The Affecting Factors for Optimization of Mesophilic Aceticlastic Methanogenesis

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Abstract
A continuous-flow methane fermentation process was operated using acetate as the sole organic substrate. The phylogenetic analysis showed that 72% of the 16S rDNA clones were affiliated with aceticlastic methanogens, Methanosaeta and Methanosarcina. The effects of pH, NH4+, acetic acid, propionic acid, S2-, Co2+, Ni2+, and NO3- concentrations on methanogenic activity were investigated in batch assays using the culture broth from the acetate reactor. The results showed that the patterns of specific gas evolution rate with and without pH control were different. Under the condition with pH control, the specific gas evolution rate decreased drastically when the pH was below 6.5 and over 7.5. The optimum pH was found to be 7 for mesophilic aceticlastic reaction. The specific gas evolution rate decreased sharply when the concentration of free NH3 exceeded 100-150 mg/l; acetic acid and propionic acid did not interfere with the stability of anaerobic treatment when their concentrations were less than 3,500 mg/l and 4,000 mg/l, respectively. S2- had a toxic effect on methanogenic activity, such that the gas production rate was nearly zero at a Na2S concentration of 300 mg/l; the additions of Ni2+ and Co2+ increased the gas production rate and they had almost no negative effects on methane fermentation even when their concentrations reached 10 mg/l. The specific gas evolution rate also decreased sharply when the concentration of NO3- exceeded 500 mg/l which corresponded to about 110 mg-N/l.

Key words: Methane fermentation, aceticlastic methanogen, specific gas evolution rate, ammonia, acetic acid, propionic acid, sulfide, cobalt, nickel, nitrate

INTRODUCTION
Methane fermentation is a long-life technology throughout a century so that researches concerning the factors affecting the treatment efficiencies of methane fermentation processes have been carried out1. A number of useful knowledge concerning the affecting factors on methane fermentation, such as temperature, composition of wastewater (C/N ratio), pH, oxidation-reduction potential, sludge concentration and inhibitory compounds, have been accumulated so far3. However, most of these results were based on experiments using anaerobic digested sludge and acclimatized sludge in small-scale batch-wise cultivations without controlling pH1). It has been, therefore, difficult to differentiate whether acidogenesis or methanogenesis was affected by these factors. And, the actual pH during cultivation has been hardly regarded.

In general, methanogenic archaea is recognized to be more sensitive to the

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environmental parameters and inhibitory compounds than acidogenic bacteria\(^{10}\). And, approximately 70% methane produced in a methane fermentation is derived from acetate\(^{2,3}\). So, the knowledge concerning kinetics of aceticlastic methanogens has been of interest and accumulated. However, most of the findings were concerning thermophilic aceticlastic methanogens, which have 2-7 times higher growth rate than that of mesophilic aceticlastic methanogens, although mesophilic methane fermentation processes are widely commercialized, compared with thermophilic processes\(^{4-9}\).

In this report, we constructed a mesophilic continuous cultivation system and enriched aceticlastic methanogens. Then, phylogenetic analysis of the microbial community in the culture broth was carried out. Using the broth, the effect of the affecting factors, such as pH and concentrations of ammonium ion, volatile fatty acids and sulfide, on aceticlastic methanogens were evaluated under pH-controlled or no controlled conditions. The influence of concentrations of Ni\(^{2+}\) and Co\(^{2+}\), which have previously demonstrated to show a stimulatory effect on aceticlastic methanogenesis\(^{10}\), was also evaluated. Moreover, the range of NO\(_3^-\) concentrations for denitrification in methane fermentation reactor were evaluated. Under pH-controlled conditions, some new knowledge obtained about the affecting factors on aceticlastic methanogens were described here. The allowance range of NO\(_3^-\) concentrations for denitrification in a methane fermentation reactor were also described.

