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The role of hydrogenotrophic methanogens in an acidogenic reactor

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Abstract

A laboratory-scale acidogenic anaerobic sequencing batch reactor was set up to test the effect of pH change on microbial community structure of the reactor biomass and process performance. No immediate performance change on acidogenesis was observed after the pH change. However, as the hydrogenotrophic methanogen population decreased, hydrogen content in biogas increased followed by a sharp decrease in volatile fatty acids (VFAs) with acetic acid (HAc) in particular. Recovery of reactor performance following pH correction was only apparent after recovery of hydrogenotrophic methanogen population. These suggested hydrogenotrophic methanogens played a very important role in performance of the acidogenic process.

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Key words Anaerobic digestion; Acidogenic phase; Volatile Fatty Acid; Hydrogen production; Hydrogenotrophic Methanogenesis

1. Introduction

High-strength organic and biodegradable wastewaters, such as food processing wastewater and young landfill leachate, are often treated anaerobically (Speece, 1983; Luostarinen et al., 2007; Onodera et al., 2007; Abu Ghunmi et al., 2010; Bialek et al., 2011). Without large energy requirement, anaerobic treatment is an efficient process to achieve substantial reductions in pollutant content, and the production of methane – an energy source. As energy cost rises, the anaerobic treatment process, as a pretreatment for high-strength organic wastewaters, becomes increasingly favored. Since multiple biological processes (i.e., hydrolysis, acidogenesis, acetogenesis and methanogenesis) are involved in the anaerobic process and these different processes require various optimal operating conditions, the idea of the two-phase (i.e., acidogenic phase and methanogenic phase) anaerobic process had been proposed (Pohland and Ghosh, 1971). Through phase separation, it was argued that optimum growth conditions could be developed for the acidogens and methanogens respectively. The substrate turnover rate therefore could be increased, which consequently would improve treatment efficiency, overall process stability and methane production (Cooney et al., 2007; Rincon et al., 2009).

As the lead phase, the acidogenic phase would not only produce the necessary substrates for the next phase, it was also anticipated to help maintain stability of the overall anaerobic process (Ince, 1998). Various microorganisms, such as acidogens, acetogens, homoacetogens and methanogens, and complex biological conversions between acetate, other volatile fatty acids (VFAs), hydrogen and carbon dioxide are involved in this phase (Mosey, 1983). Acidogens, as one of the key microbial groups in the acidogenic phase, have been extensively investigated. Intuitively it would seem reasonable that only a few studies have investigated methanogens in the acidogenic phase (Mizuno et al., 1998; Shimada et al., 2011). However, methanogens, such as hydrogenotrophic methanogens, do exist and may also play an important role in the acidogenic phase. Results of Shimada (2011) showed that hydrogenotrophic methanogens constitute the major archaeal microbes in the acidogenic reactors of two-phase anaerobic digesters, and the methane generation did not necessarily result in lower production of acetic acids (HAc).

Hydrogen, which is an inevitable byproduct during the acidogenic process, has a major impact on acid fermentation as it plays an essential role in keeping the redox balance within the acidogenic phase and so affect composition of the acid products generated from fermentation of organic matters (Mosey, 1983; Madigan and Brock, 2009). Mosey (1983) suggested increased concentration of hydrogen slows down glycolysis but speeds up conversion from pyruvate to butyrate and propionate in acidogenesis reactions by

lowering the ratio of $[NAD^+]/[NADH]$. It may also inhibit acetogenesis of butyrate and propionate to acetate by acetogenic bacteria due to thermodynamic reasons. So for a healthy acidogenic phase, production of hydrogen need to be kept as low as possible.

Hydrogenotrophic methanogen can use hydrogen and carbon dioxide as substrate to produce methane. Its role in single-phase anaerobic digestion has never been underestimated and has been suggested to contribute to 28-34% of methane production in single-phase anaerobic digestion (Conrad, 1999). However, its role in the acidogenic phase, where pH is usually less than 6.0, is yet to be studied. It should be noted, due to the heterogeneous nature of acidogens, acidogens are perceived as being more robust and adaptable than methanogens; methanogens in the acidogenic phase where conditions favor the acidogens, are therefore likely more vulnerable and thus potentially more sensitive to environmental changes in the reactor. Environmental condition changes, e.g. pH, may therefore have a larger impact on resident methanogens than acidogens in the acidogenic reactor. A change in the microbial structure in terms of the methanogenic community may then go on to affect the acidogenic process performance. Therefore, research on methanogens therein is essential for a better understanding of acidogenic reactor performance.

