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Acetic acid inhibition on methanogens in a two-phase anaerobic process

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Highlights

We explore the effect of acetate on acidogenic and methanogenic methanogens in a two-phase anaerobic system;
 The methanogens were present in both acidogenic and methanogenic phases;
 The acidogenic phase methanogens could tolerate higher acetate concentration than methanogenic phase methanogens;
 The methanogenic phase methanogens still tolerated higher undissociated acetic acid than those in the single stage anaerobic digester;
 The high concentration of undissociated acetic acid may still inhibit acidogenic and methanogenic phase methanogens.

Abstract

The inhibitory effect of acetic acid on methanogens in a two-phase anaerobic process was evaluated. The results in this study showed that some methanogens still existed in the acidogenic phase although their dominance in the total microbial community was only 1% compared to 9.6% in the methanogenic phase. The inhibition threshold of acetic acid on acidogenic phase methanogens was, however, higher than that on methanogenic phase methanogens. At pH 6.00, acetic acid inhibition on methanogenic phase methanogens was observed when acetic acid concentration was higher than 1619.47 mg HAc/L although there was no obvious inhibition on acidogenic phase methanogens in the range of 1646.47-2781.19 mg HAc/L. There was also no acetic acid inhibition on acidogenic phase methanogens at pH 5.50, 6.00 and 6.50 in the range of 565.29-2781.19 mg HAc/L. However, for methanogenic phase methanogens, the inhibition was obvious and a second order substrate inhibition model, $q_s = q_m S / [K_s + S + (S^2/K_i)]$, could be adapted to describe

the inhibition kinetics and mechanism of undissociated acetic acid on methanogenic phase methanogens. The results showed substrate saturation constant K_s , substrate inhibition constant K_i , and maximum specific utilization rate of acetic acid q_m , were 1.66 mg unHAc/L, 145.17 mg unHAc/L, and 3.53 mg HAc/L.g MLVSS.h, respectively.

Keywords

Two-phase; Anaerobic; Acetic Acid; Inhibition; Methanogen

Nomenclature

Ac_i	initial acetic acid concentration
COD	chemical oxygen demand
CSTR	continuous stirred tank reactor
C_T	total acetic acid concentration
F/M	Food/Microorganism
HAc	acetic acid
HRT	hydraulic retention time
MLVSS	mixed liquor volatile suspended solids
qPCR	quantitative polymerase chain reaction
TCOD	total chemical oxygen demand
unHAc	undissociated acetic acid
VFAs	volatile fatty acids
VS	volatile solids

1. Introduction

Conventional bioconversion of sludge in anaerobic digestion systems is usually characterized by hydrolysis, acidogenesis, acetogenesis and methanogenesis [1]. The imbalanced growth of acidogens and methanogens in a single-stage anaerobic reactor can result in process failure due to accumulation of volatile fatty acids (VFAs), which would cause pH decrease and inhibition of methanogen activity. The two-phase anaerobic process has physical separation of hydrolysis-acidogenesis from methanogenesis in two reactors [2]. Complex organic compounds are converted into simpler forms becoming soluble chemical oxygen demand (COD) and thereafter as VFAs in the acidogenic phase; the VFAs are then converted into biogas by methanogenic phase methanogens [3]. In the two-phase system, the acidogenic phase protects the methanogenic phase from rapid acidification and sharp pH declines [4]. The two-phase process seeks to provide optimum conditions for acid- and methane-formers with its better control of acidogenesis; therefore, it can achieve high organic loading rates and higher volatile solids (VS) and COD removal efficiencies than the traditional single-stage system [5].

The activities of methanogenic communities are affected by VFA concentrations and pH [6]. During hydrolysis and acidogenesis, acetic acid is the main VFA product [7]. Many studies have been carried out to explore the inhibition effect of acetic acid on methanogens [8, 9] and the inhibitory mechanisms caused by high concentrations of acetic acid in the single-stage anaerobic digester [10,11]. However, it is noteworthy that all previous studies and results were based on the single-stage anaerobic system.

