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Effects of ammonium and/or sulfide on methane production from acetate or propionate using biochemical methane potential tests

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The inhibitory effects of ammonium and sulfide on the methane production using acetate or propionate as a carbon source were investigated under different pH and temperature conditions. The methane production rate, duration of the lag phase, and inhibition threshold limit during methane production were estimated using the Gompertz equation and inhibitor mathematical model. The methane production rates at 53°C were 2.3–2.7 times higher than those at 35°C in the case of non-inhibitors. Increasing the NH_4^+ and/or S^{2-} concentration decreased the methane production rate and increased the duration of the lag phase. For methane fermentation that was not acclimated to high NH_4^+ concentration, the critical NH_4^+ concentration beyond which methane fermentation would stop was 4000–5650 mg/L, depending on the pH, temperature, and carbon source. When NH_4^+ and S^{2-} were coexistent, the critical NH_4^+ concentration decreased to approximately 3800 mg/L when propionate was used and to approximately 4450 mg/L when acetate was used. However, no synergistic effect of NH_4^+ and S^{2-} on the methane production rate was found at an NH_4^+ concentration of < 5000 mg/L and S^{2-} concentration of 50 mg/L.

[**Key words:** Methane fermentation; Biogas; Ammonia; Sulfide; Inhibition]

Conversion of organic waste to biogas via methane fermentation is an effective approach for minimizing environmental pollution from the untreated waste and conserving fossil fuel. From the perspective of environmental sustainability, methane fermentation is regarded as a promising process for renewable energy generation and waste stabilization (1).

When treating organic waste with a low carbon to nitrogen (C/N) ratio and/or low carbon to sulfur (C/S) ratio, methane fermentation is easily inhibited by ammonium (NH_4^+) and/or sulfide (S^{2-}) ions because free ammonia (NH_3) and hydrogen sulfide (H_2S) can diffuse into the cell membrane (2–5). Moreover, the quality of biogas is reduced by the presence of NH_3 and H_2S (6). The anaerobic digestion process will be more difficult when NH_4^+ and S^{2-} are coexistent (7,8). Hansen et al. (7) studied the anaerobic digestion of swine manure, and found that when the ammonia concentration was 4.6 g-N/L, even a very low concentration of sulfide (23 mg S^{2-}/L) has an inhibitory effect on the anaerobic digestion process. Lauterbock et al. (8) found that when the ammonia concentration in the reactor was 5.7 g-N/L, only 28–37 mg/L S^{2-} resulted in a 50% reduction (IC50) in the methane yield. However, the S^{2-} concentration ≥50 mg/L may cause IC50 inhibition of methanogens in the presence of S^{2-} inhibition alone (2). These results indicate that NH_4^+ and S^{2-} have a potential synergistic inhibitory effect on anaerobic digestion.

To alleviate inhibition of methane fermentation by S^{2-} and increase the biogas quality, a methane fermentation process with micro-aeration was developed in our previous study (9), and the phylogenetic diversity of microorganisms in the fermenter was analyzed (10). Furthermore, a methane fermentation process with micro-aeration and biogas recirculation was developed to simultaneously alleviate the inhibitory effects of NH_4^+ and S^{2-} (11,12). During the methane fermentation process with micro-aeration and biogas recirculation, H_2S in biogas was primarily oxidized to sulfur and/or sulfate with micro-aeration. The biogas exhausted was washed with water before recirculation, and NH_3 was fixed in the water tank mainly as NH_4HCO_3 by reaction of NH_3 and CO_2. Recirculation of the water-washed biogas into the fermenter enhanced stripping of NH_3 and H_2S. Therefore, the inhibitory effects of NH_4^+ and/or S^{2-} were alleviated (11). The state of NH_3 and H_2S in the liquid phase is pH- and temperature-dependent. Generally, NH_3 preferentially exists at high pH, while H_2S preferentially exists at low pH. Therefore, the inhibitory effects of NH_4^+ and/or S^{2-} could potentially be alleviated by controlling the pH and temperature during the methane fermentation process (13). In our previous study, the efficiency of NH_3 removal increased 1.23-fold with an increase of the temperature of the methane fermentation process from 53°C to 60°C (12). However, methane fermentation is easily affected by pH and/or temperature changes. Moreover, volatile fatty acids (VFAs), especially acetic acid and propionic acid, were accumulated when the inhibitory effects of NH_4^+ and/or S^{2-} were

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expressed (11,14,15), causing failure of methane fermentation. To directly alleviate the inhibitory effects of NH_4^+ and/or S^{2-}, it is important to investigate the effects of NH_4^+ and/or S^{2-} on methane production under various pH and temperature conditions.

In this study, biochemical methane potential (BMP) tests are carried out to investigate the effects of the individual species and synergistic inhibition by NH_4^+ and S^{2-} on methane production under different pH and temperature conditions using acetate or propionate as a carbon source. The Gompertz equation and inhibitor mathematical model are used to estimate the methane production rate, duration of the lag phase, and inhibition threshold limit for methane fermentation.

MATERIALS AND METHODS

Acclimation of inoculum sludge Sludge from a thermophilic anaerobic digester for treating the stillage of ethanol fermentation was acclimated in an anaerobic digester with a working volume of 8 L (MDL-10L; B.E. Marubishi, Chiba, Japan) at 53°C and 85 rpm by using canteen garbage (volatile total solid (VTS), 201 g/kg, wet base) as a feed. The VFAs, gas evolution, NH_4^+, SO_4^{2-}, and pH were monitored during acclimation. Sludge at steady-state was used for the BMP tests.

Experimental set-up The BMP tests used an Automatic Methane Potential Analysis System II (AMPTS II; Bioprocess Control Sweden AB, Sweden) and were carried out in a 600 mL vial with a working volume of 400 mL. The conditions used for each experimental vial are summarized in Table 1. A 230 mL aliquot of nutrient solution, 0.1 mol/L phosphate buffer (60 mL; pH 7.0 or 8.0), and inoculum sludge (20 mL) were mixed. NH_4Cl solution (0–50 mL; $[NH_4^+]$ = 40 g/L) and/or Na_2S solution (0–60 mL; $[S^{2-}]$ = 2 g/L) were added according to the designated inhibitor concentrations. Sodium acetate or sodium propionate solution (10 mL), with a total organic carbon (TOC) concentration of 40 g/L, was added as a carbon source. Finally, distilled water was added to make a working volume of 400 mL. The carbon source added was equivalent to an acetate or propionate concentration of 2500 mg/L or 2050 mg/L, respectively, which would not inhibit methane fermentation according to our previous study (16). When necessary, the initial pH was adjusted to 7.0 or 8.0 using 10% (*w/w*) HCl solution or 10% (*w/w*) NaOH solution. A control vial was prepared by the same method as outlined above, except that distilled water was substituted for NH_4Cl solution and Na_2S solution and the initial pH was adjusted to 7.0. A blank vial was prepared by adding nutrient solution, phosphate buffer, inoculum sludge, and distilled water. The applied inoculum sludge had a pH of 8.1, a TS content of 5.5%, a VTS content of 3.3%, and a NH_4^+ content of 800 mg/L. The 230 mL nutrient solution contained KH_2PO_4, 69 mg; $KHCO_3$, 920 mg; NH_4Cl, 230 mg; NaCl, 130 mg; $MgCl_2\cdot 6H_2O$, 180 mg; $CaCl_2$, 15 mg; L-cysteine hydrochloride anhydrous, 20 mg; and trace element solution, 1.5 mL. The trace element solution consisted of $FeCl_3\cdot 6H_2O$, 1350 mg/L; $ZnCl_2$, 100 mg/L; $NiCl_2\cdot 6H_2O$, 410 mg/L; $MnCl_2\cdot 4H_2O$, 100 mg/L; $Na_2MoO_4\cdot 2H_2O$, 24 mg/L; $CuCl_2\cdot 2H_2O$, 25 mg/L; $CoCl_2\cdot 6H_2O$, 140 mg/L; and H_3BO_3, 10 mg/L.