In the present study, a laboratory-scale acidogenic anaerobic system was set-up to study the effects of pH changes on the performance of the acidogenic reactor. Microbial community structure was analyzed by quantitative real-time PCR during these periods of stress to understand the role of methanogens in the acidogenic phase.

2. Materials and methods

2.1 AnSBR setup and substrate

A laboratory-scale acidogenic anSBR system (R_A) was operated using a programmable logic controller (PLC) system (Fig. 1). The seed sludge for R_A was obtained from an anaerobic digester at a municipal wastewater treatment plant. The raw seed sludge was filtered through a 600 μm sieve and stored at 4 °C before seeding in the reactors. The anSBR system was fed with synthetic feed simulating high strength organic wastewater. The composition of the synthetic feed is shown in Table 1. pH, feeding, mixing, desludging, settling and decanting processes were controlled with the PLC. Detailed operating parameters and the operating schedule are shown in Table 2 and Table 3.

2.2 pH change test on R_A

In order to suppress the methanogenic population and investigate performance change of R_A , on Day 180, the pH was decreased from 5.5 ± 0.2 to 4.5 ± 0.2 and the culture was

incubated under low pH for 10 d. Then the pH was changed back to 5.5 ± 0.2 to allow R_A to recover. Other operation details were the same as normal during the pH change test.

2.3 Analytical methods

Influent and effluent samples were collected from feed tanks and reactors routinely for chemical analysis. Volatile fatty acids (VFAs, C_2 - C_8) were measured using gas chromatography (Agilent Technologies 7890A GC system, US) with Zebron ZB-FFAP 30 m $\times$ 320 μ m $\times$ 0.5 μ m column and a flame ionization detector (FID). Prior to analysis, 0.1 ml of 10% formic acid was added to each 0.9 ml of samples and standards for better dissolution. COD, MLSS, and MLVSS were determined in accordance with Standard Methods (APHA, 1998). MLSS and MLVSS inside the reactors and in the discharge were tracked in order to monitor the SRT.

Biogas was collected using a 3 L or 10 L gasbag (TEDLAR, US), and the volume of biogas generated each day was estimated from the gas bag. Methane, carbon dioxide and hydrogen in the biogas was analyzed using gas chromatography (Agilent Technologies 7890A GC system, US) with (1) an Agilent HayeSep R 0.9 m $\times$ 1/8" $\times$ 2.0 mm packed column, (2) an Agilent HayeSep C 3.0 m $\times$ 1/8" $\times$ 2.0 mm packed column, (3) an Agilent MolSieve 5A 3.0 m $\times$ 1/8" $\times$ 2.0 mm packed column, (4) an Agilent HayeSep Q 0.9 m $\times$ 1/8" $\times$ 2.0 mm packed column, and (5) an Agilent MolSieve 13 $\times$ 3.0 m $\times$ 1/8" $\times$ 2.0 mm

packed column with two thermal conductivity detectors (TCD, a front detector for measuring methane and carbon dioxide, and a back detector for measuring hydrogen). Helium was the reference gas for Column 1-3 for detection of methane and carbon dioxide and argon was the reference gas for Column 4-5 for detection of hydrogen.

2.4 DNA extraction

0.5 mL sludge samples were collected in 2 mL plastic tubes, centrifuged at 10000 rpm for 30 sec, followed by decantation of the supernatant. The sludge was then washed twice with 1 mL phosphate buffer solution (PBS 1X). The pellets were stored at 4 °C before DNA extraction. Before extraction, the sludge samples were diluted 5 times to reach cell concentration of around 10^{10} /mL. Total DNA was then extracted from samples using an automated nucleic acid extractor (MagNA Pure Compact, Roche, Germany). The purified DNA was then stored at -20 °C before analysis.

