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CHARACTERISTICS OF BIOHYDROGEN PRODUCTION AND MICROBIAL COMMUNITY AS A FUNCTION OF SUBSTRATE CONCENTRATION

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Abstract : The feasibility of hydrogen production with a raw seed sludge through direct acclimation of feedstock was investigated at acidogenic stage, and methane was harvested at followed methanogenic stage in an anaerobic two-stage process. Hydrogen content was higher than 57% at all tested organic loading rates (OLRs) and the yield of hydrogen ranged from 1.5 to 2.4 mol H_2/mol hexose $_{consumed}$ and peaked at 6 $gVSI^{-1}day^{-1}$. Normal butyrate and acetate were main volatile fatty acids (VFAs), whereas the concentration of propionate was insignificant. The hydrogen-producing bacteria, *Clostridium thermosaccharolyticum*, was detected with strong intensity at all tested organic loading rates (OLRs) by denaturing gradient gel electrophoresis (DGGE) of the polymerase chain reaction (PCR) analysis. From COD balance in the process, the fraction of the feed-COD converted to the hydrogen-COD at acidogenic stage ranged from 7.9% to 9.3% and peaked at 6 $gVSI^{-1}day^{-1}$, whereas the fraction of feed-COD converted to the methane-COD at methanogenic stage ranged from 66.2% to 72.3% and peaked at 3 $gVSI^{-1}day^{-1}$.

Key Words : *clostridium thermosaccharolyticum*, food waste, hydrogen, methane, volatile fatty acids (VFAs)

INTRODUCTION

Hydrogen has been considered as a promising alternative to fossil fuels due to its clean, renewable and high energy yielding.[1~3] Biological hydrogen production by anaerobic fermentation is an environmentally friendly and energy saving process, and reduces the wastes to be treated as well.[4,5]

For the production of biohydrogen, H_2-producing bacteria were usually obtained by heat-treated sewage sludge to inhibit the bioactivity of methanogenesis which scavenge hydrogen released by H_2-producing bacteria during the anaerobic fermentation of organic wastes.[1,2,5,6] The seed sludge was used to acclimate with to readily biodegradable pure substances such as sucrose, starch or glucose at short hydraulic retention time (HRTs) and /or low pH.[1~6] However, if the inoculum consisted of spore-formers, growth on pure substances, might face difficulties not found with complex feedstocks, since spore germination, like sporulation, could require species-specific nutrients.[7] Thus, for a viable technology, continuous processes using non-sterile fermentable organic feedstocks are necessary.

Organic solid wastes need HRTs longer than

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3 days to allow an efficient acidogenesis during which hydrogen consumers such as methanogenesis may proliferate. Because of this reason, they have been considered so far for the production of methane which has only about one third of the energy of hydrogen.[8] It was reported that more hydrogen could be produced under thermophilic condition (55°C) than mesophilic condition (37°C)[9] and temperatures in the range of thermophily had an inhibitory effect on most methanogenesis.[10] Possibility of efficient hydrogen production from thermophilic condition is, therefore, deserved to study.

This study was thus conducted to study the feasibility of extraction of H_2-producing bacteria from a raw seed sludge through direct acclimation of feedstock without heat pre-treatment and acclimation in pure substances steps. Furthermore, it was investigated the production of biohydrogen from carbohydrate-rich organic solid waste at HRT compatible with acidification of organic solid waste. Methane was then obtained from acidogenesis by-products such as acetate and butyrate relying on a separate methanogenic step within a two-phase process.

Food waste, a carbohydrate-rich organic solid matrix, was used as substrate in this work. The microbial community and their dynamic behavior as a function of organic loading rates (OLRs) were examined by denaturing gradient gel electrophoresis (DGGE) of the polymerase chain reaction (PCR)-amplified V3 region of 16S rDNA.