Each experimental vial was flushed with N_2 for 3 min before assembling the rubber stoppers and the mechanical agitators. The gas pipelines were then connected and the vials were incubated at 35°C or 53°C in a water bath. The control vials were incubated at 53°C. Agitation was set at 85 rpm with repeated ON-OFF (85 s On and 15 s Off) cycles throughout the tests. The biogas produced was passed through a 3 mol/L NaOH solution (80 mL) to fix CO_2 and H_2S, allowing only methane to be detected. The methane volume is measured through water displacement using flow cells which give a signal for approximately every 10 mL of produced gas. Internal temperature and pressure sensors are used to normalize the gas volume to 0°C, 1 atm and dry gas conditions at each measurement point. The BMP tests at 35°C were carried out for 185 h, while the BMP tests at 53°C were carried out for 50 h. The BMP test under each condition was independently carried out twice, and the data presented are the average values.

Data analysis The modified Gompertz equation (Eq. 1) was used to fit the experimental methane production curve to determine the methane production performance (17).

$$P(t) = P_m \cdot \exp\left\{ -\exp\left[\frac{V \cdot e}{P}(\lambda - t) + 1\right]\right\} \quad (1)$$

where $P(t)$ (mL/L) is the accumulated methane production at time t (h); P_m (mL/L) is the maximum accumulated methane production during the test period, which was fixed at P (185) at 35°C and P (50) at 53°C for model prediction; V (mL/L/h) is the methane production rate, which was defined as the volume of methane produced per liter sludge in 1 h; and λ (h) is the duration of the lag phase.

The effect of the NH_4^+ or S^{2-} concentration on the methane production rate under specific pH and temperature conditions was determined by using the inhibitor mathematical model (Eq. 2) (17):

$$V_C = V_0\left(1 - \frac{C}{C^*}\right)^n \quad (2)$$

where C (mg/L) is the inhibitor (NH_4^+ or S^{2-}) concentration; V_C (mL/L/h) is the methane production rate at an inhibitor concentration C; V_0 (mL/L/h) is the methane production rate in the absence of an added inhibitor; C^* (mg/L) is the critical inhibitor concentration, beyond which methane fermentation would stop. Origin Pro 8.0 was used for curve-fitting with a minimum residual sum of squared errors between the experimental data and model curve.

The inhibition ratio (R) is defined as follows (Eq. 3):

$$R = \frac{(V_{Con} - V_E)}{V_{Con}} \times 100\% \quad (3)$$

where V_{Con} (mL/L/h) is the methane production rate of the control vial (in the absence of an added inhibitor at pH 7.0 and 53°C); V_E (mL/L/h) is the methane production rate for the experimental vials with different inhibitor concentrations.

The synergistic effect of NH_4^+ and S^{2-} on the methane production performance was analyzed using the method reported by Greetham et al. (18). The synergistic effect was determined from the epsilon values (ε) as calculated using Eq. 4.

$$\varepsilon = (1 - R_{N-S}) - (1 - R_N) \times (1 - R_S) \quad (4)$$

where $R_{N\text{-}S}$ is the inhibition ratio with the co-existence of NH_4^+ and S^{2-}; R_N is the individual inhibition ratio of NH_4^+; R_S is the individual inhibition ratio of S^{2-}.

Analytical methods Acclimation sludge (10 mL) was centrifuged at 12,000 rpm and 4°C for 10 min. The supernatant was filtered through a 0.45 μm membrane filter (Jinteng, Tianjin, China) and the filtrate was collected. The concentrations of VFAs, SO_4^{2-}, and NH_4^+ in the filtrate were analyzed. The VFAs were analyzed using an HPLC equipped with a UV detector (at 450 nm) and a Shimpack SCR-101H (Shimadzu, Kyoto, Japan) column (19). The NH_4^+ concentration was analyzed with an ion chromatography system (ICS-1500; Dionex, Sunnyvale, CA, USA) equipped with a CS-12A column and a CG-12A guard column. The SO_4^{2-} concentration was measured using a different ion chromatography system (ICS-1100; Dionex) equipped with an AS-12A column and an AG-12A guard column. The pH was measured using a pH meter (HM-25G; TOA-DKK, Tokyo, Japan).

RESULTS AND DISCUSSION

Acclimation of inoculum sludge As shown in Fig. 1, acclimation of the inoculum sludge was initiated at a VTS loading rate of 2 g/L/day. The propionate concentration increased to

TABLE 1. Conditions of BMP tests.

Solution	Volume (mL)				
	Individual inhibition		Synergistic inhibition	Control[c]	Blank
	NH_4^+ inhibition[a]	S^{2-} inhibitions[b]			
Nutrient solution	230	230	230	230	230
0.1 mol/L phosphate buffer (pH 7.0 or 8.0)	60	60	60	60	60
Inoculum sludge	20	20	20	20	20
NH_4Cl solution ($[NH_4^+]$ = 40 g/L)	0, 10, 20, 30, 40, or 50	0	0, 10, 20, 30, 40, or 50	0	0
Na_2S solution ($[S^{2-}]$ = 2 g/L)	0	0, 10, 20, 30, 40, or 60	10	0	0
Sodium acetate or sodium propionate solution ([TOC] = 40 g/L)	10	10	10	10	0
Distilled water	Water was added to total volume of 400 mL				

[a] The concentrations of NH_4^+ in the experimental vials ranged from 0 to 5000 mg/L.
[b] The concentrations of S^{2-} in the experimental vials ranged from 0 to 300 mg/L.
[c] Control vial was carried out at pH 7.0 and 53°C.