2.5 16S ribosomal RNA gene real-time quantitative PCR (qPCR)

16S rRNA gene quantifications of the DNA samples were performed on LightCycler 480 II (Roche, Germany). The primer and probe sets specific for two domains: Bacteria (BAC) and Archaea (ARC); two order-level Archaea: Methanomicrobiales (MMB) and Methanobacteriales (MBT); and two family-level Archaea: *Methanosarcinaceae* (MSC) and *Methanosaetaceae* (MST) were used (Yu et al., 2005; Bialek et al., 2011). Most

bacteria and methanogens in anaerobic reactors were expected to be covered by these primer and probe sets. MMB and MBT are hydrogenotrophic methanogens, which utilize only H₂ and CO₂ or formate to produce methane; MST only utilize acetate, and MSC utilize acetate as well as various other methyl compounds and hydrogen (Madigan and Brock, 2009). The reaction was performed with a total volume of 20 µL mixture: 10 µL of 2 X LightCycler 480 Probes Master, 4 µL of PCR-grade water, 2 µL of TaqMan probe (final concentration 200 nM), 1 µL of each forward and reverse primer (final concentration 500 nM), and 2 µL of template DNA. The operation processes consisted of a predenaturation step of 10 min at 95 °C, amplification of 55 cycles (10 s) at 95 °C and 30 s at 60 °C, and cooling for 10 s at 40 °C. Standard curves were constructed using those strains corresponding to primer and probe sets used in this experiment (Table 4). A 10-fold dilution series from 10¹-10¹⁰ copies/µL of standard solution was established and analyzed by qPCR in duplicate to construct the standard curve for the corresponding primer and probe set.

3. Results

3.1 Performance of R_A

After 110 d of operation, stable acidogenesis performance was achieved in R_A. From Day 110 to Day 179, total VFA (TVFA) concentration in the effluent was 16.2 ± 1.4 gCOD/L, with 4.7 ± 0.6 gCOD/L of HAc. Soluble COD in the effluent was 17.1 ± 1.4 gCOD/L

(Fig. 2a). This suggested residual COD in the effluent was largely in the form of VFAs and successful acidogenesis was achieved. Production rate of biogas was 4.9 ± 1.1 L/d with $39 \pm 4\%$ of methane and $61 \pm 4\%$ of carbon dioxide (Fig. 2b).

3.2 Effect of pH change

After pH was dropped to 4.5, there was no significant change in the reactor's performance during the first 10 d (Fig. 2 a, b) (Day 180 - Day190). However, from Day 202, hydrogen content in the biogas increased sharply from 0 to 22%. Meanwhile, COD in the effluent increased from 17.1 ± 1.0 to 21.5 ± 1.8 g/L with substantial reduction of TVFA (15.1 ± 2.5 to 4.0 gCOD/L) and HAc (6.1 ± 1.0 to 1.6 gCOD/L) concentrations (Fig. 2a) which indicated that acidogenesis was disturbed in R_A . Biogas production increased to 10.5 ± 2.8 L/d but with hydrogen content of 25% to 38% till Day 281. On Day 281, hydrogen content decreased to 0% and methane content increased to 17%. Meanwhile, TVFA and HAc concentration in effluent also increased. TVFA concentration increased from 12.7 gCOD/L (Day 281) to 21.1 gCOD/L (Day 294) and HAc concentration increased from 3.0 gCOD/L to 7.0 gCOD/L respectively. Increased organic acids production indicated acidogenic performance of R_A was recovering from pH effect.

3.3 Microbial community dynamics in R_A

The qPCR assays revealed 16S rRNA gene concentration of the domain Bacteria (BAC) and Archaea (ARC) in the acidogenic reactor at different periods (Fig. 2c). Bacteria was

predominant and remained relatively stable with an average gene concentration of $3.9 \pm 1.9 \times 10^{10}$ copies/mL during the whole process. For the domain of Archaea, hydrogenotrophic MBT was dominant in the Archaea domain, while MMB and MST constituted very small portion of Archaea population. From Day 135 to Day 179, no substantial change in Archaea domain was observed. However, after 10 d of pH change, a major change in community structure took place (Fig. 2c). While the BAC population remained relatively stable, ARC population decreased significantly. 16S rRNA gene concentration of ARC decreased from 7.53×10^7 to 1.83×10^7 copies/mL and kept decreasing to 9.43×10^5 copies/mL till Day 230. Within the domain of Archaea, population of the two hydrogenotrophic methanogens declined greatly. Gene concentration of MBT decreased from 8.06×10^7 to 5.64×10^5 copies/mL on Day 230, and MMB became undetectable (less than 9×10^4 copies/mL). Population of MST was less affected by the pH change, but became undetectable (less than 1.7×10^4 copies/mL) after Day 230. After Day 230, ARC population started to recover. However, only MBT was detectable since then.