MATERIALS AND METHODS

Seed Sludge

The seed sludge was obtained from an anaerobic digestion tank at a municipal sewage treatment plant. It had total suspended solid (TSS), volatile suspended solid (VSS) and pH of 23.6 gl^{-1}, 14.1 gl^{-1} and 7.3, respectively. The seed sludge was allowed to acclimate to the feed substrate without heat-pretreatment for 3 months in the thermophilic-acidogenic fermenter at pH, OLR, HRT and temperatures of 5.5±0.1, 1.5 $gVSl^{-1}day^{-1}$, 5 days and 55±1°C and mesophilic-methanogenic fermenter as well. The mesophilic-methanogenic fermenter operated at 20 days HRT, 35±1°C. Organic loading rates (OLRs) were increased stepwise to 3 $gVSl^{-1}day^{-1}$, 6 $gVSl^{-1}day^{-1}$ and 8 $gVSl^{-1}day^{-1}$ after steady-state was reached.

Feedstock

Food waste collected from a dining hall was ground after removal of animal bones and clamshells. Since such a food waste released water as a consequence of squeezing due to compaction during collection, de-ionized water was added at the ratio of 1 : 2 (food waste : deionized water) in order to get a liquid-phase feed after grinding. This feed was then stored at 4°C to prevent pre-acidification. Average total solid (TS), volatile solid (VS), total carbohydrate, pH and C/N of the food substrate were 67 gl^{-1}, 63 gl^{-1}, 25 gl^{-1}, 5.5 and 16, respectively.

Experimental Apparatus and Operation

Continuous production of hydrogen and methane was conducted using the lab-scale anaerobic two-stage process (Figure. 1). The working volume of thermophilic-acidogenic fermenter was 3 l (internal diameter, 13 cm; height, 30 cm) that was fed with the food waste by draw and fill mode once every 24 hours. The working volume of mesophilic-methanogenic fermenter was 12 l (internal diameter, 19 cm; height, 51 cm). The pH of thermophilic-acidogenic fermenter was automatically adjusted with 2 N KOH and 2 N HCl to 5.5±0.1, but that of mesophilic-methanogenic fermenter was not controlled. To maintain thermophilic or mesophilic condition, the acidogenic and methanogenic fermenter were covered with an external water jacket that kept the temperature at thermophilic (55±1°C) or mesophilic (35±1°C) conditions. Both fermenters were stirred at a constant 60 rpm to ensure a thorough mixing. The biogas produced was collected by a downstream displacement of acidified water (0.05 M H_2SO_4). The gas volume was corrected to standard temperature (0°C) and pressure (760

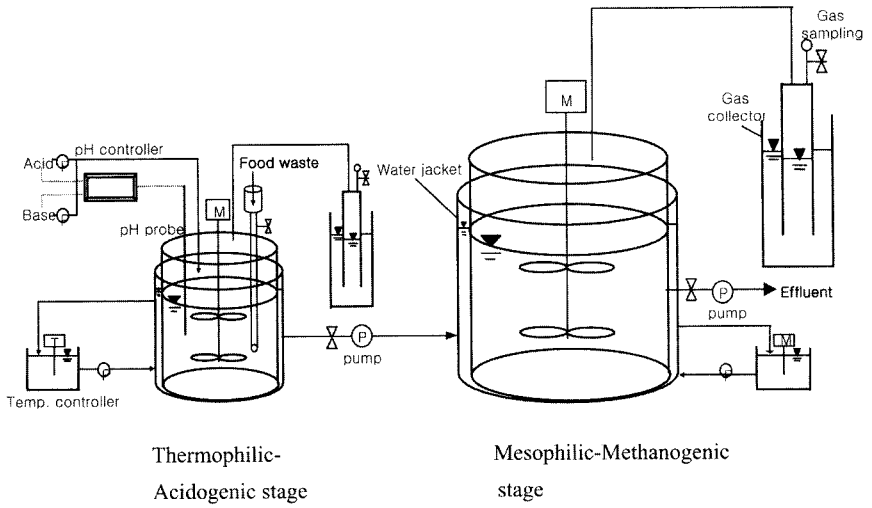

Figure 1. Schematic description of an anaerobic two-stage process for continuous hydrogen and methane production.

mmHg) (STP). Experiments were run at different OLRs, namely 3 $gVSl^{-1}day^{-1}$, 6 $gVSl^{-1}day^{-1}$ and 8 $gVSl^{-1}day^{-1}$. At each OLR, the experiments were carried out for 2 months. Steady-state condition was defined when the variation range of product concentration was less than 10% at least for 1 week.

