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Enhanced volatile fatty acids (VFAs) production in a thermophilic fermenter with stepwise pH increase – investigation on dissolved organic matter transformation and microbial community shift

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Abstract

In this study, a mixture of primary and wasted activated sludge was fermented in a semi-continuous reactor aiming for enhanced volatile fatty acids (VFAs) production. The reactor was subjected to a stepwise pH increase from 7 to 10 during approximately 130 days of operation. The result revealed that the maximum acidification was obtained at pH 8.9 (21%) resulting in the maximum production of VFAs (423.22±25.49 mg COD/g VSS), while the maximum hydrolysis efficiency was observed at pH 9.9 (42%). The high pH was effective in releasing dissolved organic matter (DOM) including protein, carbohydrate, building blocks and low molecular weight (LMW) neutrals. More LMW DOMs were released than high molecular weight (HMW) DOMs fractions at higher pH. pH 9.9 favored hydrolysis of HMW DOMs while it did not enhance the acidogenesis of LMW DOMs. The microbial community analysis showed that the relative abundance of phyla *Actinobacteria* and *Proteobacteria* increased with the increased pH, which may lead to the maximum hydrolysis at pH 9.9. At pH 8.9, class *Clostridia* (59.16%) was the most dominant population where the maximum acidification (21%) was obtained. This suggested that the dominance of *Clostridia* was highly related to acidification extent. The relative abundance of *Euryarchaeota* decreased significantly from 58% to 2% with increased pH.

Keywords: VFAs production; progressive pH increase; dissolved organic matter; microbial community.

1 Introduction

Large amount of waste sludge (primary and wasted activated sludge) is generated all over the world from wastewater treatment plants (WWTPs) and direct discharge of sludge raises serious environmental concerns (Carrere et al. 2010). Due to its high organic content (such as carbohydrate and protein), waste sludge can be treated through anaerobic fermentation to produce volatile fatty acids (VFAs) or methane (Zhang et al. 2010a). Clearly, VFAs have a wide range of applications such as carbon sources for biological nutrient removal from wastewater (Li et al. 2011, Wang et al. 2016), synthesis of long chain fatty acids (Ren et al. 2015), hydrogen generation and electricity generation in bioelectrochemical systems (BES) (Liu et al. 2012b, Teng et al. 2010). Typically, anaerobic sludge digestion involves three steps: hydrolysis, acidification of hydrolyzed products, and methane generation. The initial hydrolysis is considered as the rate-limiting step (Zhang et al. 2015). Hence, various pretreatment methods have been developed to enhance the release of organics and improve VFA production from sludge (Neumann et al. 2016, Wan et al. 2016).

Alkaline pH was recommended to enhance VFAs production from waste activated sludge anaerobic fermentation as more soluble COD (sCOD) can be generated and methanogens are inhibited under high pH conditions (Wu et al. 2010, Yu et al. 2008, Yuan et al. 2006, Zhao et al. 2015). However, it is noteworthy that the culture used in previous studies was not adapted to sudden pH shocks, that is, alkaline conditions were directly applied to the digestion process. Thus, the results could not reflect the microbial community's acclimation status and optimal performance. In fact,

stepwise pH change with long-term acclimation period was recommended to reduce the pH shock effect on microorganisms (Madigou et al. 2016). Temperature is also an important operational factor to improve VFAs production. For example, Hao and Wang (2015) described that thermophilic fermentation led to 10-fold more VFAs compared to mesophilic fermentation without pH control, and Zhang et al. (2009) also reported that higher degree of hydrolysis can be obtained under thermophilic conditions compared to mesophilic fermentation. To date, it is not clear how stepwise pH increase would affect VFA production and microbial population under thermophilic conditions.

Complex dissolved organic matter (DOM) is an important substrate for VFA generation. A large amount of DOM could be released from sludge solids into sludge supernatant after pretreatments (Guo et al. 2014), which consists of different molecular weight fractions ranging from low molecular weight (LMW) substances (e.g. amino acids, carboxylic acids, alcohols, aldehydes, etc.) to high molecular weight (HMW) compounds such as humic substances, polysaccharides, and proteins (Tran et al. 2015). Thus, identification of composition and characteristics of DOM are useful for studying transformation and fate of many sludge components in the anaerobic digestion processes (Zhang et al. 2013). DOM composition of the fermentation liquid was previously examined using three-dimensional excitation–emission matrix (3D-EEM) (Wu et al. 2016, Yu et al. 2015) to determine protein (tyrosine-like and tryptophan-like) and humic acid-like organics. Nevertheless, this method can only semi-quantify DOM. Recently, size exclusion chromatography (SEC)

coupled with organic carbon detection and organic nitrogen detection (LC-OCD-OND) have been successfully applied to measure DOMs changes in the fermentation liquid (Li et al. 2016). Hence, this new method can provide insights into the transformation of DOMs in sludge treatment process.