It has been pointed out that it was difficult to completely separate acidogenesis from methanogenesis [12], and that some methanogenic activities in the acidogenic phase were necessary to support the syntrophic interaction between different trophic groups of microorganisms [13]. Researchers have identified presence of methanogens in the acidogenic phase of a two-phase anaerobic digestion system [14]. In this study, the two-phase anaerobic process referred herein also had some methanogens in the acidogenic phase. As is known, the amount of acetic acid-utilizing methanogens in traditional single-stage anaerobic digesters was only 10%-50% of that in the methanogenic phase of the two-phase system [5]. Thus, the acetic acid utilization by acidogenic phase methanogens (methanogens cultivated in the acidogenic phase) and methanogenic phase methanogens (methanogens cultivated in the methanogenic phase) of the two-phase system may be different from that cultivated in the conventional single-stage anaerobic digestion system. Previous research has shown methanogens in the single-stage anaerobic system were severely inhibited by the action of undissociated VFAs [15] and undissociated acetic acid (unHAc) was the uncoupler of the plasma membrane [16]. The effect of acetic acid concentration on methanogens was through the undissociated acetic acid form. To date, the degradation of acetic acid and its effect on acidogenic methanogens and methanogenic methanogens of the two-phase system have not been studied in detail.

This study aims to (1) identify the existence of methanogens in the acidogenic phase and their abilities to degrade acetic acid; (2) explore the effect of pH and acetic acid

concentration on acetic acid utilization by acidogenic phase and methanogenic phase methanogens in a two-phase anaerobic process; and (3) investigate the possible kinetic parameters associated with the effect of undissociated acetic acid on acidogenic phase and methanogenic phase methanogens.

2. Materials and Methods

2.1 Culture source

The culture for the study was drawn from a laboratory-scale continuous stirred tank reactor (CSTR) two-phase anaerobic sludge digestion system. Nitrogen gas was sparged into the headspace to maintain anaerobic conditions whenever sludge was withdrawn. The system was fed with concentrated mixed primary sludge and secondary sludge (total chemical oxygen demand (TCOD) of 46.90 ± 9.00 g/L) collected from a local sewage treatment plant. The CSTR system had been operated for 113 days with a hydraulic retention time (HRT) of 3 days and pH of 5.50 ± 0.30 for the acidogenic phase, and a HRT of 17 days and pH of 7.00 ± 0.20 for the methanogenic phase. The system displayed good performance with VS reduction of 41.46% and biogas yield of 0.96 L/g VS_{destroyed} before the experiments described in this paper were carried out. The highest concentrations of acetic acid that the acidogenic and methanogenic culture experienced prior to these experiments were 1125 and 1172 mg HAc/L, respectively. The term acetic acid is used here to indicate the chemical species in all its forms (generic form); i.e. dissociated acetic acid as well as undissociated acetic acid.

2.2 Experimental set-up: acetic acid inhibition on acidogenic and methanogenic phase methanogens

Sludge for this study was withdrawn from both acidogenic phase and methanogenic phase reactors. Serum bottles (120 mL) containing 50 mL culture and 50 mL synthetic feed media (Table 1) were incubated in an incubator (Sartorius Stedim Biotech, Germany) (35 ± 2 °C and 150 rpm). Prior to addition of the synthetic feed and acetic acid, the culture from the methanogenic phase was incubated at room temperature overnight without additional carbon source to allow degradation of residual VFAs (20-30 mg VFAs /L) in the culture. Residual VFAs from the acidogenic culture were removed by centrifugation ($12857 \times g$, 10 mins) and washing (with COD free synthetic feed).

A baseline concentration of acetic acid which did not inhibit was chosen in order to evaluate the normal activity of the methanogens in the two phases. Previous researchers have demonstrated that 500 mg HAc/L did not show inhibitory effect on methanogens from the single-stage anaerobic digestion system [15]. Hence, 500 mg HAc/L acetic acid was added in each serum bottle as baseline carbon source for the two cultures. To determine the effect of initial acetic acid concentration (Ac_i) and pH on acetic acid utilization by the acidogenic phase and methanogenic phase methanogens, different amounts of additional Ac_i were then added into the serum bottles with various pre-set pH values (Table 2). The concentrations of acetic acid added to the serum bottles with culture from the acidogenic phase (Condition 1) varied from 65.29 to 2281.19 mg HAc/L with pH ranging from 4.50 to 6.50. The concentrations of acetic acid added to the serum

bottles with culture from the methanogenic phase varied from 46.08 to 4279.01 mg HAc/L (Condition 2) with pH ranging from 6.00 to 7.70. The desired pH in each serum bottle was adjusted by addition of 1N HCl or 1 N NaOH before the start of the experiment.