**MATERIALS AND METHODS**

**Continuous cultivation of acetate-degrading methanogens** Continuous cultivation was conducted at 37°C using a continuous stirred tank reactor (CSTR) with a working volume of 1.7 l operated at a dilution rate of 0.6 d\(^{-1}\). The reactor had been fed with synthetic wastewater containing acetate as a sole carbon source for over one year of operation. A modified methanothrix medium (DSMZ medium 334 provided by Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH [DSMZ], Braunschweig, Germany) was used as the synthetic wastewater, which consisted of (per liter of distilled water): 5.46 g sodium acetate, 16.0 g acetic acid, 0.3 g K\(_2\)HPO\(_4\), 4.0 g KHCO\(_3\), 1.0 g NH\(_4\)Cl, 0.6 g NaCl, 0.82 g MgCl\(_2\)-6H\(_2\)O, 0.08 g CaCl\(_2\)-2H\(_2\)O, and 0.1 g cystein-HCl•H\(_2\)O; 10 ml of trace element solution containing 21.3 mg/l of NiCl\(_2\)-6H\(_2\)O and 24.7 mg/l of CoCl\(_2\)-6H\(_2\)O; and 10 ml of vitamin solution (without B\(_12\)). For use as the seed sludge, a mesophilic digested sludge from Kumamoto-Tobu sewage works (Kumamoto-City) was acclimatized with a thickened surplus sludge at an organic matter-loading rate of 1.0 g/l•d at 37°C for 6 months. A 1.7-l portion of the acclimatized digested sludge was washed with the synthetic wastewater under anaerobic conditions and diluted to give a final OD\(_{600}\) of 3.0 prior to addition in a CSTR.

**Determination of specific gas evolution rate without pH control** The specific gas evolution rate was determined according to the method of Kida et al\(^{10}\) as follows: 0.2 ml of sodium acetate solution (292.85 mg/l) was added into a vial containing a stirring bar to a final concentration of 70 mM. To evaluate the effects of NH\(_4^+\), acetate, propionate, S\(^{-}\), Ni\(^{2+}\), Co\(^{2+}\) and NO\(_3^-\) on methanogenic activity, (NH\(_4\))\(_2\)HPO\(_4\), acetic acid, propionic acid, Na\(_2\)S•9H\(_2\)O, NiCl\(_2\)-6H\(_2\)O, CoCl\(_2\)-6H\(_2\)O and NaNO\(_3\) solutions, respectively, were added to the test vials. The vials were sealed with a rubber stopper and an aluminum cap and then a needle was penetrated into the rubber stopper to allow for N\(_2\) purging to create anaerobic conditions. A 10 ml aliquot of culture broth from the reactor was transferred to each vial and the vials were then connected using a vinyl tube to a volumetric pipette immersed in a thermostated water-bath as shown in Fig. 1. The gas evolution rate was then measured during incubation at 37°C under agitation at 100 rpm.

**Determination of specific gas evolution rate with pH control** The determination of specific gas evolution rate with pH control was carried out using the same type of CSTR at 37°C with mixing at 300 rpm (Fig. 2). 1.7 l of an anaerobic acetate-degrading methanogens culture broth was added to the reactor, then 34 ml of sodium acetate solution (292.85 mg/l) was added to a concentration of 70 mM.
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pH in the reactor was regulated automatically by a 1 N HCl solution using a pH controller (HB-96K2; Denki Kagaku Keiki Co. Ltd., Tokyo). Evolved gas was collected in a wet-gas holder and used to measure the gas evolution rate.

**DNA extraction and analysis of the 16S rDNA clone library** DNA from the continuous cultivation broth was extracted according to the previous report\(^\text{11}\). Amplification of 16S rDNA from the extracted DNA was performed by PCR using AmpliTaq Gold (Applied Biosystems, Foster City, Calif.) according to the manufacturer’s instructions with the universal primer 530F (5’-GTGCCAGCMGCCGCGG-3’; 514-529, *Escherichia coli* position) and prokaryote-specific primer 1490R (5’-GGTACCTTGTTA CGACTT-3’; 1491-1509 *E. coli* position). The amplified fragment was ligated with pT7-Blue T vector (Novagen) and introduced into *E. coli* DH5α cells to form a 16S rDNA clone library. Cloned 16S rDNA segments were prepared from randomly selected recombinants and used as templates for sequencing. Sequencing was performed using a DNA sequencer (ABI 373S-18, Applied Biosystems) with a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Bioscience, Piscataway, NJ). 16S rDNA sequences with a range of 950-970 bases were obtained for all clones. All sequences were checked manually for chimeric artifacts by using the CHIMERA_CHECK program version 2.7 in the Ribosomal Database Project II (RDP-II)\(^\text{12}\). Searching for similar sequences was done using the BLAST program\(^\text{13}\). Identical sequences (with 100% similarity) were recognized as an operational taxonomic unit (OTU). The nucleotide sequences for OTUs AHU01-AHU28 were deposited in the DDBJ database under the accession numbers AB092886-AB092913.