4. Discussions

4.1 Effects of pH on performance and microbes in acidogenic reactor

The results suggested that low pH (4.5) could have caused the following performance changes to the acidogenic reactor: (1) decrease of CH₄ content in biogas; (2) increase of

H₂ content in biogas; (3) decrease of TVFA production; and (4) decrease of HAc production. The performance change (1) was observed during the pH change test. This was because methanogens were very sensitive to pH change, especially under acidic environment. However, performance changes (2)-(4) was observed not during the pH change test but 12 d after that. Similarly, the performance of R_A changed on Day 280 when pH was stabilized at 5.5 ± 0.2 . This suggested that pH was not the direct cause of performance change (2)-(4) in present study though it was an important factor of acidogenesis process.

pH was reported to be closely related to substrate degradation and organic acid products distribution (Ren et al., 1997; Yu and Fang, 2002, 2003). Yu et al. (2003) showed that overall acidogenesis performance improved with increasing pH in the pH range of 4.0 - 6.0. As shown in Table 5, at pH 4.5, concentrations of HAc and TVFA were 47% and 26% lower as compared to those at pH 5.5. However, data from other reports suggested otherwise (Li et al., 2010; Wu et al., 2010). Results of Wu et al. (2010) showed concentration of TVFA was 28% higher at pH 4.5 than pH 5.5, while that of HAc was 3% lower (Table 5). For the present study, as shown in Table 5 and Figure 2, in the same pH range (5.5), performance of R_A was drastically different before and after the pH change whereby the system was in steady-state in both cases. These results indicated that other

than pH, there may still be other factors influencing the acidogenesis process, even when the pH was the unique operating variable.

From Fig. 2c, after the pH change, a substantial reduction in the hydrogenotrophic methanogen population was observed in R_A . This observation was in accordance with the performance of the acidogenic reactor when methane content decreased to 0% and hydrogen content built up to 20-30%. As the hydrogenotrophic methanogen population declined, consumption of hydrogen was also substantially reduced. As the hydrogenotrophic methanogen population gradually recovered (between 10^7 to 10^8 copies/mL in present study), consumption of hydrogen was regained, leading to decrease in the hydrogen content and increase in methane content from Day 281. This indicated that the pH change (5.5 to 4.5) had a profound impact on the hydrogenotrophic methanogens which then led to the performance change of R_A .

4.2 Effect of hydrogen on acidogenesis

It was observed during operation of R_A under normal conditions, more than 60% COD was converted to VFA COD, indicating healthy acidogenesis in R_A . However, production of TVFA and HAc in R_A declined after the pH of the system was changed.

This may be attributed to two reasons: low pH and/or high hydrogen content in biogas. Low pH may affect fermentation by the acidogens; hydrogen content can affect the redox balance within the acidogenic phase. However, as is shown in Figure 2a and 2b, pH had no immediate impact on production of TVFA and HAc. Furthermore, substantial reduction in TVFA and HAc was observed once hydrogen content started to build in the system on Day 202; similarly, as hydrogen decreased on Day 280, TVFA and HAc started to increase in the system when pH remained unchanged. This indicated that the reduction of TVFA and HAc was more likely to be caused by the accumulated hydrogen in R_A . Hydrogen was reported to have impact on acidogenic and acetogenic bacteria which are two important bacteria groups involved in the production of HAc. (Mosey, 1983).

Accumulation of HAc in the acidogenic reactor of a two-phase system, which would be the carbon source for the following methane generation process, is a key indicator of a robust acidogenic reactor. Therefore in order to increase the HAc production, it is crucial to create an environment with low hydrogen presence and so guarantee an efficient performance of acidogenic reactor.