The reaction periods for sludge from the acidogenic phase and methanogenic phase were 97 h and 70 h, respectively. The sampling intervals for the acidogenic phase experiment were at 0th h, 22th h, 28th h, 53th h and 97th h and for the methanogenic phase experiment were at 0th h, 19th h, 26th h, 32th h, 44th h, 50th h, 56th h and 70th h, respectively. Acetic acid utilization rate was calculated using linear regression of the measured acetic acid concentrations during 22th h to 97th h for the acidogenic phase methanogens test, and 19th h to 70th h for the methanogenic phase methanogens test. These periods were chosen based on the estimated adaption period for methanogens to new conditions and the need for maintenance of buffering capacity in order that pH change was within the range of 0.10-0.20 pH units. The specific rate of acetic acid degradation was calculated by the utilization rate against biomass concentration.

2.3 Analytical methods

To determine VFAs, 1 mL mixed liquor was taken from each serum bottle at the pre-set sampling times and immediately centrifuged ($12857 \times g$, 10 mins). The supernatant was filtered through a 0.2 μ m sterilized nylon membrane filter and then 0.90 mL was added into a GC vial with 0.10 mL of 10% formic acid. Analysis was made with a gas chromatograph (Agilent Technologies Inc., USA) after the method described by

Zhou et al. [18] and with a DB-FFAP 15 m × 0.53 mm × 1.0 μm (length × ID × film) column. Temperature of the injector block and FID detector was 250 °C and 300 °C, respectively. Helium was used as the carrier gas. Other measurements were in accordance with standard methods [19].

2.4 Kinetic analysis

A second-order substrate model (equation 1 [20]) was adapted to describe the inhibition kinetics and mechanism of undissociated acetic acid on methanogens. This model obeyed the Haldane equation, which was widely utilized to describe substrate inhibition kinetics [20]. The data-fitting procedure was based on the non-linear least-squares regression method.

$$q_s = q_m S / [K_s + S + (S^2 / K_i)] \quad (1)$$

where q_s (mg HAc/L.g MLVSS.h): the specific acetic acid utilization rate;

S (mg unHAc/L): the initial concentration of undissociated acetic acid;

q_m (mg HAc/L.g MLVSS.h): the maximum value of q_s ;

K_i (mg unHAc/L): the substrate inhibition constant;

K_s (mg unHAc/L): the substrate saturation constant.

2.5 Microbial profiles

The biomass sample was washed with phosphate buffered saline (pH=7.00) and DNA was then extracted by an automated nucleic acid extractor (MagNA Pure, Roche Diagnostics GmbH, Germany). Quantitative polymerase chain reaction (qPCR) was performed following the protocol established by Yu et al. [21]. The microbial

communities from the acidogenic phase and methanogenic phase were analyzed by qPCR using the primer/probes shown in Table 3.

3. Results

3.1 Microbial population profiles of methanogens in acidogenic and methanogenic cultures

The qPCR results confirmed the existence of methanogens in the acidogenic phase had 1% dominance of the total microbial communities (Fig. 1a). These methanogens could have degraded acetic acid under acidogenic conditions. The methanogenic phase culture had more abundant methanogens (9.6%) against the whole community (Fig. 1b).

Methanobacteriales (hydrogenotrophic methanogen), *Methanomicrobiales* (hydrogenotrophic methanogen), *Methanosaetaceae* (acetoclastic methanogen) and *Methanosarcinaceae* (hydrogenotrophic, acetoclastic, methylotrophic methanogen) were found in the acidogenic phase and methanogenic phase communities (Fig. 1) with *Methanomicrobiales* (hydrogenotrophic methanogen) being the most dominant methanogen in both phases (Fig.1). Differences in abundances of the various methanogens in the acidogenic and methanogenic communities may result in different degradation mechanisms of acetic acid and this shall be discussed further.

