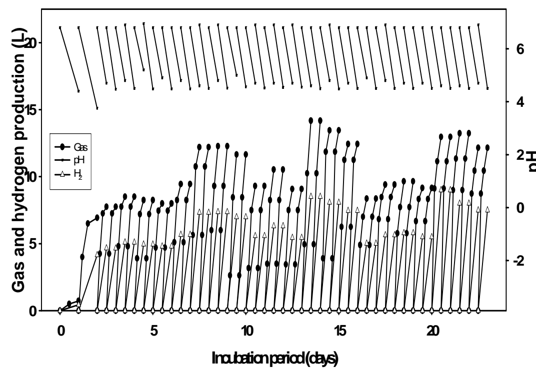


Fig. 2 Hydrogen production by the mixed-culture prepared from heat-treated sewage sludge during semi-continuous anaerobic fermentation in a 5 L stirred-tank fermenter. Symbol: ●, gas; △, H_2 and •, pH

glucose at 37°C under the anaerobic condition. Hydrogen was started to be produced from the early exponential growth phase and then the rate of production was rapidly decreased at the early stationary growth phase. The initial pH 7.0 decreased to 5.5 in 5 – 6 h of fermentation and 4.8 in 9 – 10 h and the rate of H_2 production decreased. The pH drop resulted in only 80% degradation of added glucose. Organic acids were also produced during the anaerobic fermentation, which were mainly 32 mM and 15 mM of butyrate and acetate, respectively. The average H_2 production rate was 1.15 L/g-dcw at this culture condition. The H_2 yield of this microflora was calculated to 1.93 mole H_2/mole-glucose assuming the one mole H_2 was equivalent to the volume of 22.4 L at the room temperature. While pH was adjusted to 5.5 during the fermentation, glucose was completely degraded and the pattern of organic acids produced was changed to 20 mM and 35 mM of butyrate and acetate, respectively. This result was in close agreement with the results obtained by Khanal et al.[8] based on the study with mixed culture of baked compost. They reported that the ratio of acetate and butyrate during anaerobic fermentation process was increased as initial pH decreased. And a lower initial pH could have driven the reaction predominantly toward acetate production resulting in higher H_2 production.

Hydrogen production continued over 25 days when Hasu C-1 was used for semi-continuous anaerobic fermentation in a 5 L stirred-tank reactor using PYG synthetic media containing 1% (w/v) glucose at 37°C. Hydrogen content of produced gas was 60 – 70%, and rest of them was CO_2. The fresh medium was replaced every 12 h, and the glucose was completely degraded for 11 – 12 h. The initial pH 7.0 was decreased to 4.5 in 11 – 12 h of fermentation and the cell concentration was maintained to 2.6 – 2.8 g-dcw/L-culture. The average H_2 and gas production rate are 10 – 15 L-gas/12 h and 0.5 – 0.7 L-H_2/g-dcw, respectively, at this culture conditions. The H_2 content of gas produced during the anaerobic fermentation was constant to 60 – 70% over 60 days (data not shown) indicating the stabilization of the microflora present in the reactor.

Table 2 Hydrogen production from Tofu wastewater by anaerobic fermentation of 'Hasu C-1'*

Time (hr)	Total gas (ml)	H_2 content (%)	H_2 production rate (ml/ml broth)	pH	Starch (g/L)
0	0	0	0	6.73	14.5
3.5	260	34	0.83	5.45	12.0
6.5	1010	46	1.69	4.64	6.0
24	1100	46	1.76	4.58	5.8

*Cultures were anaerobically grown at 37°C in a 1 L stirred-tank type fermenter.

Tofu wastewater, containing 14 – 15 g-starch/L and 0.6 – 0.8 g-sugars/L generated 1.76 L-H_2/L-wastewater, along with some organic acids, during 24 h of fermentation(Table 2). Ammonium sulfate and peptone were added as the nitrogen sources. The H_2 production from Tofu wastewater using Hasu C-1 reached about 0.9 mole of H_2/mole of glucose, assuming that the conversion factor of starch to sugar is 1.2.