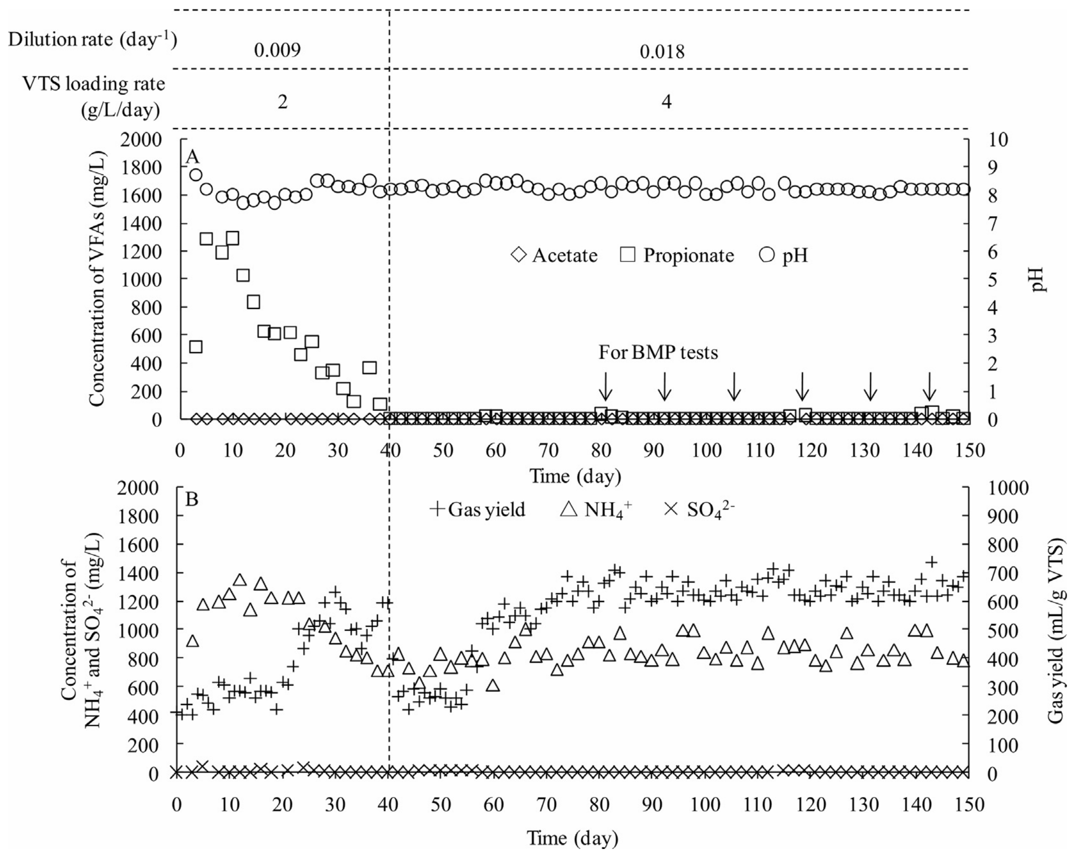

FIG. 1. Acclimation of inoculum sludge for BMP tests.

approximately 1300 mg/L in the first 5 days, causing a decrease in the pH from 8.7 to 7.9. Meanwhile, the NH_4^+ concentration increased to approximately 1200 mg/L. The propionate concentration decreased gradually to zero after the 12th day, and the gas yield increased after the 20th day. The pH increased to 8.5 after 24 days due to the decrease in the propionate concentration, associated with the decrease in the NH_4^+ concentration due to stripping of more NH_3 at higher pH. In our previous study on continuous methane fermentation with a complete stirring tank reactor (CSTR) using acetate as the sole carbon source, it was found that the methanogenic activity was significantly affected by the dilution rate, and increased linearly at a dilution rate below 0.12 day^{-1} (20). The dilution rate at the VTS loading rate of 2 g/L/day was calculated to be 0.009 day^{-1} in this study. To achieve higher methanogenic activity sludge for the BMP tests, the VTS loading rate was increased to 4 g/L/day after the propionate decreased to zero. The VFAs, pH, and NH_4^+ concentration were relatively stable after increasing the VTS loading rate. Although the gas yield decreased after increasing the VTS loading rate, it increased after the 56th day and then stabilized again. At the steady-state, the gas yield at the VTS loading rate of 4 g/L/day was approximately 640 mL/g VTS, and was higher than 536 mL/g VTS on average for the 25th–40th days at the VTS loading rate of 2 g/L/day, indicating that the methanogenic activity increased with increasing VTS loading rate. Acetate was negligibly detected throughout the acclimation. The sludge samples at the 80th, 91st, 107th, 120th, 131st, and 141st day were used for the BMP tests. For each BMP test vial, the NH_4^+ concentrations derived from the inoculum sludge and nutrient solution were approximately 40 mg/L and 190 mg/L, respectively, which were much lower than the amount of NH_4^+ added. Therefore, NH_4^+ from the inoculum sludge and nutrient solution is ignored in the following discussions.

Effect of temperature on the methane production rate Fig. 2 shows the time-courses of methane production in the BMP tests under various conditions. The methane production rate, which can be used to express the methanogenic activity, is a useful indicator for evaluating the methane production performance under specific conditions (17). To determine the effect of temperature on methane fermentation, the methane production rates without addition of NH_4^+ were estimated from Eq. 1 and are presented in Table 2. The methane production rates at 53°C were 2.3–2.7 times higher than those at 35°C in the absence of inhibitors. This phenomenon was probably due to higher temperature improved the activity of microorganism (21). Moreover, the methane production rates achieved when acetate was used as a carbon source were higher than those achieved using propionate as the carbon source under the same pH and temperature conditions. In our previous study, the maximum dilution rate was 0.6 day^{-1} in an anaerobic chemostat where acetate was continuously fed as the sole carbon source (22), while the maximum dilution rate was 0.3 day^{-1} when propionate was fed as the sole carbon source (23), indicating that the specific growth rate of acetate-degrading microbes was faster than that of the propionate-degrading microbes. The higher methane production rate achieved using acetate as the carbon source in this study was consistent with our previous results.

As reported by Gillooly et al. (24), the effect of temperature on the metabolic rate within the biologically relevant temperature range can be approximated by Eq. 5.

$$B = A_0 e^{-E_i/kT} \tag{5}$$

where B is the metabolic rate, which is reflected as the methane production rate in this study; A_0 is a constant; E_i (J/mol) is the average activation energy for the enzyme-catalyzed biochemical