4.3 Role of methanogens in acidogenic reactor

As shown in Fig. 2c, hydrogenotrophic methanogen MBT consisted majority of methanogen population in R_A , while acetotrophic methanogens (MST and MSC)

consisted only minor population of methanogen. This is because short HRT, low pH and high HAc concentration conditions are more inhibitory to acetotrophic methanogens. All acetotrophic methanogens belong to the order Methanosarcinales comprising the two families, *Methanosaetaceae* and *Methanosarcinaceae*. Their growth rates are rather low with minimum doubling time of 2-3 d, and they are very sensitive to pH change. *Methanosaetaceae* is adapted for utilizing low concentrations of acetate (threshold < 600 µg/L), while *Methanosarcinaceae* is only able to convert acetate at higher concentrations (threshold of 12-72 mg/L) (Fey and Conrad, 2000; Klocke et al., 2008; Shimada et al., 2011). In the present study, however, pH was lower than 5.5 and HAc concentration in acidogenic reactor was always above 1 g/L, which is inhibitory to the *Methanosaetaceae* family. On the other hand, unlike acetotrophic methanogens, hydrogenotrophic methanogens' growth rates are relatively faster with minimum doubling times of around 6 hours. Previous studies have also reported existence of hydrogenotrophic methanogen in acidogenic reactors (Raskin et al., 1995; Liu et al., 2002; Shimada et al., 2011). These suggested that methane production from acetate was not favored in the acidogenic phase, even though this process is considered to be responsible for most methane production (66-72%) in single-phase (combining acidogenic phase and methanogenic phase) anaerobic digestion (Schink, 1997).

In this sense, the role methanogens played in RA was more likely to convert hydrogen to methane (by hydrogenotrophic methanogen) and not to use HAc to produce methane (by acetotrophic methanogen). This may also explain why higher population of methanogen did not result in lower HAc production but higher HAc production. It was also noted that acetotrophic methanogens (MST and MSC) both became undetected after Day 230. Therefore, keeping a proper methanogenic activity in acidogenic phase can enhance the acetic acid production by consuming hydrogen without interfering with the acidogenesis process.

5. Conclusions

In summary, methanogens, especially hydrogenotrophic methanogens existed and played an important role in the acidogenic reactor. While production of hydrogen weakened acidogenesis, methane production in the acidogenic reactor, which was always ignored, can effectively remove the hydrogen leading to higher organic acid production, HAc production in particular. With more HAc produced in the acidogenic phase, which is the desired substrate for the following methanogenic phase, the overall stability and efficiency of a two-phase anaerobic process is expected to be improved.

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Figure Captions

Fig. 1 Schematic of the laboratory-scale two-phase anSBR

Fig. 2 Performance and microbial community structure of R_A . (a) COD, VFA variation: COD of influent into R_A (IN) and effluent from R_A (EF A); COD of TVFA in influent into R_A (VFA IN) and effluent from R_A (VFA EF A); COD of HAc in effluent (HAc EF A); (b) Biogas: CH_4 , CO_2 and H_2 content of biogas from R_A . (c) Quantification of 16S rRNA gene concentration of methanogenic communities and bacteria in the R_A .

Figures

Fig. 1 Schematic of the laboratory-scale two-phase anSBR

Fig. 2 Performance and microbial community structure of RA. (a) COD, VFA variation: COD of influent into RA (IN) and effluent from RA (EF A); COD of TVFA in influent

into R_A (VFA IN) and effluent from R_A (VFA EF A); COD of HAc in effluent (HAc EF A); (b) Biogas: CH_4 , CO_2 and H_2 content of biogas from R_A . (c) Quantification of 16S rRNA gene concentration of methanogenic communities and bacteria in the R_A .

Table 1 Synthetic feed composition

Component	Concentration, mg/L
Organics:	
Sucrose	20000
Ethanol	1200
Sodium Acetate	1100
Propionic Acid	540
Butyric Acid	260
Starch	1100
Cellulose	1100
Macronutrients (inorganics):	
NH ₄ HCO ₃	1885
NH ₄ Cl	1276
K ₂ HPO ₄	250
MgCl ₂ ·6H ₂ O	125
FeSO ₄ ·7H ₂ O	180
CaCl ₂	100
Na ₂ SO ₄	800
Micronutrients (inorganics):	
CoCl ₂ ·6H ₂ O	2.5
MnCl ₂ ·4H ₂ O	2.5
Na ₂ MoO ₄ ·2H ₂ O	0.5
H ₃ BO ₄	0.5
NiCl ₂ ·6H ₂ O	3.5
ZnCl ₂	1
CrCl ₃ ·6H ₂ O	2
CdNO ₃	0.3
PbCl ₂	1.4
CuSO ₄ ·5H ₂ O	1.8