3.3 Stirring and gas removal

The continuous stirring and gas removal to reduce the partial pressure of H_2 gas and to increase the mass-transfer between cells seemed to be the important factors for operating fermenter. In our study, stirring and gas removal have an influence on H_2 productivity during the

Table 3 Effect of stirring and continuous gas removal on H_2 production during 8 - 10 h of anaerobic fermentation of the mixed culture 'Hasu C-1'*

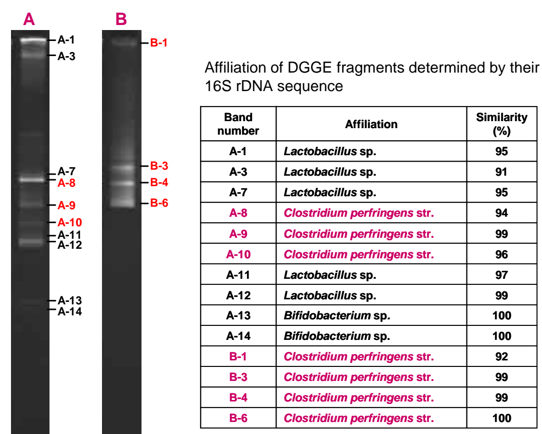
	Total gas (mL)	H_2 content (%)	H_2 production rate (mL/mL broth)
No stirring and no gas removal	32.7	36.7	1.77
Gas removal only	36.7	43.3	1.85
Stirring only	48.3	44.5	2.10
Stirring and gas removal	52.7	45.7	2.17

*Cultures were anaerobically grown at 37°C in a 150 mL serum bottle.

anaerobic fermentation of Hasu C-1(Table 3). Continuous stirring and gas removal increased the H_2 production rate by 1.2 times compared to the culture without treatments. In batch culture, the continuous stirring of broth showed more positive effect on the average H_2 production rate by increasing 1.77 to 2.10 L-H_2/L-broth compared to the gas removal only which increased to 1.85 L-H_2/L-broth. It have been reported and observed by many studies that H_2 partial pressure in the liquid phase is one of the key factors affecting H_2 production. A decrease in H_2 concentration will favor H_2 formation and permit bacteria to metabolize acetyl-CoA through the energy-efficient path leading to acetate and ATP formation. Thus a decrease in partial pressure of H_2 should give an enhanced H_2 yield. Nitrogen sparging, proper substrate concentration, and agitation regime[2,10,11,12] were reported to lower dissolved H_2..

3.4 DGGE Analysis

The microbial communities in sewage sludge itself and heat-treated Hasu C-1 culture were analyzed and compared by PCR-DGGE analysis, and the DGGE profiles are shown in Fig. 3. The



Band number	Affiliation	Similarity (%)
A-1	*Lactobacillus* sp.	95
A-3	*Lactobacillus* sp.	91
A-7	*Lactobacillus* sp.	95
A-8	*Clostridium perfringens* str.	94
A-9	*Clostridium perfringens* str.	99
A-10	*Clostridium perfringens* str.	96
A-11	*Lactobacillus* sp.	97
A-12	*Lactobacillus* sp.	99
A-13	*Bifidobacterium* sp.	100
A-14	*Bifidobacterium* sp.	100
B-1	*Clostridium perfringens* str.	92
B-3	*Clostridium perfringens* str.	99
B-4	*Clostridium perfringens* str.	99
B-6	*Clostridium perfringens* str.	100

Fig. 3 Affiliation of DGGE fragments determined by their 16S rRNA sequence from the band
A; Non heat-treated sludge
B; Heat-treated sludge, Hasu C-1

major bands in the DGGE gels were excised and purified to determine the sequence. The sequence affiliation was determined by the BLAST. The number of bands detected from heat-treated culture was decreased compared to that of non-heat treated culture. A simple heat-shock treatment removed the non-spore forming bacteria from culture. The microorganisms closely related to *Lactobacillus* sp. and *Bifidobacterium* sp., which were known to produce lactate from carbohydrates, were isolated and detected with strong intensity by PCR-DGGE analysis from the non heat-treated culture (Fig. 3, A). In contrast, the H_2-producing bacterium, *Clostridium* sp. were mainly existed in heat-treated culture (Fig. 3, B). And it was closely related to *Clostridium* strain.

4. Conclusion

A stable and long-term fermentative H_2-producing microbial community, Hasu C-1, was obtained through the simple heat-treatment

of sewage sludge. Hydrogen productivity of the microbial consortium was mainly due to *Clostridium* sp. as determined by PCR-DGGE method. Hydrogen productivity of Hasu C-1 was 1.9 mole-H_2/mole of glucose at 37°C from the PYG synthetic media containing 1% (w/v) glucose, and 0.9 mole H_2 per glucose mole from Tofu wastewater. Future work will be the optimization of culture condition, especially the composition of culture media and application of heat-treated sewage sludge to various kinds of food wastes or industrial waste/wastewater.

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