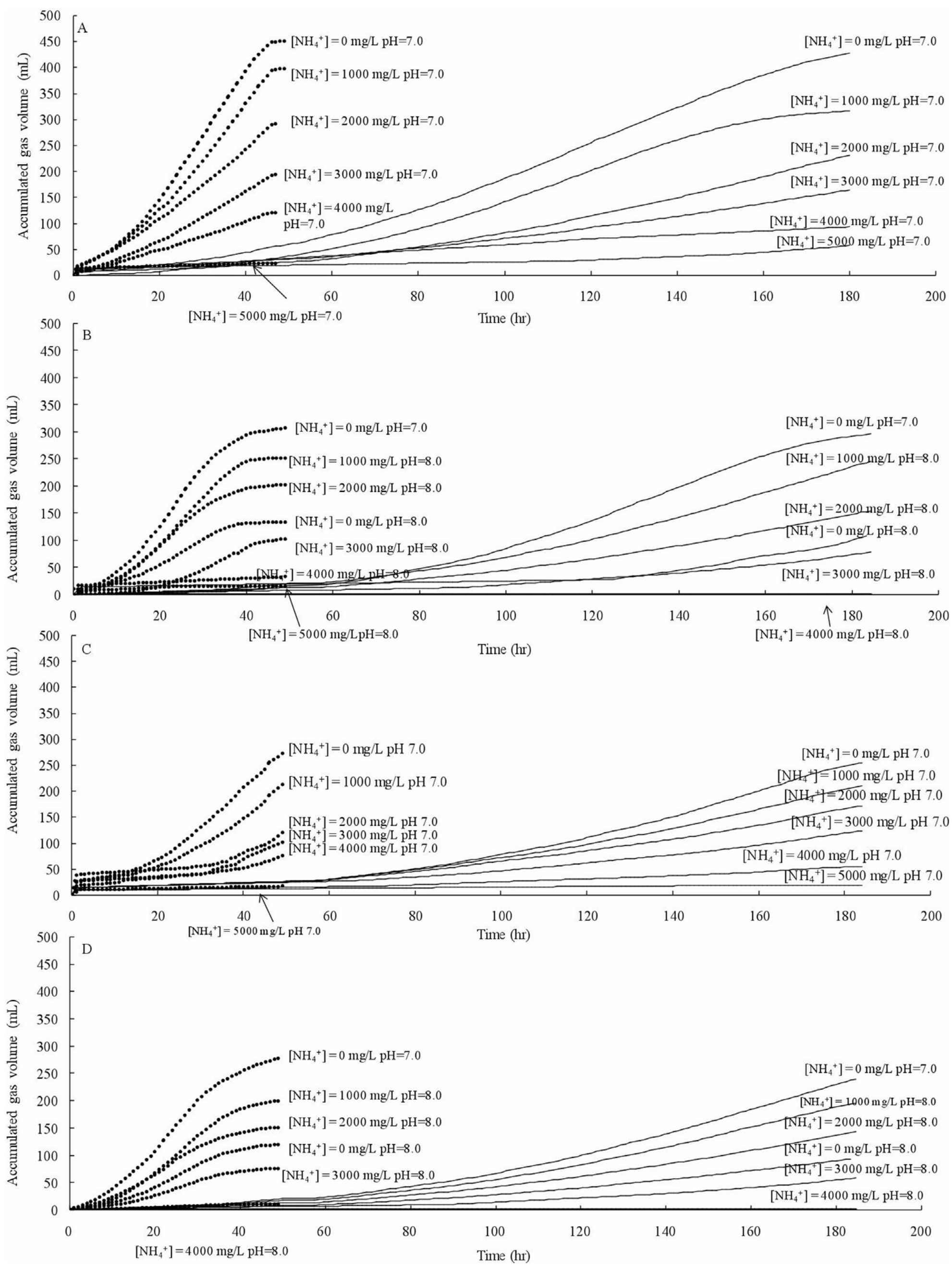

FIG. 2. Time courses for methane production in BMP tests under various conditions. Solid line: 35°C; dotted line: 53°C. (A) BMP tests with addition of NH_4^+ using acetate as carbon source at pH 7.0; (B) BMP tests with addition of NH_4^+ using acetate as carbon source at pH 8.0; (C) BMP tests with addition of NH_4^+ using propionate as carbon source at pH 7.0; (D) BMP tests with addition of NH_4^+ using propionate as carbon source at pH 8.0.

reactions of metabolism; k is Boltzmann's constant, which is 8.31 J/mol/K (25); T (K) is the absolute temperature. Substituting the absolute temperature (308 K (35°C) and 326 K (53°C)) and the methane production rates at different temperatures into Eq. 5, E_i was calculated to be 37.8–44.8 kJ/mol, which was in the reported range, where E_i varies between 19.3 and 115.6 kJ/mol (24). Therefore, the significantly higher methanogenic activity under thermophilic conditions than under mesophilic conditions is reliable.

Effect of NH_4^+ on methane production using acetate/propionate as the carbon source As shown in Fig. 3A, the methane production rate generally decreased with an increase in the NH_4^+ concentration using acetate as the carbon source, irrespective of pH and temperature. However, the methane production rate decreased much faster at 53°C than at 35°C, which is consistent with the reports that thermophilic methane fermentation was more sensitive to NH_4^+ than mesophilic methane fermentation (26). At pH 8.0, the methane production rate without addition of NH_4^+ was lower than that with addition of 1000 mg/L NH_4^+, which is similar to the data reported by Sung and Liu (26),

TABLE 2. Effect of temperature on methane production rate in absence of inhibitors.

Conditions		Methane production rate (mL/L/h)		V_{53}/V_{35}[a]
Carbon source	pH	53°C	35°C	
Acetate	7	27.8	12.3	2.3
Acetate	8	11.3	4.3	2.6
Propionate	7	24.3	9.0	2.7
Propionate	8	8.8	3.8	2.3

[a] V_{53}/V_{35} is the ratio of the methane production rate at 53°C to that at 35°C.

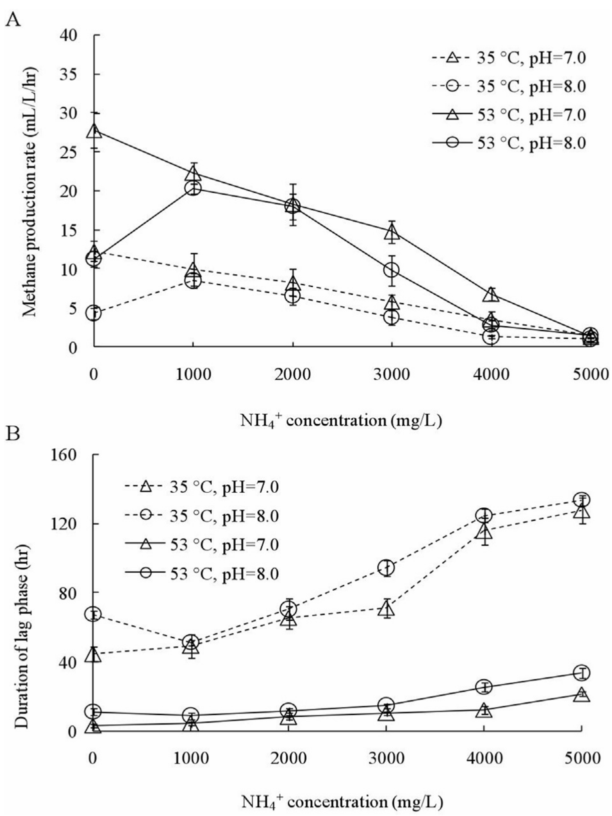

FIG. 3. Effect of NH_4^+ on (A) the methane production rate and (B) the duration of the lag phase using acetate as carbon source. X-axis shows the concentration of added NH_4^+.