Analyses

The gas composition was analyzed using a gas chromatograph (Gow Mac series 580, USA) with a thermal conductivity detector and two columns. The methane and carbon dioxide were detected with a column packed with porapak Q (80/100 mesh), and the hydrogen was detected with a column packed with molecular sieve 5A. The temperature of the injector, detector and column were kept at 80°C, 90°C and 50°C, respectively. Helium was used as a carrier gas. Volatile fatty acids (VFAs) were quantified by a high-performance liquid chromatography (Spectra-system P2000, USA) with an ultraviolet (210 nm) detector and an Aminex HPX-97H (300 mm × 7.8 mm) column after pretreatment with a 0.45 μm membrane filter. H_2SO_4 of 0.005 M was used as a mobile phase. Carbohydrate was measured using the calorimetric ferric-cyanide method.[11] Measurements of total solid (TS), volatile solid (VS) and pH were performed according to the Standard Methods.[12]

In order to identify the responsible hydrogen-producing bacteria and investigate their dynamic behavior in response to OLRs, DNA in the microbial community at each tested OLR was extracted at steady-state by using the Ultraclean DNA Kit (Cat # 12800-50; Mo Bio Laboratory Inc., USA). For the amplication of 16S rDNA fragments, EUB 357f(5'-CCTACGGGAGGCAG CAG-3') and UNIV518r(5'-ATTACCGCGGCTG CTGG-3') with a GC clamp were used as a forward and reverse primer, respectively. PCR products were purified using MultiScreen Vacuum Manifold (MILLIPORE com., USA), and search of the GenBank database was conducted using the BLAST program.

RESULTS AND DISCUSSION

Biohydrogen Production and Composition of Metabolites at Acidogenic Stage

The OLR of 3 $gVSl^{-1}day^{-1}$, 6 $gVSl^{-1}day^{-1}$ and 8 $gVSl^{-1}day^{-1}$ was adjusted by adding deionized water to food waste stored at 4°C before feeding. Table 1 shows the steady-state analysis for biogas and organic acids production at thermophilic-acidogenic stage. The biogas pro-

Table 1. Steady-state analysis for biogas and organic acids production

OLR (gVS/ L-day)	Gas content (%)			H_2 (LH_2/ L-day)	H_2 yield (mol-H_2/ mol-hexose	Organic acids (mgl^{-1} as COD)				
	CH_4	CO_2	H_2			TOA	HLa	HAc	HPr	n-HBu
3	ND	42.4	57.6	0.3	1.5	7234	32	965	187	5978
6	ND	41.0	59.0	0.7	2.4	13127	40	3731	345	8526
8	ND	40.2	59.8	0.9	2.1	17348	159	4782	605	11360

ND-non-detectable, gas content<0.1%;
TOA-total organic acids; HLa-lactate; HAc-acetate; HPr-propionate; *n*-HBu-normal butyrate

duced was mostly composed of hydrogen and carbon dioxide but free of methane at all tested OLRs. The hydrogen content was higher than 57% at all tested OLRs and insensitive to the tested OLRs. The hydrogen production rates increased from 0.3 l H_2/l-day at 3 $gVSl^{-1}day^{-1}$ to 0.9 l H_2/l-day at 8 $gVSl^{-1}day^{-1}$, whereas the hydrogen yield ranged from 1.5 to 2.4 mol H_2/mol hexose $_{comsumed}$ consumed and peaked at 6 $gVSl^{-1}day^{-1}$. The efficiency of the carbohydrate decomposition ranged 87.6% to 94.1% and peaked at 6 $gVSl^{-1}day^{-1}$ like the case of hydrogen yield.