3.2 Effect of acetic acid concentration and pH on acetic acid degradation by acidogenic phase and methanogenic phase methanogens

The utilization rates of acetic acid under different initial acetic acid concentrations and pH by acidogenic phase methanogens and methanogenic phase methanogens are

shown in Fig. 2 and Fig. 3, respectively. Fig. 2 shows the utilization rates of acetic acid by acidogenic phase methanogens under each of the pre-set acetic acid concentration were relatively similar at pH 5.50, 6.00 and 6.50. The rates increased with increasing concentrations of Ac_i in the range of 565.29 to 2781.19 mg HAc/L. The exception was at pH 5.00; it increased initially and then decreased with the increase of Ac_i concentrations. Hardly any utilization of acetic acid was observed at pH 4.50. The maximum acetic acid utilization rate (1.93 mg HAc/L.g MLVSS.h) was at pH 5.50 when the initial total acetic acid concentration was 2781.19 mg HAc/L.

However, the effect of acetic acid on acetic acid utilization by methanogenic phase methanogens was different. Fig. 3 illustrates that at all the pH values tested, at each pH value, the utilization rates of acetic acid increased initially and then decreased as Ac_i concentration increased. At pH 6.00, acetic acid utilization rates declined sharply when the concentration of Ac_i was more than 1619.47 mg HAc/L, and completely stopped at Ac_i concentration of 3000 mg HAc/L. The maximum utilization rate (3.30 mg HAc/L.g MLVSS.h) of acetic acid was obtained at pH 6.80 when the concentration of Ac_i was 2703.23 mg HAc/L. Fig. 2 and Fig. 3 suggested that inhibition of the acidogenic phase and methanogenic phase methanogens was associated with high acetic acid concentration and low pH.

3.3 The effect of undissociated acetic acid on acidogenic phase and methanogenic phase methanogens

Acetic acid can be present in two forms, dissociated and undissociated (free acetic acid). Initial concentration of acetic acid and pH would affect concentration of the undissociated acid [15]. The concentrations of undissociated acetic acid with different Ac_i concentrations and pH in above two studies were calculated and are listed in the Table 4 (acidogenic phase methanogens) and Table 5 (methanogenic phase methanogens). The formula used for the calculation is as follows (C_T means the total acetic acid concentration) [24]:

$$UnHAc = C_T [H^+] / (K_a + [H^+]) \quad (pK_a: 4.76, 35^\circ C) \quad (2)$$

From Table 4 and 5, it was noted that the concentration of undissociated acetic acid was higher at low pH value when the initial acetic acid concentration was at the same level. At pH 5.50, 6.00 and 6.50, the undissociated acetic acid (10.10-428.16 mg unHAc/L) had no obvious inhibition on acidogenic phase methanogens. However, the effect of undissociated acetic acid on methanogenic phase methanogens was obvious. The correlation between specific acetic acid utilization rate and initial undissociated acetic acid in the methanogenic phase methanogens experiments was modeled with the second-order substrate inhibition model using equation 1 [20].

Fig. 4 shows the specific acetic acid utilization rate of methanogenic phase methanogens under various undissociated acetic acid concentrations ($pK_a=4.76, 35^\circ C$). The best fitting curve was found using the non-linear least-squares regression method and the second order substrate inhibition model. The kinetic constants were found as follows: $K_s = 1.66$ mg unHAc/L, $K_i = 145.17$ mg unHAc/L and $q_m = 3.53$ mg HAc/L.g MLVSS.h.

Although all the cultures from acidogenic phase and methanogenic phase were buffered with bicarbonate, there were still some changes between initial adjusted pH and the final pH during the experiments, especially at the lower initial pH where the inhibition effect was more obvious. The changes of pH for all the above experiments were in the range of 0.10-0.20 pH units during the reaction period for acidogenic phase methanogens (97 h) and methanogenic phase methanogens (70 h). Sergio et al. [25] reported that change of 0.10-0.20 pH units might have insignificant influence on the final utilization rate of acetic acid.

4. Discussion

It was reported that the acidogenic phase in a two-phase system may protect methanogenic phase methanogens from pH shocks and the establishment of acidogenic phase was more favored with high organic loading, short HRT and low pH [5]. Some observations showed that there were no methanogens in the acidogenic phase [26], while others stated that the purpose of phase separation was to strengthen the ecological relationship among trophic groups of microorganisms in each phase instead of completely separating them [5] and it was also impractical to completely separate acidogenesis from methanogenesis in the acidogenic phase. Brummeler et al. [27] suggested the possibility *Methanosarcinaceae* growth at pH values as low as 5.00 and 4.68 and isolation of *Methanosarcinaceae* at such low pH values was achieved [28]. Shimada et al. (2011) also reported the existence of methanogenic activity confirmed by the 20% methane production in the acidogenic phase of a two-phase anaerobic digestion

