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Developing a New Technology for the Two Phase Methane Fermentation Sludge Recirculation Process

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Methane fermentation is an effective way to treat wet organic wastes. Since methane fermentation is a multistage process, intermediates are likely to accumulate and cause fermentation inhibition. Two phase methane fermentation process enables further accurate control of the environmental conditions to suit each reaction and this will prevent fermentation inhibition. Hydrogen-methane two phase fermentation process can produce both H_2 and CH_4, and increase total energy quantity. Partial recirculation of methane fermentation sludge will act as inoculum compensation and pH control for the hydrogen fermentation process because the sludge includes hydrogen producing bacteria. Since methane fermentation sludge includes also hydrogen consuming microbes such as methanogenic bacteria and those that compete with hydrogen producing bacteria for substrate, making hydrogen producing bacteria dominant is necessary for a fine hydrogen-methane process. Heat treatment to the methane fermentation sludge has been used for effectively making the hydrogen fermentation bacteria dominant. This research aimed to develop a new treatment technology for effectively dominating the hydrogen producing bacteria. Hot compressed water (HCW) treatment was employed as a new treatment technology and was compared with heat treatment. Hydrogen fermentation was performed by using treated seed sludge and the effectiveness of the treatment was evaluated. Mesophilic methane fermentation sludge from food waste was used as seed sludge. Heat treatment was operated by a water bath at 95 °C for 2 h. HCW treatment was operated at 150 °C for 40 min as retention time under three different pressures 0.5, 2.5 and 4.5 MPa. Hydrogen fermentation was performed with a 100 ml batch reactor adding 0.375 g glucose as substrate in an incubator at 37 °C for 150h. Gas generation and composition were measured every 6 h with GC. The organic acid and glucose concentration was measured with HPLC after fermentation. Microbial community structure was analyzed with a next-generation DNA sequencer on the hydrogen fermentation sludge. HCW treatment at 0.5 MPa was effective and high hydrogen yield over 1.5 mol-H_2/mol-glucose was obtained in two of three replicates, which was 4 times higher than non-treatment, and 3 times higher than heat treatment. Microbial community structure analysis showed that HCW treatment at 0.5 MPa was more effective than heat treatment for eliminating methanogenic bacteria and dominating Clostridium.

1. Backgrounds

1.1 Two-phase methane fermentation

Anaerobic digestion is a key technology to promote the use of biomass. Methane fermentation is for collecting CH_4 as a final product, and hydrogen fermentation is for collecting H_2. CH_4 and H_2 both could be converted into energy. Also, methane fermentation sludge contains much nutrient contents such as inorganic nitrogen, phosphorus, and potassium and is used as a liquid fertilizer. Various kinds of anaerobic bacteria act in this multistage process and generate several intermediate products. Volatile fatty acids (VFAs) are one of the main intermediate products. Accumulation of VFAs will decrease the pH and lead to fermentation inhibition so environmental condition control will be important for a fine and smooth process. To prevent these fermentation inhibitions, there is an idea to divide the reaction process into two phases, hydrogen fermentation and

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methane fermentation, by using two tanks. This will enable further accurate control and enable fermentation efficiency improvement. Furthermore, separating hydrogen fermentation in the first tank from the sequence of methane fermentation will enable to collect both H_2 and CH_4 and increase the total energy production.
Hydrogen fermentation is similar to methane fermentation but performed in different conditions for generating H_2 as a final product. Hydrogen fermentation sludge contains much VFAs and secondary treatment such as methane fermentation is usually necessary. In Hydrogen-methane two phase fermentation, hydrogen fermentation sludge will flow into the second methane fermentation tank and be finely degraded.

1.2 Pretreatment to the seed sludge for making hydrogen producing bacteria dominant

Partial sludge recirculation from the second tank in this two phase fermentation will work as pH control and inoculum compensation for hydrogen fermentation. However, the second tank sludge also contains hydrogen consuming bacteria such as methanogen and others that compete with the hydrogen producing bacteria for substrate. These bacteria may inhibit hydrogen fermentation or lower H_2 yield. Making hydrogen producing bacteria dominant in the sludge before feeding the hydrogen fermentation tank will be desirable for improving hydrogen fermentation efficiency.
In the aim of inactivating the competitive bacteria and selecting the hydrogen producing bacteria, several pretreatments have been examined to the seed sludge (Jia et al., 2013). Clostridium is one of the hydrogen producing bacteria which has an ability to form spores that is durable to physicochemical stress. Heat treatment is known to be effective for selecting Clostridium (Tosaka et al., 2005) and is mostly used for its easy dominating operation.
This study aimed to develop a new technology for making hydrogen producing bacteria dominant. We employed hot-compressed water (HCW) treatment as a new pretreatment to the seed sludge. The effect of HCW treatment on hydrogen fermentation efficiency was compared with fermentation by non-treatment seed sludge and heat treatment seed sludge.