where acclimation of sludge at an NH_4^+ concentration of 1200 mg/L resulted in the peak specific methanogenic activity at pH 8.0. Stripping of NH_3 preferably occurred at the relatively high pH of 8.0 (27); thus, no NH_4^+ was detected in the experimental vial without addition of NH_4^+ in the present BMP test. The lack of nitrogen source of the sludge restricted growth of the microbes, resulting in the low methane production rate at pH 8.0 (28). The change in the methane production rate with an increase in the NH_4^+ concentration from 1000 mg/L to 4000 mg/L was almost linear at 35°C. Under the conditions of 53°C and pH 7.0, the methane production rate also decreased almost linearly with an increase in the NH_4^+ concentration if the methane production rate at an NH_4^+ concentration of 3000 mg/L is ignored. On the other hand, under the conditions of 53°C and pH 8.0, the methane production rate decreased slightly when the NH_4^+ concentration increased from 1000 mg/L to 2000 mg/L, but decreased sharply when the NH_4^+ concentration was >2000 mg/L. Generally, increasing the pH from 7 to 8 would lead to an 8-fold increase in the NH_3 concentration under mesophilic conditions, and this increase would be even higher under thermophilic conditions (13,29). Therefore, it is commonly recognized that thermophilic methane fermentation is more sensitive to pH changes. The difference in the methane production rate at different pH values was more significant at 53°C than at 35°C when the NH_4^+ concentration was >2000 mg/L, which might be because NH_3 inhibition became dominant at this NH_4^+ level.

Acclimation of methane fermentation to the inhibitor can be evaluated based on the effect of the inhibitor on the duration of the lag phase in a batch experiment (17). As shown in Fig. 3B, the duration of the lag phase increased with an increase in the NH_4^+ concentration, and was significantly shorter at 53°C than at 35°C. The shorter lag phase at 53°C might be caused by the higher metabolic rate under thermophilic conditions. Besides, it is also possible that the thermophilically acclimated inoculum sludge used for the mesophilic BMP tests prolonged the lag phase. In future work, mesophilic BMP tests using mesophilically acclimated inoculum sludge should be carried out to evaluate the lag phase of mesophilic methane fermentation and the sensitivity of mesophilic methane fermentation to NH_4^+ and pH.

TABLE 3. Effect of inhibitor threshold limit on methane production rate.

Inhibitor	Carbon source	Conditions	n	C_{50}[a] (mg/L)	C^*[b] (mg/L)
NH_4^+	Acetate	pH 7.0, 53°C	0.85	2854.9	5120.2
		pH 7.0, 35°C	0.97	2868.5	5617.8
		pH 8.0, 53°C	0.80	2472.6	4266.4
		pH 8.0, 35°C	0.87	2401.0	4371.8
	Propionate	pH 7.0, 53°C	0.89	2536.7	4688.5
		pH 7.0, 35°C	0.72	2475.2	4004.2
		pH 8.0, 53°C	1.04	2228.3	4580.4
		pH 8.0, 35°C	0.73	2452.4	4000.2
S^{2-}	Acetate	pH 7.0, 53°C	1.32	129.8	317.8
	Propionate	pH 7.0, 53°C	1.52	103.6	282.9
NH_4^+ with 50 mg/L S^{2-}	Acetate	pH 7.0, 53°C	0.63	2963.8	4442.1
	Propionate	pH 7.0, 53°C	0.66	2466.4	3793.7

[a] C_{50} (mg/L) is the critical inhibitor concentration beyond which the methane production rate decreased by 50% compared with the control vial.
[b] C^* (mg/L) is the critical inhibitor concentration beyond which methane fermentation would stop.

From an engineering viewpoint, it is important to understand the inhibitor limitation concentration (17). As shown in Table 3, C_{50} and C^* indicate the inhibitor concentration at which the methane production rate dropped by 50% and 100%, respectively. Using acetate as a carbon source, the C_{50} and C^* values were 2400–2900 mg/L and 4250–5650 mg/L, respectively. The C_{50} value was relatively constant at a specific pH, irrespective of temperature, and was higher at pH 7.0 than at pH 8.0. Similarly, the C^* value at pH 7.0 was also higher than that at pH 8.0. Besides, the C^* value at 35°C was slightly higher than that at 53°C at a given pH. These results support the observation that methane fermentation at lower pH and lower temperature was less sensitive to an increase in the NH_4^+ concentration (26).

When propionate was used as a carbon source, as shown in Fig. 4A and B, the changes in the methane production rate and the duration of the lag phase with increasing NH_4^+ concentration were similar to those achieved using acetate as the carbon source. However, the methane production rate achieved using propionate as the carbon source was lower than that obtained using acetate as the carbon source under the same pH and temperature conditions because propionate oxidation was thermodynamically unfavorable due to the positive free energy change (30). The methane production rate was lower without addition of NH_4^+ than with addition of 1000 mg/L NH_4^+ at pH 8.0 due to the lack of nitrogen source. The lag phase at 35°C was also significantly longer than that at 53°C. With the same amount of NH_4^+, the lag phase was slightly longer when propionate was used as the carbon source than when acetate was used as the carbon source under the same pH and temperature conditions. The change in the methane production rate with an increase in the NH_4^+ concentration when propionate was used as the carbon source was slower than that achieved using acetate as the carbon source. The difference in the methane production rate caused by difference of the pH at 53°C was more significant than that at 35°C, regardless of the NH_4^+ concentration. This observation indicated that thermophilic methane fermentation was more sensitive to changes in the pH.

As shown in Table 3, the C_{50} and C^* values were 2200–2550 mg/L and 4000–4700 mg/L, respectively, when propionate was used as the carbon source. The C_{50} and C^* values obtained using acetate as the carbon source were lower than those obtained when propionate was used as the carbon source under the same pH and temperature conditions. Using propionate as the carbon source, the C_{50} and C^* values were relatively constant at different pH values for a given