system [29]. The results in this study showed that the dominance of methanogens in the total microbial communities was 1% and 9.6% in the acidogenic phase and methanogenic phase, respectively. And in both acidogenic and methanogenic phases, the *Methanomicrobiales* (hydrogenotrophic methanogen) was the most dominant methanogen. Similar conclusion was reported by Shimada et al.(2011) [29] who also found that the main archaea groups were hydrogenotrophic methanogens in acidogenic phase (*Methanobacteriales*) and methanogenic phase (*Methanomicrobiales* and *Methanobacteriales*) of a two-phase anaerobic digestion system. The total number of the acetic acid-utilizing methanogens, namely *Methanosarcinaceae* and *Methanosaetaceae* was 0.016% of the total community in the acidogenic phase. These observations showed that acetic acid degradation by acetic acid-utilizing methanogens was possible at pH 5.00-6.50 when initial acetic acid concentration ranged from 565.29 to 2781.19 mg HAc/L (Fig. 2).

Although the number of acetic acid-utilizing methanogens, such as *Methanosarcinaceae* and *Methanosaetaceae*, in the methanogenic phase (0.335%) was higher than in the acidogenic phase (0.016%), the acetic acid utilization rate by acidogenic phase methanogens was higher than that by methanogenic phase methanogens at pH 6.00 (1.63 mg HAc/L.g MLVSS.h vs 0.22 mg HAc/L.g MLVSS.h) (Fig. 2 and Fig. 3). At pH 6.00, initial acetic acid concentration higher than 1619.47 mg HAc/L inhibited the methanogenic phase methanogens, however, initial acetic acid concentration ranging from 1646.47 mg HAc/L to 2781.19 mg HAc/L had no inhibitory effect on acidogenic

phase methanogens. It seemed that the long term acclimation (113 days) of acidogenic phase methanogens to high VFAs concentrations and low pH setpoints in the acidogenic phase may have resulted in their better tolerance of high undissociated acetic acid.

Fukuzaki et al. [30] and Mawson et al. [15] studied the combined effect of pH and acetic acid concentration on degradation of the latter and found undissociated acetic acid was a major factor affecting degradation rate. It was pointed out that undissociated acetic acid acted as uncouplers of the plasma membrane and that passive diffusion of undissociated acetic acid into the cell was at expense of ATP since that diffusion resulted in intracellular acidification and extra protons needed to be pumped out to maintain the intracellular balance [16]. Based on this theory, this study investigated the concentration of undissociated acetic acid in acidogenic phase and methanogenic phase. Results in Table 4 and 5 showed that at the same Ac_i concentration, lower pH resulted in higher undissociated acetic acid concentration. In the acidogenic phase, the corresponding undissociated acetic acid at pH 5.00 and initial acetic acid concentration of 1646.47 mg HAc/L was 601.46 mg unHAc/L, and this caused 40.90% inhibition when compared to maximum acetic acid degradation rate obtained in this study. This value was higher than that at pH 5.50-6.50 when acetic acid concentration ranged from 565.29 to 2781.19 mg HAc/L (Table 4). Thus, inhibition by acetic acid on acidogenic phase methanogens was associated with high concentration of undissociated acetic acid. However, acidogenic methanogens were also significantly inhibited at pH 4.50 when the undissociated acetic acid was 364.84 mg unHAc/L (Ac_i = 565.29 mg HAc/L) and 532.15 mg unHAc/L

($Ac_i=824.53$ mg HAc/L). These values were both lower than 601.46 mg unHAc/L. It is possible at such low pH in the acidogenic phase, low pH determined the acetic acid degradation rate rather than the concentration of undissociated acetic acid; and the second-order substrate inhibition model (equation 1), which describes substrate inhibition kinetics and so is related to substrate concentration, would not be suitable to demonstrate undissociated acetic acid inhibition on the acidogenic phase methanogens. Therefore, the inhibition model (equation 1) developed in this study was only used to analyze undissociated acetic acid inhibition on methanogenic phase methanogens.