2. Materials and methods

2.1 Seed sludge acclimation

Methane fermentation sludge was taken from Ogawa-machi biogas plant in Saitama prefecture, Japan. It was generated from mesophilic fermentation, and raw material of methane fermentation was food waste. This sludge was first acclimated by feeding dried soybean curd refuse before the seed sludge pretreatment.

2.2 Seed sludge pretreatment

Two kinds of pretreatments, heat treatment and HCW treatment, were operated to the seed sludge. Heat treatment was performed for 2 h using a water bath set to be at 95 °C. HCW treatment was operated at constant temperature of 150 °C, under three different pressures of 0.5 MPa, 2.5 MPa, and 4.5 MPa, and for the same retention time of 40 min. A 300 mL stainless steel batch reactor (Parr, Parr 4766) was used. 100mL fermented sludge was put into the reactor and was purged with N_2 gas and pressurized. The reactor was heated by using a band heater and hot plate, and temperature inside of the reactor was measured by a T-type thermocouple. Once the temperature reached 150 °C, temperature was held at 150 °C for 40 min by using a controller (KEYENCE, TF4-10V). Once the treatment ended, the reactor was cooled by using water and seed sludge was collected.

2.3 Hydrogen fermentation

A 100 mL glass bottle was used as a batch reactor. 100 mL culture medium, 5mL of seed sludge, and 0.375 g of glucose as substrate was put into each bottle and covered with a butyl rubber stopper and aluminum cap. Hydrogen fermentation was examined with 5 different groups; which used non-treatment seed sludge (control), heat treatment seed sludge (heat), HCW 0.5MPa treatment seed sludge (0.5MPa HCW), HCW 2.5MPa treatment seed sludge (2.5MPa HCW), and HCW 4.5MPa treatment seed sludge (4.5MPa HCW) by three replicates for each group. The bottles were always shook at about 70 rpm using a shaker (EYELA, MMS-310) in an incubator (EYELA, LTI-601SD) set to be 37 °C. The experiment ended when the gas generation of all groups got under 1 mL, which was after 150 h. Gas generation and gas composition were measured every 6 h, and the hydrogen fermentation sludge was collected and analyzed after fermentation. H_2 yield was calculated from the generated H_2 (mol) divided by the input glucose (mol) and fermentation efficiency was evaluated.

2.4 Gas analysis

Gas volume was measured by using a plastic syringe. Gas composition was determined by a TCD gas chromatograph (Shimadzu, GC-14B) with Porapack Q column (Agilent Technologies).

2.5 Hydrogen fermentation sludge analysis

The pH of hydrogen fermentation sludge was measured by a pH meter (TOADKK, HM-21P). Organic acid analysis was performed on lactic acid, formic acid, acetic acid, propionic acid, and butyric acid by HPLC (Shimadzu, UFLC Prominence) with column: Shim-pack SCR-102H (Shimadzu GLC) and detector: CDD-6A (Shimadzu). Glucose was analyzed using HPLC (Shimadzu, UFLC Prominence) with column: Shim-pack ISA-07/S2504 (Shimadzu GLC) and detector: RF-10AXL (Shimadzu).

2.6 Microbial community structure analysis

Microbial community structure was analyzed with a next generation sequencer by NIPPON STEEL & SUMIKIN Eco-Tech Corporation, Japan. The analysis was performed on three seed sludge; non-treatment (control), heat treatment, 0.5MPa HCW, and on hydrogen fermentation sludge of 0.5MPa HCW pretreatment.
First, the eubacterial 16S rDNA gene amount was measured by real time PCR method. Then, V4-V5 region of the 16S rDNA gene was amplified by PCR using primers U515F and 926R which contain sequences necessary for sequencing analysis. After purifying the obtained PCR amplification product, the concentration was measured using PicoGreen ds DNA Assay Kit (Invitrogen). The concentration of the PCR amplification products were adjusted and sequence analysis was performed with MiSeq (Illumina). Approximately 250 bases from both sides of the PCR amplification product were analyzed (pair end analysis), and the ends of the two sequence analysis data were overlapped to obtain base sequence information about 410 bases. The obtained sequence data was analyzed using the QIIME (Quantitative Insights Into Microbial Ecology) pipeline. For each representative OTU sequence, homology search was performed on Silva's Living Tree 16S rRNA gene database to estimate the lineage classification.