The main VFAs were normal butyrate, with a content ranging from 65% to 82.6%, and acetate, with a content ranging from 13.3% to 28.4%, whereas, propionate was insignificant. Glucose in organic substances gives a maximum yield of $4H_2$ per glucose when acetate is the by-product. Half of this yield per glucose is obtained with butyrate as the fermentation end product.[7] On the other hand, Zoetemeyer *et al.* (1982)[13] reported that a decrease of the hydrogen partial pressure coincided with the start of propionate production. Vavilin *et al.* (1995)[14] suggested, on the basis of Zoetemeyer's results, that the following chemical stoichiometric equation for the production of propionate from hexose, shows that this involves the consumption of H_2.

$$Glucose + 2H_2 \rightarrow 2propionate + 2H_2O.$$

The high concentration of acetate and n-butyrate but low concentration of propionate might contribute to the high yield of hydrogen. Ueno *et al.* (1996)[10] reported that methane was produced and increased with an increase in HRTs when hydrogen was produced from sugary wastewater at a pH of 6.8, and at 60°C for various HRTs of 0.5 day, 1 day, 2 days and 3 days. They speculated that the fermentation pattern might shift to methanogenic fermentation if the HRT of the wastewater increased. The incomplete inhibition of methanogenesis in their study might have been caused by the short period of cultivation, which was less than 1 month, and a relatively high pH of 6.5, even though the experiments were conducted under thermophilic conditions. Another study reports that at pH values lower than 6.3, the methanogenesis rate decreases or stops,[15] suggesting that the thermophilic condition, operation at a low pH and for a long period of acclimation of seed sludge inhibited hydrogen-utilizing methanogenesis. As a result, the operated conditions inhibited the growth of methanogenesis even though at the tested long HRT.

Furthermore, through the production of hydrogen, the bacteria re-oxidize the reduced ferredoxin and hydrogen-carrying coenzymes, and these reactions are less favorable when the hydrogen concentration in the liquid rises.[7] Thermophilic condition reduces the dissolved hydrogen concentration. Therefore, thermophilic condition is more effective in producing hydrogen than mesophilic condition in acidogenic fermenter. The maximum hydrogen yield obtained in this study, namely 2.4 mol H_2/mol hexose $_{consumed}$, is comparable to the hydrogen yield obtained from wastewater.[4,10]

Microbial Community and Their Behavior at Acidogenic Stage

The microbial community and their dynamic behavior in response to OLRs were examined by PCR-DGGE analysis targeted at eubacterial 16S rDNA. Figure 2 and Table 2 show the DGGE profiles and the results of sequence affiliation determined by the BLAST, respectively.

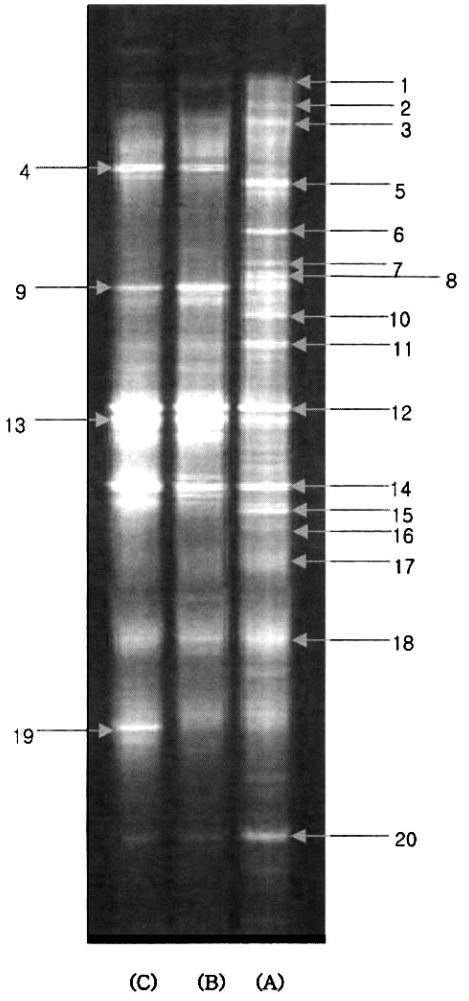

Figure 2. Denaturing gradient gel electrophoresis (DGGE) profiles of the PCR-amplified 16S rDNA extracted from the microbial community at the tested OLRs at steady-state.
(A) : 3 $gVSl^{-1}day^{-1}$; (B) : 6 $gVSl^{-1}day^{-1}$; (c) : 8 $gVSl^{-1}day^{-1}$