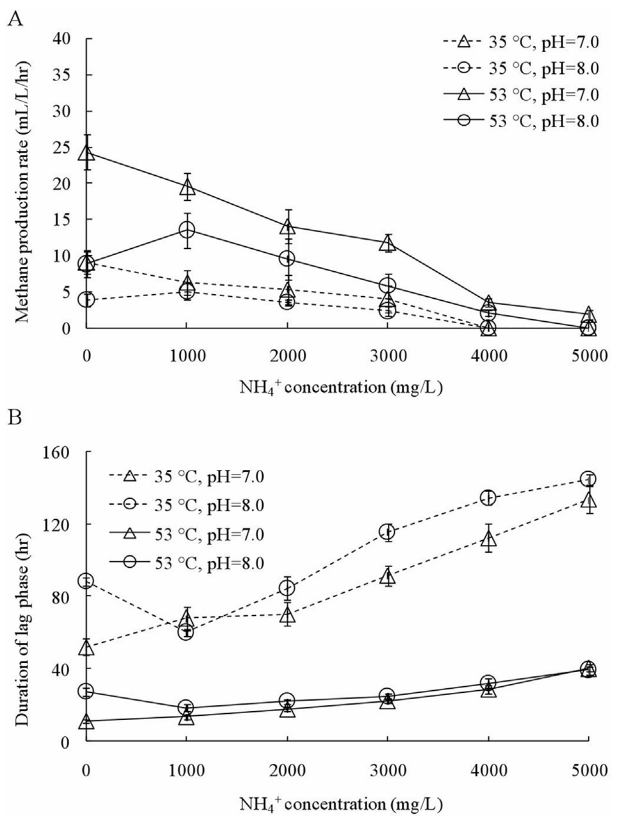

FIG. 4. Effect of NH_4^+ on (A) the methane production rate and (B) the duration of the lag phase using propionate as carbon source. X-axis shows the concentration of added NH_4^+.

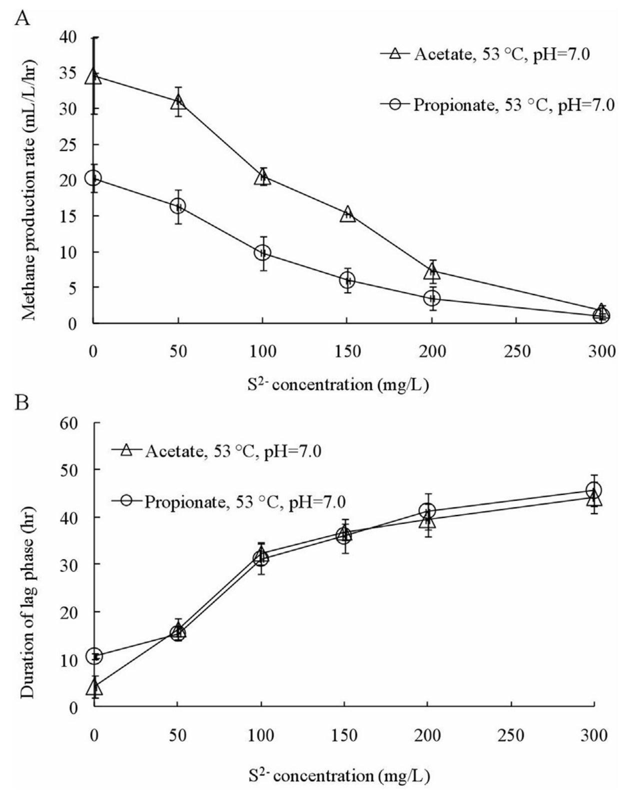

FIG. 5. Effect of S^{2-} on (A) the methane production rate and (B) the duration of the lag phase at 53°C and pH 7.0 using acetate or propionate as carbon source. X-axis shows the concentration of added S^{2-}.

temperature, except that the C_{50} was higher at pH 7.0 and 53°C than at pH 8.0 and 53°C. On the other hand, the C^* of approximately 4600 mg/L at 53°C was significantly higher than that of approximately 4000 mg/L at 35°C for a given pH. Anaerobic propionate oxidation is an endothermic reaction that involves two catabolic reactions: H_2 production from propionate and methanogenesis from H_2 and CO_2 (31). Higher temperature is beneficial for the endothermic reaction and results in a higher methane production rate. The optimum pH range for propionate oxidation was 7–8; thus propionate oxidation was faster under thermophilic conditions than under mesophilic conditions (32). Therefore, although thermophilic methane fermentation is generally more sensitive to increases in the NH_4^+ concentration, enhanced propionate oxidation under thermophilic conditions conferred greater resistance to an increase in the NH_4^+ concentration during thermophilic methane fermentation.

Overall, for methane fermentation that was not acclimated to high NH_4^+ concentration, the critical NH_4^+ concentration beyond which methane fermentation would stop was 4000.2–5617.8 mg/L, depending on the pH, temperature, and carbon source. Generally, the inhibitory effect of NH_3 would lead to accumulation of VFAs like acetic acid and propionic acid during methane fermentation (13), and accumulation of VFAs would further inhibit methane fermentation (28). Therefore, the NH_4^+ concentration should be controlled to <4000 mg/L for methane fermentation that was not acclimated to high NH_4^+ concentration to prevent the risk of fermentation failure.

Effect of S^{2-} on methane production performance at 53°C and pH 7.0 Since the methane production rate and the lag phase at 53°C and pH 7.0 were much superior to those under the other pH and temperature conditions, the effect of S^{2-} on the methane production performance was investigated at 53°C and pH 7.0.

Fig. 5 shows the effect of S^{2-} concentrations ranging from 0 to 300 mg/L on the methane production performance. The methane production rate declined constantly with an increase in the S^{2-} concentration, which is consistent with our previous study showing that the specific gas evolution rate decreased steadily with an increase in the S^{2-} concentration (16). Similarly, Hilton and Oleszkiewicz (33) reported that complete anaerobic utilization of acetate required a longer retention time with an increase in the S^{2-} concentration. The duration of the lag phase increased with increasing S^{2-} concentration, but the carbon source did not influence the duration of the lag phase. Compared with the BMP test at a specific S^{2-} concentration using acetate as the carbon source, the methane production rate from propionate was significantly lower due to the positive free energy change of propionate oxidation, while the duration of the lag phase was similar. The change in the methane production rate with an increase in the S^{2-} concentration was relatively smooth, regardless of the carbon source used. As shown in Table 3, the C_{50} and C^* values were slightly higher when acetate was used as the carbon source than when propionate was used. Methane fermentation stops when the S^{2-} concentration reaches approximately 300 mg/L.

Synergistic effect of NH_4^+ and S^{2-} on methane production performance Because the methane production rate was inhibited even at a S^{2-} concentration of 50 mg/L, the synergistic effect of NH_4^+ and S^{2-} (50 mg/L) on the methane production performance was investigated. As shown in Fig. 6, the methane production rate decreased and the duration of the lag phase increased with an increase in the NH_4^+ concentration, regardless of the carbon source. With the same amount of NH_4^+, the methane production rate was higher and the lag phase was shorter when acetate was used as the carbon source.