3. Results and discussion

3.1 Gas generation and hydrogen yield

The average H_2 generation and yield of control was 17.6mL and 0.38 mol-H_2/mol-glucose, respectively and CO_2 generation amount was larger than that of H_2. Heat treatment seemed to be effective for inactivating the hydrogen consuming bacteria and two of the three replicates generated less CO_2 than H_2. The highest H_2 generation and yield of heat was 23.82 mL and 0.51 mol-H_2/mol-glucose, respectively. Although there were differences between the replicates, high H_2 yield was observed in two of the three replicates in 0.5MPa HCW (0.5MPa HCW2 and 3). The H_2 generation and yield of 0.5MPa HCW2 and 3 were 70.0, 71.8 mL and 1.50, 1.54 mol-H_2/mol-glucose, respectively. This amount was about 4 times larger than control and about 3 times larger than heat. CO_2 generation was inhibited in all three replicates of 0.5MPa HCW. HCW treatment seemed not to be effective at 2.5 MPa and 4.5 MPa. (Table 1).
Seed sludge pretreatment lead to delay the start of gas generation. This dormant period was predicted to have occurred since the pretreatment had also decreased the number of hydrogen producing bacteria and cell growth period was necessary (Sano et al., 2006). By shortening the treatment time may enable to shorten the delay. The 0.5MPa HCW 2 and 3 which had the highest H_2 yield generated H_2 in two parts. The second gas generation started from about 90 h and generated 2 times larger amount of H_2 than in the first generation (Figure 1, 2, 3).

Table 1: Total H_2 and CO_2 generation and H_2 yield

Treatment	Sample No.	H_2 (mL)	CO_2 (mL)	H_2 yield (mol-H_2/mol-glucose)
Control	1	16.92	19.21	0.36
	2	18.26	22.36	0.39
	3	17.63	21.23	0.38
Heat	1	23.82	9.21	0.51
	2	13.61	5.20	0.29
	3	16.34	19.96	0.35
0.5MPa HCW	1	23.16	9.09	0.50
	2	71.80	36.92	1.54
	3	70.03	37.34	1.50
2.5MPa HCW	1	29.11	10.58	0.62
	2	15.77	4.69	0.34
	3	16.13	18.23	0.35
4.5MPa HCW	1	19.89	10.28	0.43
	2	11.99	4.59	0.26
	3	15.76	18.64	0.34

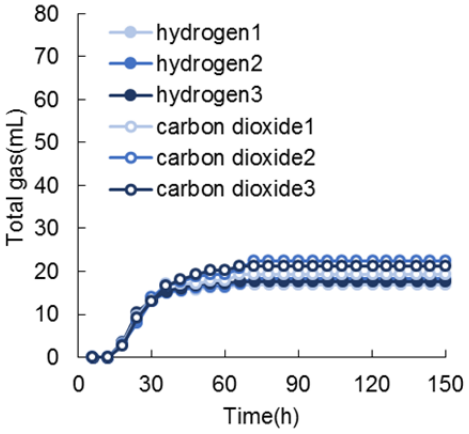

Figure 1: Gas generation during the hydrogen fermentation with non-treatment seed sludge

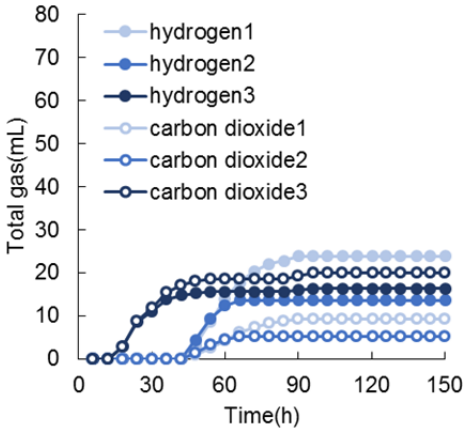

Figure 2: Gas generation during the hydrogen fermentation with heat treatment seed sludge