**Other analytical methods** pH was measured by using a pH meter (HM-25G, DKK-TOA Co., Tokyo). Volatile suspended solids (VSS) were determined in accordance with Standard Methods\(^\text{14}\).

**RESULTS AND DISCUSSION**

**Phylogenetic analysis of the 16S rDNA clones** A mesophilic acetate-degrading methanogenic consortium was cultivated continuously using the CSTR at a dilution rate of 0.6 d\(^{-1}\). At a steady state, DNA was extracted from the culture broth and 16S rDNA clone library was constructed and analyzed. A total 92 rDNA clones with 28 different sequences (OTUs) were obtained. Within the rDNA clones, 66 clones (72% of total clones) with 7 OTUs were affiliated with the domain *Archaea* and the remainders were affiliated with the domain *Bacteria* (Fig. 3). In the domain *Archaea*, 47 clones (51% of total clones) with 5 OTUs and 19 clones (21%) with 2 OTUs were closely related to the genera *Methanosarcina* and *Methanosaeta*, respectively, which were both known as aceticlastic methanogens. In the domain
Bacteria, clones were distributed in the four phyla: Firmicutes (Low G+C Gram-positive bacteria), Bacteroidetes, the candidate division OP12\textsuperscript{30} and Proteobacteria. These results indicated that the aceticlastic methanogens with two genera Methanosarcina and Methanosaeta were dominant in the continuous cultivation system. The cultivation conditions with acetate as the sole carbon source at the dilution rate of 0.6 d\(^{-1}\) were considered to be suitable for the two kinds of the aceticlastic methanogens to growth dominantly. The culture broth mainly consisted of Methanosarcina and Methanosaeta was used in the following experiments.

**Effect of pH on methanogenic activity**

Determinations of methanogenic activity without pH control were conducted at initial pH values of 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5. As shown in Fig. 4, the specific gas evolution rate increased sharply with an increase in the initial pH up to 6.0 where it reached a maximum of 2.2 ml/h/10 ml-cultured broth. With further increases in initial pH, the rate of gas production decreased slowly to 1.5 ml/h/10 ml-cultured broth at an initial pH of 8.0. However, gas production dropped to nearly zero with an initial pH of 8.5. The final pH increased for all the conditions studied (Table 1) due to the conversion of acetate to biogas by aceticlastic methanogens. The final pH with an initial pH of 6.0 was 7.4, reflecting the highest volume of biogas evolved.

Experiments with pH control were conducted at pH levels of 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5, respectively. As shown in Fig. 4, the rate of specific gas evolution was nearly zero when the pH was held at 5.5 and only 0.4 ml/h/10 ml-cultured broth at a pH of 6.0, which is only 20% of the rate without pH control at an initial pH of 6.0. The maximum specific gas evolution rate of 2.5 ml/h/10 ml-cultured broth occurred at a pH of 7.0. However, when the pH was held constant at levels greater than 7.0 the specific gas evolution rate decreased sharply and was only 0.55 ml/h/10 ml-cultured broth at a pH of 8.5. Thus the effects of strategies to control or not to control culture pH were demonstrated, with the greatest differences being evident at pH values below 6.5 and over 7.5. It was found that mesophilic aceticlastic methanogenic activity was
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sharply inhibited by the culture pH and the optimum pH was narrow range, just 7.0. Clarens et al. reported that the optimum pH for growth of a thermophilic aceticlastic methanogen Methanosarcina sp. MSTA-1 at 55°C was a relatively wide range of 6.5-7.5 compared with our result. The two temperature conditions, mesophilic and thermophilic, possibly caused this difference of optimum pH ranges.