Table 2 Operating parameters of two-phase anSBR

Parameters	R _A
Working volume (L)	5
Total volume (L)	6
Feed	Synthetic feed
Feed SCOD (g/L)	25.3 ± 1.9 ^a
Feed VFA (g/L)	1.4 ± 0.1
Feed HAc (g/L)	0.9 ± 0.1
pH	5.3 ± 0.2
T (°C)	40 ± 1
ORP (mV)	-400 ± 50
SRT(d)	25

^a During startup (Period I) the feed for R_A is diluted synthetic feed with COD of around 12.5 g/L.

Table 3 Operating schedule of R_A

Period	Day	Feed COD, g/L	Vol/Cycle, L	Cycle	HRT _{RA} , d	OLR g/L/d ^a
I	1-15	12.5	0.5	2	5	0.8
II	16-53	25	0.5	2	5	1.7
III	54-80	25	1	2	2.5	3.3
IV	81-300 ^b	25	1	3	1.7	5

a OLR (Organic Loading Rate) = Feed COD × (Vol/Cycle) × Cycle / Vol(R_A)

b The data in the first 100 days are not included in the present study as the system was in acclimation stage.

Table 4 Primer and probe sets for qPCR (Yu et al., 2005; Bialek et al., 2011)

Name	Function	Target group	Sequence (5'-->3')	Representative strains
ARC787F	F primer	Archaea	ATTAG ATACC CSBGT AGTCC	MMB+MBT+MSC+MST
ARC915F	TaqMan		AGGAA TTGGC GGGGG AGCAC	
ARC1059R	R primer		GCCAT GCACC WCCTC T	
BAC338F	F primer	Bacteria	ACTCC TACGG GAGGC AG	Escherichia coli K12
BAC516F	TaqMan		TGCCA GCAGC CGCGG TAATA C	
BAC805R	R primer		GACTA CCAGG GTATC TAATC C	
MMB282F	F primer	Methanomicrobiales	ATCGR TACGG GTTGT GGG	Methanospirillum hungatei JF1 (DSM 864)
MMB749F	TaqMan		TYCGA CAGTG AGGRA CGAAA GCTG	Methanomicrobium mobile BP (DSM 1539)
MMB832R	R primer		CACCT AACGC RCATH GTTTA C	
MBT857F	F primer	Methanobacteriales	CGWAG GGAAG CTGTT AAGT	Methanobacterium formicicum M.o.H. (DSM 863)
MBT929F	TaqMan		AGCAC CACAA CGCGT GGA	Methanobrevibacter arboriphilicus DH1 (DSM 1536)
MBT1196R	R primer		TACCG TCGTC CACTC CTT	
Mst702F	F primer	Methanosaetaceae	TAATC CTYGA RGGAC CACCA	Methanosaeta concilli GP6 (DSM 3671)
Mst753F	TaqMan		ACGGC AAGGG ACGAA AGCTA GG	
Mst862R	R primer		CCTAC GGCAC CRACM AC	
Msc380F	F primer	Methanosarcinaceae	GAAAC CGYGA TAAGG GGA	Methanosarcina acetivorans C2A (DSM 2834)
Msc492F	TaqMan		TTAGC AAGGG CCGGG CAA	Methanosarcina barkeri MS (DSM 800)
Msc828R	R primer		TAGCG ARCAT CGTTT ACG	Methanosarcina mazei Go1 (DSM 3647)

Table 5 Effects of pH on acidogenesis

References	VFA	Conc. at pH 5.5, mg/L	Increment →	Conc. at pH 4.5, mg/L	Increment →	Conc. at pH 5.5, mg/L
Yu et al, 2003	HAc	373	-47%	196	N.A. ^b	N.A.
	TVFA	1404	-26%	1034	N.A.	N.A.
Wu et al, 2010	HAc	594 (pH 5.6)	-3%	578 (pH 4.4)	N.A.	N.A.
	TVFA	1083 (pH 5.6)	28%	1388 (pH 4.4)	N.A.	N.A.
Present study	HAc	5889 ^a	1%	5951	-68%	2007
	TVFA	10110	0%	10098	-46%	5480

^a Averaged by data from Day 170 to Day 180.

^b Not available.