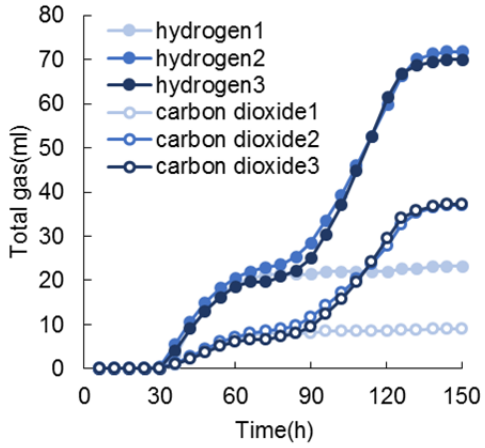

Figure 3: Gas generation during the hydrogen fermentation with 0.5MPa HCW treatment seed sludge

3.2 pH, Organic acids and glucose concentrations

In 0.5MPa HCW 2 and 3, which had the highest H_2 yield, the acetic acid and butyric acid concentration in the hydrogen fermentation sludge was higher than the other groups. Especially, butyric acid was in very high concentration. Even the H_2 yield was low, control also contained high acetic acid in the hydrogen fermentation sludge. It was predicted that high acetic acid concentration and low H_2 yield of control was because of the existence of hydrogen consumers that produce acetic acid. Acetic acid/butyric acid concentration ratio around

0.5 seemed to be good for obtaining a high H_2 yield. Though in very low concentration, glucose was still contained in some samples. The substrate consumption didn't rely on the H_2 yield (Table 2).

Table 2: Glucose and organic acid concentrations of the hydrogen fermentation sludge

Treatment	Sample No.	H_2 yield (mol-H2/ mol-glucose)	pH	Glucose (mg/L)	Lactic acid (mg/L)	Formic acid (mg/L)	Acetic acid (mg/L)	Propionic acid (mg/L)	Butyric acid (mg/L)	Acetic/ Butyric
Control	1	0.36	4.69	0.00	4.4	0.0	554.7	6.2	73.6	7.54
	2	0.39	4.73	0.00	0.0	0.0	667.3	10.2	130.8	5.10
	3	0.38	4.72	0.00	0.0	0.0	613.2	6.9	71.6	8.56
Heat	1	0.51	4..45	2.62	103.4	11.8	204.0	9.8	357.2	0.57
	2	0.29	4.29	3.25	113.6	138.3	331.3	12.7	278.0	1.19
	3	0.35	4.61	0.00	108.2	69.9	77.7	17.6	0.00	
0.5MPa HCW	1	0.50	4.59	4.83	30.8	14.0	330.8	15.2	780.2	0.42
	2	1.54	4.17	0.01	19.4	5.8	531.5	13.2	1086.4	0.49
	3	1.50	4.14	0.00	30.7	8.9	666.0	17.7	1291.6	0.52
2.5MPa HCW	1	0.62	4.39	2.90	116.5	0.0	423.8	13.6	555.8	0.76
	2	0.34	4.46	3.11	121.2	12.4	196.8	7.7	263.9	0.75
	3	0.35	4.43	0.00	115.3	58.4	79.7	0.0	0.0	
4.5MPa HCW	1	0.43	4.36	1.03	93.2	58.9	302.0	11.7	325.1	0.93
	2	0.26	4.27	0.87	228.5	24.5	195.1	8.2	219.3	0.89
	3	0.34	4.46	0.02	106.1	27.6	69.3	0.0	0.0	

3.3 Microbial community structure

Euryarchaeota, to which methanogenic bacteria belongs was still 0.5% detected in heat treatment seed sludge but not detected in 0.5MPa HCW seed sludge. This shows that HCW treatment can more effectively eliminate methanogenic bacteria. Pretreatments acted differently on the microbial community structure and heat treatment increased Bacteroidetes and 0.5MPa HCW treatment increased Proteobacteria. HCW1 and 2 was mostly consisted of Firmicutes, to which Clostridium belongs (Figure 4).