The model developed showed the substrate saturation constant of methanogenic methanogens was 1.66 mg unHAc/L which is lower than that reported by Fukuzaki et al. [30] who indicated the K_s value for a culture of *M.barkeri* without acetic acid-acclimatization and in a single-stage anaerobic digester was 6.25 mg unHAc/L. In other words, the substrate concentration associated with a rate that is one-half of the maximum rate in this study's sludge is lower than that in a culture of *M.barkeri* [30]. These values suggested that the methanogenic phase methanogens in the two-phase anaerobic system have higher affinity for substrate. The relatively high substrate inhibition constant K_i (145.17 mg unHAc/L) calculated from the model demonstrated that the methanogenic phase methanogens in this study's system could tolerate quite high concentration of undissociated acetic acid and the experimental data also showed the methanogenic phase methanogens can degrade acetic acid without inhibition at relative high concentration of undissociated acetic acid (88.10 mg unHAc/L); whereas Fukuzaki

et al. [30] reported acetic acid utilization by cultures of both *M.barkeri* and acclimatized sludge in the single-stage anaerobic digester was completely stopped at 0.29 mg unHAc/L and 0.005 mg unHAc/L, respectively. Thus, it seems that the two-phase anaerobic digestion system has higher tolerance to undissociated acetic acid when compared with the single-stage anaerobic digester. The mechanism of how undissociated acetic acid inhibited methanogenic phase methanogens shall be investigated in further study.

The experiment results have demonstrated the "multi-faceted" role of acetic acid in the anaerobic process. Acetic acid-utilizing methanogens utilized acetic acid to produce methane, thus, the effect of acetic acid on these methanogens would be determined by its concentration which was affected by the environmental parameter pH. Acetic acid would be a promotor to methanogens when its concentration was lower than the inhibition threshold. But when acetic acid concentration was higher than the inhibition threshold, especially at low pH environment, which induced high concentration of undissociated acetic acid, the activities of acetic acid-utilizing methanogens were inhibited consequently. Therefore, acetic acid became inhibitor of methanogens. In order to maintain the activities of methanogens and the stable performance of the two-phase anaerobic process, the suitable organic loading in terms of F/M ratio was important. Steven and Logan (2005) [31] have demonstrated that the hydrogen yield from fermentation of glucose was significantly inhibited by high concentration of undissociated acetic acid (inhibition threshold of 1141 mg unHAc/L). In this study, the

acetic acid-utilizing methanogens and hydrogen-utilizing methanogens were proved to exist in the acidogenic and methanogenic phases. The inhibition threshold of undissociated acetic acid on hydrogen-utilizing methanogens in the acidogenic and methanogenic phases was not clear and might be different from the values reported previously.

5. Conclusions

The study explored the effect of acetic acid on the acidogenic phase and methanogenic phase methanogens. The results showed that methanogens were present in the acidogenic and methanogenic phases; and the acidogenic phase methanogens could tolerate higher acetic acid concentration than methanogenic phase methanogens. However, the methanogenic phase methanogens in this study still tolerated higher undissociated acetic acid concentration than the methanogens in the single-stage anaerobic digester, and the parameters achieved in the model which was developed for methanogenic phase methanogens in this study further demonstrated it. Nevertheless high concentrations of undissociated acetic acid may still inhibit acidogenic and methanogenic phase methanogens. The results of this study did, however, showed greater tolerance of high undissociated acetic acid in the two-phase anaerobic system with stable performance at higher VFA loading; and both acidogenic phase methanogens and methanogenic phase methanogens degraded acetic acid in the two-phase anaerobic system. This points to the possibility of a need to reconceptualize the two-phase anaerobic system where acidogenic

phase methanogens are seen as an integral part of this phase and that it is not necessary (nor desirable) to attempt complete elimination of methanogens in the acidogenic phase.

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Figure Captions

Fig. 1 Relative abundance of microorganisms by qPCR in the acidogenic (a) and methanogenic (b) phases.

Fig. 2 The correlation between specific acetic acid utilization rate and different initial acetic acid concentrations under different pH conditions by acidogenic phase methanogens.

Fig. 3 The correlation between specific acetic acid utilization rate and different initial acetic acid concentrations under different pH conditions by methanogenic phase methanogens.

Fig. 4 Specific utilization rate of acetic acid by methanogenic phase methanogens versus initial concentration of unHAc under different pH.