Effect of NH₄⁺ on methanogenic activity

The influence of NH₄⁺ on the anaerobic treatment process was investigated at pH values of 7.5, 7.8 and 8.0. As shown in Fig. 5, with pH held at 7.5, NH₄⁺ at concentrations up to 5,000 mg/l did not inhibit methanogenic activity, which was nearly stable at about 1.75 ml/h/10 ml-cultured broth. However, with the pH maintained at 7.8, the gas evolution rate decreased slowly with an increase in NH₄⁺ up to 5,000 mg/l; furthermore, with pH maintained at 8.0, the specific gas evolution rate decreased sharply with an increase in NH₄⁺ and was nearly zero at NH₄⁺ concentrations of 3,500 mg/l and greater.

Equations (1) and (2) show the dissociation of NH₄⁺ to free NH₃ and the dissociation constant Kₐ, respectively. Free NH₃ concentration at each pH investigated was calculated using equation (3).

\[
\text{NH}_4^+ \rightleftharpoons \text{NH}_3 + \text{H}^+ \quad \text{(1)} \\
K_\text{a} = \frac{[\text{NH}_3][\text{H}^+]}{[\text{NH}_4^+]} \quad \text{(2)} \\
[\text{NH}_3] = K_\text{a} \frac{[\text{NH}_4^+]}{[\text{H}^+]^\text{pH} - \text{pK}_\text{a}} \quad \text{(3)}
\]

Figure 6 shows the effect of free NH₃ concentration on the specific gas evolution rate at each pH studied. Specific gas evolution rate decreased sharply when the concentration of free NH₃ was more than 100-150 mg/l.

The pH control at near to 7 should be better to avoid the inhibition of free NH₃, considering the optimum pH described above.

Effect of acetic acid on methanogenic activity

Acetic and propionic acids are the major volatile fatty acids (VFAs) formed during anaerobic treatment of organic waste. These main substrates in the terminal step of methanogenesis are inhibitory to the process at high concentrations (Boone et al., 1987).

In this study, the effect of acetic acid on methanogenic activity without pH control was investigated using an initial pH of 6.0. As shown in Fig. 7, the gas production rate was nearly constant at 1.75 ml/h/10 ml culture broth at acetic acid concentrations up to 3,000 mg/l; however, the rate decreased linearly with increases in acetic acid greater than 3,000 mg/l. The final pH of this experiment was 7.0.

The effect of acetic acid on methanogenic activity with pH control was investigated at a pH of 6.5. Acetic acid concentrations up to
2,000 mg/l did not interfere with the specific gas evolution rate; however, the rate decreased sharply from 1.50 ml/h/10 ml-cultured broth at an acetic acid concentration of 2,000 mg/l to 0.25 ml/h/10 ml-cultured broth at 6,000 mg/l (Fig. 7).

From the above results, it is evident that the inhibitory effects of acetic acid on methanogenic activity with or without pH control followed the same pattern, and that acetic acid had a greater inhibitory effect when pH was maintained at 6.5.

**Effect of propionic acid on methanogenic activity** The effect of propionic acid on methanogenic activity without pH control was investigated using an initial pH of 6.0 and with pH control at a pH of 6.5. As shown in Fig. 8, no significant effects of propionic acid on the specific gas evolution rate with and without pH control were seen at propionic acid concentrations up to 4,000 mg/l. However, the specific gas evolution rate without pH control was consistently about 35% higher than that with pH control. Dhaked *et al.*[19] investigated the effect of propionate on biodegradation of human waste at 30°C with initial pH values of 6.0, 7.0, and 8.0. Their results showed that increasing the propionate concentration up to 14,800 mg/l resulted in increased toxicity to biomethanation at all pH levels tested and the biogas evolution decreased 77% and 70% at pH levels of 7.0 and 8.0, respectively. Kugelman *et al.*[20] also reported that propionate is more toxic than other VFAs. However, our results indicated that acetic acid inhibited methanogenic activity more strongly than propionic acid.

**Effect of S^{2-} on methanogenic activity** In the methane fermentation process, it is well known that S^{2-} at 50 mg/l accelerate the gas evolution rate, but S^{2-} at more than 100 mg/l generally inhibit completely[21]. To further evaluate the effect of S^{2-} on methane fermentation, experiments were conducted without pH control. As shown in Fig. 9, the specific gas evolution rate steadily decreased...
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with an increase in $S_2^-$ concentration and it was nearly zero at a Na$_2$S concentration of 300 mg/l. The effect of $S_2^-$ could become critical with scale-up to a reactor with greater liquid depth, which would result in higher concentrations of hydrogen sulfide dissolved in solution.