Bands affiliated with *Clostridium thermosaccharolyticum* (band no. 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 18, 19),[16] *Bacillus farraginis* (band no. 2),[17] *Thermoanaerobacterium bryantii* (band no. 17),[18] *Desulfotomaculum geothermicum* (band no. 1, 20),[19] which were known as hydrogen-producing bacteria, were detected. The number of bands detected was higher at lower OLR. *Clostridium thermosaccharolyticum* that was known as a hydrogen-producing microorganism, was detected with strong intensity at all tested OLRs. *Clostridium thermosaccharolyticum* is a thermophilic saccharolytic microorganism involved in acetate/butyrate fermentation that leads to produce large amount of hydrogen from carbohydrates.[16] From the characteristic study of *C. thermosaccharolyticum*,[10] it was reported that the maximum growth of *C. thermosaccharolyticum* was at the pH range from 5 to 6, and the optimum temperature for growth was 60°C. The yield of hydrogen production from *C. thermosaccharolyticum* was 2.4 mol-H_2/mol-glucose nearly equivalent hydrogen production ability to that of *Clostridium butyricum* which had hydrogen production yield of 2.4 mol-H_2/mol-hexose. *Desulfotomaculum geothermicum* is a thermophilic, fatty acid-degrading, sulfate-reducing bacterium.[19]

COD Balance for Hydrogen and Methane Conversion at Entire Process

Methane production was performed using organic acids at mesophilic-methanogenic stage. The average operating results along with the OLRs of 3 $gVSl^{-1}day^{-1}$, 6 $gVSl^{-1}day^{-1}$ and 8 $gVSl^{-1}day^{-1}$ which refer to the OLRs applied to the acidogenic-stage, were shown in Table 3. The pH and alkalinity increased slightly as the OLRs increased. The methane content was higher than 65% at all tested OLRs and reached a maximum of 68.8% at 6 $gVSl^{-1}day^{-1}$. The efficiency in removing the VS and COD in the entire process decreased as the OLRs increased ranging from 87.1% to 84.0% and from 85.2% to 81.5%, respectively.

Table 4 shows COD balance and the fraction of the feed-COD converted to the hydrogen-COD and the methane-COD at acidogenic and methanogenic stage, respectively. The influent

Table 2. Affiliation of denaturing gradient gel electrophoresis (DGGE) fragments determined by their 16S r DNA sequence

Band No.	Sequence Determined (bp)	Affiliation	Similarity[a] (%)	Accession No.
1	1468	*Desulfotomaculum geothermicum*	96	AJ294428
2	1401	*Bacillus farraginis*	90	AY443037
3	1524	*Clostridium thermosaccharolyticum*	90	M59119
4	1524	*Clostridium thermosaccharolyticum*	94	M59119
5	1458	*Clostridium thermoamylolyticum*	91	X76743
6	1524	*Clostridium thermosaccharolyticum*	99	M59119
7	1524	*Clostridium thermosaccharolyticum*	97	M59119
8	1524	*Clostridium thermosaccharolyticum*	98	M59119
9	1524	*Clostridium thermosaccharolyticum*	100	M59119
10	1524	*Clostridium thermosaccharolyticum*	98	M59119
11	1524	*Clostridium thermosaccharolyticum*	98	M59119
12	1524	*Clostridium thermosaccharolyticum*	98	M59119
13	1524	*Clostridium thermosaccharolyticum*	98	M59119
14	1504	*Clostridiaceae bacterium rennanqilyf12*	94	AY 332392
15	1392	*Desulfotomaculum thermocisternum*	93	U33455
16	191231	*Mus musculus*	95	AC126277
17	1329	*Thermoanaerobacterium bryantii*	93	AY 140670
18	1524	*Clostridium thermosaccharolyticum*	98	M59119
19	1524	*Clostridium thermosaccharolyticum*	95	M59119
20	1468	*Desulfotomaculum geothermicum*	99	AJ294428