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Fig. 4 Specific utilization rate of acetic acid by methanogenic phase methanogens versus initial concentration of undissociated acetic acid under different pH.

Table 1 Composition of stock solution of nutrients and trace elements (0.2 mL/L) for the synthetic feed [17]

Nutrient	(g/L)	Trace Element (g/L)	
$(\text{NH}_4)_2\text{HPO}_4$	0.024	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	1.25
NH_4HCO_3	0.34	H_3BO_3	1.25
KCl	0.002	$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	3.057
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	0.166	$\text{Na}_2\text{MoO}_4 \cdot 4\text{H}_2\text{O}$	0.1
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.166	$\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$	1.25
$\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$	0.006	ZnCl_2	1.25
NaHCO_3	0.5	Thiamine	1.945

Table 2 Initial added acetic acid concentrations for the acidogenic and methanogenic tests

Measured initial substrate and added acetic acid concentrations and pH under acidogenic conditions			Measured initial substrate and added acetic acid concentrations and pH under methanogenic conditions		
Condition 1			Condition 2		
Substrate	Ac _i		Substrate	Ac _i	
(mg HAc/L)	pH	(mg HAc/L)	(mg HAc/L)	pH	(mg HAc/L)
	4.50	65.29		6.00	46.08
	5.00	324.53		6.40	681.65
	5.50	603.11		6.80	1119.47
	6.00	1146.47		7.30	2203.23
Ac _i =500	6.50	2281.19	Ac _i =500	7.70	4279.01

Table 3 Primer/probes used for identifying bacteria, archaea and specific methanogens
[22, 23]

Target group	Sequence (5'-->3')
Bacteria	F: ACTCC TACGG GAGGC AG
	T: TGCCA GCAGC CGCGG TAATA C
	R: GACTA CCAGG GTATC TAATC C
	F: ATTAG ATACC CSBGT AGTCC
	T: AGGAA TTGGC GGGGG AGCAC
Archaea	R: GCCAT GCACC WCCTC T
	F: CGWAG GGAAG CTGTT AAGT
	T: AGCAC CACAA CGCGT GGA
<i>Methanobacteriales</i>	R: TACCG TCGTC CACTC CTT
	F: ATCGR TACGG GTTGT GGG
	T: TYCGA CAGTG AGGRA CGAAA GCTG
<i>Methanomicrobiales</i>	R: CACCT AACGC RCATH GTTTA C
	F: GAAAC CGYGA TAAGG GGA
	T: TTAGC AAGGG CCGGG CAA
<i>Methanosaetaceae</i>	R: TAGCG ARCAT CGTTT ACG
<i>Methanosarcinaceae</i>	F: TAATC CTYGA RGGAC CACCA

T: ACGGC AAGGG ACGAA AGCTA GG

R: CCTAC GGCAC CRACM AC

F, T and R indicate forward primer, TaqMan probe and reverse primer, respectively.

Table 4 Concentrations of undissociated acetic acid (mg unHAc/L) under different initial acetic acid concentrations (mg HAc/L) and pH in acidogenic phase methanogens study

Undissociated acetic acid concentration at different initial acetic acid concentration (mg unHAc/L)					
Initial acetic acid concentration (mg HAc/L)	pH=4.50	pH=5.00	pH=5.50	pH=6.00	pH=6.50
565.29	364.84	206.50	87.03	30.75	10.10
824.53	532.15	301.20	126.94	44.85	14.73
1103.11	711.95	402.97	169.82	60.01	19.71
1646.47	1062.63	601.46	253.47	89.57	29.42
2781.19	1794.98	1015.97	428.16	151.30	49.70

Table 5 Concentrations of undissociated acetic acid (mg unHAc/L) under different initial acetic acid concentrations (mg HAc/L) and pH in methanogenic phase methanogens study

Initial acetic acid concentration (mg HAc/L)	Undissociated acetic acid concentration at different initial acetic acid concentration (mg unHAc/L)				
	pH=6.00	pH=6.40	pH=6.80	pH=7.30	pH=7.70
546.08	29.71	12.23	4.94	1.57	0.63
1181.65	64.28	26.47	10.68	3.40	1.36
1619.47	88.10	36.28	14.64	4.66	1.86
2703.23	147.06	60.55	24.44	7.77	3.10
4779.01	259.98	107.05	43.20	13.74	5.48