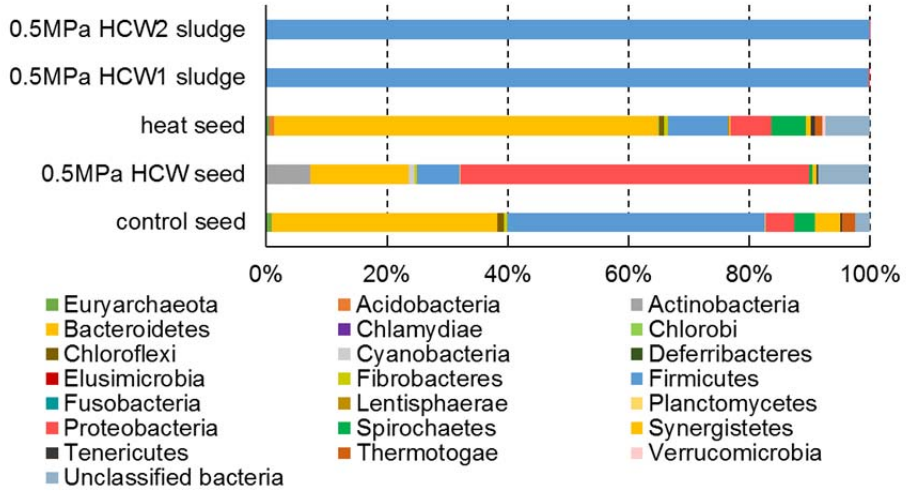

Figure 4: Phylum composition in the microbial community of seed sludge and hydrogen fermentation sludge

As mentioned above, H_2 yield and organic acid concentrations in 0.5MPa HCW1 and 2 were different. Both 0.5MPa HCW1 and 2 were consisted of mostly Clostridium which was over 95%, though the species composition of Clostridium was different. 0.5MPa HCW1 sludge was mainly consisted of C.roseum, though an unclassified species was dominant in 0.5MPa HCW2 sludge. There were also 2.4% C. cellulovorans, and 0.4% C.felsineum in the 0.5MPa HCW2 sludge which was not detected in 0.5MPa HCW1 sludge. The unclassified species was also detected in 0.5MPa HCW1 sludge and the closest related species at BLAST was C. felsineum and the homology was 97.8%. C. cellulovorans are able to degrade cellulose and cellobiose and produce H_2, CO_2, acetate, butyrate, formate, and lactate. They can also use xylan, pectin, glucose, maltose, galactose, sucrose, lactose and mannose for growth, and enable further use of woody biomass

(Sleat et al., 1984). C. felsineum is phylogenetically closely related to C. acetobutylicum (Tamburini et al., 2001), and is known as a pectinolytic microorganism of plant materials during retting (Potter and McCoy, 1952). It was suggested that the involvement of C. felsineum and C. cellulovorans enabled further use and decomposition of the material and led to a high H_2 yield in 0.5MPa HCW2. 0.5MPa HCW treatment was effective for dominating Clostridium, but the composition of the species seem to differ during the fermentation (Figure 5).

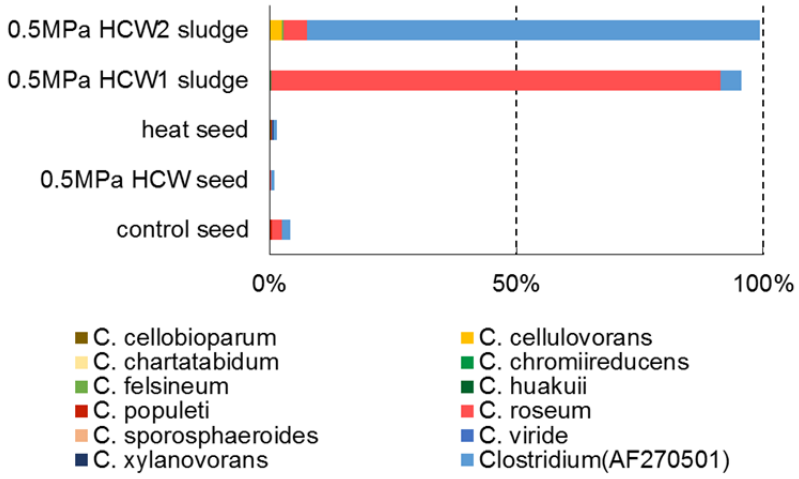

Figure 5: Species composition of Clostridium genus

4. Conclusions

HCW treatment was examined as a new seed sludge pretreatment method and compared with heat treatment. HCW treatment at 150°C and 0.5MPa for 40 min was effective than heat treatment for eliminating the methanogenic bacteria. Two of the three replicates of 0.5MPa HCW (0.5MPa HCW2 and 3) had H_2 yield over 1.50 mol-H_2/mol-glucose, which was about 3 times larger than that of the heat treatment and about 4 times larger than control. 0.5MPa HCW2 and 3 had higher acetic acid and butyric acid concentrations than the others and the acetic acid/butyric acid ratio was around 0.50. 0.5MPa HCW treatment was effective for dominating Clostridium and hydrogen fermentation sludge was consisted of over 95% Clostridium. However, species composition may differ during the fermentation and lead to different H_2 yields. The involvement of C. felsineum and C. cellulovorans seem to enable further decomposition of the material and lead to a high H_2 yield.

Acknowledgments

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