[a] Percentage similarity to the closest relative according to the BLAST comparison

Table 3. Average operating results at methanogenic stage at steady-state

OLR ($gVSl^{-1}day^{-1}$)	3	6	8
pH	7.3	7.5	7.7
Alkalintiy (g/L as $CaCO_3$)	3.1	3.6	3.8
CH_4 (%)	65.6	68.8	68.4
CH_4 (L/day)	2.05	3.87	5.0
VS removal (%)	87.1	86.4	84.0
COD removal (%)	85.2	83.6	81.5

COD value of food waste at 3 $gVSl^{-1}day^{-1}$ was 8.1 $gCODday^{-1}$ (that is, 3 $gVSl^{-1}day^{-1}$ × 3 L × 0.9 gCOD/ gVS). The conversion of H_2 and CH_4 to COD (g/day) was calculated as follows: because 1 mol (22.4 L) of hydrogen is equivalent to 16 g of COD, the COD of the hydrogen was calculated as the volume (L) of the $H_{2\ produced}$ × 0.714 $gCOD/LH_2$. Similarly, the COD of the

Table 4. COD balance at acidogenic and methanogenic stages

Stage	OLR ($gVSl^{-1}day^{-1}$)	COD_{in} ($gday^{-1}$)	COD_{out} ($gday^{-1}$)	H_2 ($gCODday^{-1}$)	COD_{H2}/COD_{feed} (%)	$COD_{recovery}$ (%)	
AGS	3	8.1	7.2	0.64	7.9	96.8	
	6	16.2	14.0	1.50	9.3	95.7	
	8	21.6	18.5	1.93	8.9	94.6	
Stage	OLR ($gVSl^{-1}day^{-1}$)	COD_{in} ($gday^{-1}$)	COD_{out} ($gday^{-1}$)	CH_4 ($gCODday^{-1}$)	COD_{CH4}/COD_{feed} (%)	$COD_{recovery}$ (%)	P $COD_{recovery}$ (%)
MGS	3	7.2	1.2	5.86	72.3	98.0	95.1
	6	14.0	2.7	11.06	68.3	98.0	94.0
	8	18.5	4.0	14.29	66.2	98.9	93.6

AGS-Acidogenic Stage; MGS-Methanogenic Stage; P $COD_{recovery}$ - the entire process COD recovery

methane was calculated as the volume (L) of the $CH_{4\ produced} \times 2.857$ gCOD/LCH_4.

The fraction of the feed-COD converted to the hydrogen-COD at acidogenic stage ranged from 7.9% to 9.3% and peaked at 6 $gVSl^{-1}day^{-1}$, whereas the fraction of feed-COD converted to the methane-COD at methanogenic stage ranged from 66.2% to 72.3% and peaked at 3 $gVSl^{-1}$ day^{-1}. The maximum hydrogen conversion value of 9.3% was higher than the corresponding value obtained by the previous study.[20]

CONCLUSIONS

From the experiments, the following conclusions were obtained.

1. It was feasible to obtain H_2-producing bacteria from a raw seed sludge through direct acclimation of feedstock without pretreatment.
2. To prevent methanogenesis at the acidogenic stage even at enough HRT for the acidification of the feedstock, the following factors are necessary as: the thermophilic condition, operation at low pH and a long period of acclimation of seed sludge to substrate in case of harvest hydrogen-producing bacteria with no heat-pretreatment of seed sludge.
3. *C. thermosaccharolyticum* was the key hydrogen-producing bacteria which played the fermentation of acetate/butyrate from carbohydrate in the food waste to the conversion of hydrogen at the acidogenic stage.
4. The fraction of feed-COD converted to the hydrogen-COD ranged from 7.9% to 9.3% at the acidogenic stage, whereas the fraction of feed-COD converted to the methane-COD ranged from 66.2% to 72.3% at the methanogenic stage.

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