The inhibition rates in the presence of the inhibitors were calculated using Eq. 3, and the epsilon values were calculated using Eq. 4. For example, the inhibition ratio with addition of NH_4^+ (1000 mg/L) and S^{2-} (50 mg/L) was 24.5% when acetate was used as the carbon source; the individual inhibition ratio with addition of

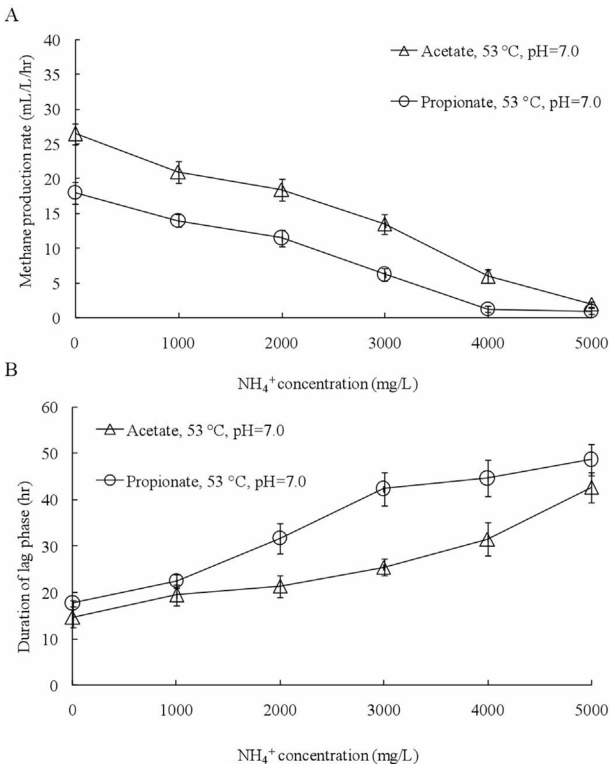

FIG. 6. Effect of co-existence of NH_4^+ and S^{2-} on (A) the methane production rate and (B) the duration of the lag phase at 53°C and pH 7.0 using acetate or propionate as carbon source. X-axis shows the concentration of added NH_4^+.

NH_4^+ (1000 mg/L) was 19.8%, and the individual inhibition ratio with addition of S^{2-} (50 mg/L) was 4.7%. The epsilon value was calculated as: $\varepsilon = (1 - 0.245) - (1 - 0.198) \times (1 - 0.047) = -0.009$.

As shown in Table 4, the epsilon values obtained when NH_4^+ and S^{2-} were coexistent ranged from −0.064 to 0.056, except for the epsilon value obtained with an NH_4^+ concentration of 3000 mg/L and S^{2-} concentration of 50 mg/L using propionate as the carbon source. As reported by Torres et al. (34), inhibitor pairings with $\varepsilon > 0.1$ are considered antagonistic effect, while pairings with $\varepsilon < -0.1$ are considered synergistic effect. Therefore, there was almost no synergistic or antagonistic effect in the tested addition amounts of NH_4^+ and S^{2-}. Thus, the methane production rate when NH_4^+ ($\leq$ 5000 mg/L) and S^{2-} (= 50 mg/L) are co-existent could be expected to follow a simple additive of the methane production rate with the individual inhibitors.

As shown in Table 3, the C_{50} achieved when NH_4^+ and S^{2-} were co-existent was 2963.8 mg/L with the use of acetate and 2466.6 mg/L

TABLE 4. Inhibition ratio and epsilon values (ε) for determining synergistic effect of NH_4^+ and S^{2-} at 53°C and pH 7.0.

Carbon source	NH_4^+ (mg/L)	Inhibition ratio (%)		ε at S^{2-} of 50 mg/L
		S^{2-} of 0 mg/L	S^{2-} of 50 mg/L	
Acetate	0	0	4.7	0
	1000	19.8	24.5	−0.009
	2000	34.2	33.5	0.038
	3000	46.8	51.4	−0.021
	4000	75.5	78.4	−0.017
	5000	95.3	93.8	0.017
Propionate	0	0	11.3	0
	1000	19.7	31.0	−0.022
	2000	42.4	43.3	0.056
	3000	51.4	69.0	−0.121
	4000	85.6	93.6	−0.064
	5000	92.6	95.1	−0.017

when propionate was used as the carbon source, which is similar to the C_{50} obtained in the presence of NH_4^+ only. On the other hand, the C^* obtained with co-existent NH_4^+ and S^{2-} was 4442.1 mg/L using acetate and 3793.7 mg/L using propionate as the carbon source, which was lower than the C^* obtained with NH_4^+ only. This observation indicates that it is more risk for methane fermentation when treating feedstock with a low C/N ratio and low C/S ratio. This result also demonstrates the importance of alleviating NH_4^+ and/or S^{2-} inhibition to enhance methanogenesis.

In summary, thermophilic methane fermentation gave rise to a higher methane production rate than mesophilic methane fermentation. The methane production rate decreased and the duration of the lag phase increased when methane fermentation was inhibited by NH_4^+ and/or S^{2-}. VFAs like acetic acid and/or popionic acid commonly accumulate during methane fermentation with NH_4^+ and/or S^{2-} inhibition. For methane fermentation that is not acclimated to high NH_4^+ concentration, the critical NH_4^+ concentration, C^*, beyond which methane fermentation would stop was 4000−5650 mg/L depending on the pH and temperature, and C^* decreased to 3800−4400 mg/L when NH_4^+ and S^{2-} were coexistent. Therefore, it is important to control the NH_4^+ concentration or/and to decrease the H_2S concentration in biogas during methane fermentation to avoid unsuccessful fermentation.

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References

1. **Botheju, D. and Bakke, R.:** Oxygen effects in anaerobic digestion - a review, Open Waste Manag. J., **411**, 1−19 (2011).
2. **Chen, Y., Cheng, J. J., and Creamer, K. S.:** Inhibition of anaerobic digestion process: a review, Bioresour. Technol., **99**, 4044−4064 (2008).
3. **Kayhanian, M.:** Ammonia inhibition in high-solids biogasification: an overview and practical solutions, Environ. Technol., **20**, 355−365 (1999).
4. **Niu, Q., Qiao, W., Qiang, H., Hojo, T., and Li, Y. Y.:** Mesophilic methane fermentation of chicken manure atawide range of ammonia concentration: stability,inhibition and recovery, Bioresour. Technol., **137**, 358−367 (2013).
5. **Dai, X., Hu, C., Zhang, D., Dai, L., and Duan, N.:** Impact of a high ammonia-ammonium-pH system on methane-producing archaea and sulfate-reducing bacteria in mesophilic anaerobic digestion, Bioresour. Technol., **245**, 598−605 (2017).
6. **Krayzelova, L., Bartacek, J., Díaz, I., Jeison, D., Volcke, E. I. P., and Jenicek, P.:** Microaeration for hydrogen sulfide removal during anaerobic treatment: a review, Rev. Environ. Sci. Biotechnol., **14**, 703−725 (2015).
7. **Hansen, K. H., Angelidaki, I., and Ahring, B. K.:** Improving thermophilic anaerobic digestion of swine manure, Water Res., **33**, 1805−1810 (1999).
8. **Lauterböck, B., Nikolausz, M., Lv, Z., Baumgartner, M., Liebhard, G., and Fuchs, W.:** Improvement of anaerobic digestion performance by continuous nitrogen removal with a membrane contactor treating a substrate rich in ammonia and sulfide, Bioresour. Technol., **158**, 209−216 (2014).
9. **Ikbal, Tang, Y., Shigematsu, T., Morimura, S., and Kida, K.:** Methanogenic activity and repression of hydrogen sulfide evolved during high rate thermophilic methane fermentation of municipal solid waste, Jpn. J. Water Treat. Biol., **39**, 17−24 (2003).
10. **Tang, Y., Shigematsu, T., Ikbal, Morimura, S., and Kida, K.:** The effects of micro-aeration on the phylogenetic diversity of microorganisms in a thermophilic anaerobic municipal solid-waste digester, Water Res., **38**, 2537−2550 (2004).
11. **Jiang, X., Hayashi, J., Sun, Z. Y., Yang, L., Tang, Y. Q., Oshibe, H., Osaka, N., and Kida, K.:** Improving biogas production from protein-rich distillery wastewater by decreasing ammonia inhibition, Process Biochem., **48**, 1778−1784 (2013).
12. **Sun, Z. Y., Yamaji, S., Cheng, Q. S., Yang, L., Tang, Y. Q., and Kida, K.:** Simultaneous decrease in ammonia and hydrogen sulfide inhibition during the thermophilic anaerobic digestion of protein-rich stillage by biogas recirculation and air supply at 60°C, Process Biochem., **49**, 2214−2219 (2014).
13. **Rajagopal, R., Masse, D. I., and Singh, G.:** A critical review on inhibition of anaerobic digestion process by excess ammonia, Bioresour. Technol., **143**, 632−641 (2013).