Effect of Ni$^{2+}$ and Co$^{2+}$ on methanogenic activity The influences of Ni$^{2+}$ and Co$^{2+}$ on methane fermentation of shochu distillery wastewater where the maximum TOC volumetric loading rate achieved under mesophilic and thermophilic conditions were 24 and 48 g/l-d, respectively, were previously determined$^{22}$. In that work, the rate of methane fermentation was enhanced 4 to 5 times with the addition of these metallic ions. To evaluate the effects of Ni$^{2+}$ and Co$^{2+}$ on anaerobic digestion, experiments were carried out without pH control. The influence of Ni$^{2+}$ was investigated using a Co$^{2+}$ concentration of 1 mg/l and for the influence of Co$^{2+}$, a Ni$^{2+}$ concentration of 1 mg/l was used. When the concentration of Ni$^{2+}$ was increased to 10 mg/l, the specific gas evolution rate increased sharply to a maximum of 1.05 ml/h/10 ml-cultured broth and then decreased slowly with subsequent increases in Ni$^{2+}$ to 25 mg/l (Fig. 10). At a Ni$^{2+}$ concentration of 50 mg/l, the specific gas evolution rate decreased to 0.4 ml/h/10 ml-cultured broth, which is the same as that without addition of Ni$^{2+}$. When concentration of Co$^{2+}$ was increased to 1 mg/l, as shown in Fig. 11, the specific gas evolution rate also increased sharply to 1.08 ml/h/10 ml-cultured broth and remained nearly stable to a Co$^{2+}$ concentration of 10 mg/l. Subsequently, the specific gas evolution rate decreased slowly to 0.65 ml/h/10 ml-culture broth at a Co$^{2+}$ concentration of 50 mg/l. These results indicated that Ni$^{2+}$ and Co$^{2+}$ concentrations up to approximately 10 mg/l do not interfere adversely with methanogenic activity.

Influence of NO$_3^-$ on methanogenic activity In a previous study, we reported that TOC and NO$_3^-$ could be simultaneously removed during methane fermentation of wastewater from a rubber thread industry using an UAFP reactor following a nitrification process, but the 13% of methane gas evolved at a volumetric loading rate of 4 g/l/d decreased by inhibition of NO$_3^-$20. In this work, to determine the maximum NO$_3^-$ concentration during methane fermentation, experiments were conducted without the pH control. As shown in Fig. 12, the specific gas evolution rate was nearly constant at 1.05 ml/h/10 ml-cultured broth up to a NO$_3^-$ concentration of 500 mg/l; however, the rate decreased sharply when NO$_3^-$ was further increased. The evolution rate dropped to 0.38 ml/h/10 ml-cultured broth at a NO$_3^-$ concentration of approximately 1,000 mg/l and then gradually approached zero as the NO$_3^-$ level was stepped up to 4,000 mg/l. These results suggest that NO$_3^-$ concentrations up to 500 mg/l will not
interfere with the stability of an anaerobic treatment process

CONCLUSION

The phylogenetic analysis of the microbial community in a continuous-flow methanogenic process using acetate as the carbon source showed that 72% of the total 16S rDNA clones were affiliated with aceticlastic methanogens, Methanosaeta and Methanosarcina. At pH levels below 6.5 and over 7.5, the patterns of specific gas evolution rates with and without pH control were significantly different and the effect of NH$_4^+$ on the anaerobic treatment process was affected by the pH of the system. The specific gas evolution rate decreased when the concentration of acetic acid or propionic acid was increased beyond 4,000 mg/l. In addition, the gas evolution rate approached zero with increases of Na$_2$S up to 300 mg/l. Ni$^{2+}$ and Co$^{2+}$ supplements had positive effects on methanogenic activity with a maximum specific gas evolution rate obtained when both metal ions were simultaneously 10 mg/l. As NO$_3^-$ concentrations up to 500 mg/l did not inhibit the specific gas evolution rate, TOC and NO$_3^-$ could be removed simultaneously during methane fermentation without inhibition up to the NO$_3^-$-N concentration of 110 mg/l.

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