14. **Fernandes, T. V., Keesman, K. J., Zeeman, G., and van Lier, J. B.:** Effect of ammonia on the anaerobic hydrolysis of cellulose and tributyrin, Biomass Bioenergy, **47**, 316–323 (2012).
15. **Ho, L. and Ho, G.:** Mitigating ammonia inhibition of thermophilic anaerobic treatment of digested piggery wastewater: use of pH reduction, zeolite, biomass and humic acid, Water Res., **46**, 4339–4350 (2012).
16. **Ikbal, Tang, Y., Fujimura, Y., Shigematsu, T., Morimura, S., and Kida, K.:** The affecting factors for optimization of mesophilic aceticlastic methanogenesis, Jpn. J. Water Treat. Biol., **39**, 189–197 (2003).
17. **Lay, J. J., Li, Y. Y., and Noike, T.:** The influence of pH and ammonia concentration on the methane production in high-solids digestion processes, Water Environ. Res., **70**, 1075–1082 (1998).
18. **Greetham, D., Hart, A. J., and Tucker, G. A.:** Presence of low concentrations of acetic acid improves yeast tolerance to hydroxymethylfurfural (HMF) and furfural, Biomass Bioenergy, **85**, 53–60 (2016).
19. **Kida, K., Sonoda, Y., Kawase, M., and Nomura, T.:** Influence of mineral nutrients on high performance during anaerobic treatment of wastewater from a beer brewery, J. Ferment. Bioeng., **72**, 54–57 (1991).
20. **Kida, K., Shigematsu, T., Kijima, J., Numaguchi, M., Mochinaga, Y., Abe, N., and Morimura, S.:** Influence of Ni^{2+} and Co^{2+} on methanogenic activity and the amounts of coenzymes involved in methanogenesis, J. Biosci. Bioeng., **91**, 590–595 (2001).
21. **Wang, G., Dai, X., Zhang, D., He, Q., Dong, B., Li, N., and Ye, N.:** Two-phase high solid anaerobic digestion with dewatered sludge: improved volatile solid degradation and specific methane generation by temperature and pH regulation, Bioresour. Technol., **259**, 253–258 (2018).
22. **Shigematsu, T., Tang, Y., Kobayashi, T., Kawaguchi, H., Morimura, S., and Kida, K.:** Effect of dilution rate on metabolic pathway shift between aceticlastic and nonaceticlastic methanogenesis in chemostat cultivation, Appl. Environ. Microbiol., **70**, 4048–4052 (2004).
23. **Shigematsu, T., Era, S., Mizuno, Y., Ninomiya, K., Kamegawa, Y., Morimura, S., and Kida, K.:** Microbial community of a mesophilic propionate-degrading methanogenic consortium in chemostat cultivation analyzed based on 16S rRNA and acetate kinase genes, Appl. Microbiol. Biotechnol., **72**, 401–415 (2006).
24. **Gillooly, J. F., Brown, J. H., West, G. B., Savage, V. M., and Charnov, E. L.:** Effects of size and temperature on metabolic rate, Science, **293**, 2248–2251 (2001).
25. **Lonhienne, T., Gerday, C., and Feller, G.:** Psychrophilic enzymes: revisiting the thermodynamic parameters of activation may explain local flexibility, Biochim. Biophys. Acta Protein Struct. Mol., **1543**, 1–10 (2000).
26. **Sung, S. and Liu, T.:** Ammonia inhibition on thermophilic anaerobic digestion, Chemosphere, **53**, 43–52 (2003).
27. **Huang, W., Zhao, Z., Yuan, T., Lei, Z., Cai, W., Li, H., and Zhang, Z.:** Effective ammonia recovery from swine excreta through dry anaerobic digestion followed by ammonia stripping at high total solids content, Biomass Bioenergy, **90**, 139–147 (2016).
28. **Prochazka, J., Dolejs, P., Maca, J., and Dohanyos, M.:** Stability and inhibition of anaerobic processes caused by insufficiency or excess of ammonia nitrogen, Appl. Microbiol. Biotechnol., **93**, 439–447 (2012).
29. **Hansen, K. H., Angelidaki, I., and Ahring, B. K.:** Anaerobic digestion of swine manure: inhibition by ammonia, Water Res., **32**, 5–12 (1998).
30. **Takaki, M., Tan, L., Murakami, T., Tang, Y. Q., Sun, Z. Y., Morimura, S., and Kida, K.:** Production of biofuels from sweet sorghum juice via ethanol-methane two-stage fermentation, Ind. Crop. Prod., **63**, 329–336 (2015).
31. **Boone, D. R. and Xun, L.:** Effects of pH, temperature, and nutrients on propionate degradation by a methanogenic enrichment culture, Appl. Environ. Microbiol., **53**, 1589–1592 (1987).
32. **Li, J. Z., Ban, Q. Y., Zhang, L. G., and Jha, A. K.:** Syntrophic propionate degradation in anaerobic digestion- A review, Int. J. Agric. Biol., **14**, 843–850 (2012).
33. **Hilton, B. L. and Oleszkiewicz, J. A.:** Sulfide-induced inhibition of anaerobic digestion, J. Environ. Eng., **114**, 1377–1391 (1988).
34. **Torres, N. P., Lee, A. Y., Giaever, G., Nislow, C., and Brown, G. W.:** A high-throughput yeast assay identifies synergistic drug combinations, Assay Drug Dev. Technol., **11**, 299–307 (2013).