This study aimed to investigate long-term effect of stepwise pH changes on sludge fermentation products and microbial community under thermophilic conditions. Briefly, the present study aimed to (i) evaluate the VFA production performance of a semi-continuous sludge digester under thermophilic conditions with stepwise pH increase; (ii) investigate the fermentation product changes (iii) analyze the bacterial and archaeal communities and reveal the relationship between the performance of sludge fermentation and microbial community.

2 Material and methods

2.1. Feedstock preparation and semi-continuous fermenter setup

The feed sludge was sewage sludge, composed of mixed primary and secondary sludge (w/w: 1:1) from a local water reclamation plant. Mixed sludge was collected weekly and stored at 4^{o}C until further use. The characteristics of mixed sludge are listed in Table 1. The inoculum was collected from a mesophilic anaerobic digester located in same plant. In order to obtain stable operation, the fermenter was operated at pH 7 under 55^{o}C for 60 days before the experiments were conducted.

A 7 L commercial fermenter (Major Science, USA) with 3 L working volume was operated in semi-continuous mode for 128 days at 55^{o}C (Figure 1). The overhead

mixer was controlled at 100 rpm throughout the entire fermentation period. 0.5 L of fermented sludge was withdrawn and replaced with the 0.5 L of feed sludge daily resulting in a SRT of 6 days. The seed anaerobic sludge was collected from the same plant. pH was adjusted automatically with 2 M NaOH from 7 to 10 in progressive step of 1 when steady state reached and held for at least 2 SRTs (12 days) at each pH.

2.2 Analytical methods

COD, TSS and VSS were measured in accordance with the standard methods (APHA 2005). Collected samples were centrifuged at 12000 rpm for 10 min and the supernatant was filtered through a 0.45 μm membrane filter for further analysis of soluble fraction of sludge samples. Soluble carbohydrate was measured by the phenol–sulfuric acid method with glucose as standard (Xiao et al. 2016). Soluble protein was determined by the modified Lowry–Folin method using the protein assay kit (Thermofisher, USA) with bovine serum albumin as standard. Ammonium was measured with the Nessler method kit (Hach, USA), which was described in (Maspolim et al. 2016).

A gas chromatograph (GC7890A, Agilent, USA) with flame ionization detector and equipped with DB-FFAP fused-silica capillary column was utilized to analyze the composition of VFAs and ethanol. The filtrate (1 mL) was collected in a 2 mL gas chromatography (GC) vial, and acidified with 10% (v/v) formic acid before analysis. The column operating temperature profiles were 80^{o}C for 1 min, then increased to 120^{o}C at 20^{o}C/min and then to 205^{o}C at 10^{o}C/min, hold for 2min. The injector and detector temperatures were 260^{o}C. The sample injection volume was 0.5 µL. Lactic

128 acid, formic acid, and succinate were measured by high performance liquid
129 chromatograph (HPLC, Agilent 1260 Infinity, CA) as described in Chen et al. (2016b)
130 study.

131 Volume of biogas produced daily was measured with a wet gas meter (Ritter,
132 Germany). The hydrogen and methane content in the headspace was determined using
133 a customized 7890 GC (Agilent, USA) equipped with dual thermal conductivity
134 detectors.

135 According to Wan et al. (2016), COD was calculated based on the following
136 coefficients: 8 g COD/g H_2, 382.25 mL CH_4/g COD at 25°C, 1.07 g COD/g acetate,
137 1.51 g COD/g propionate, 1.81 g COD/g butyrate, 2.04 g COD/g valerate, 1.06 g
138 COD/g carbohydrate, 1.50 g COD/g protein. Based on these coefficients, the extent of
139 hydrolysis and acidification were calculated according to (Wu et al. 2015) and briefly
140 described in Eq. (1) and (2). The VSS destruction efficiency is shown in Eq. (3)

141 $$\text{extent of hydrolysis (\%)}=\frac{COD_{CH4}+COD_{H2}+sCOD_o-sCOD_i}{tCOD_i-sCOD_i}\times 100\%$$

142 (1)

143 $$\text{extent of acidification (\%)}=\frac{COD_{CH4}+COD_{H2}+COD_{VFAs}-sCOD_i}{tCOD_i-sCOD_i}\times 100\%$$

144 (2)

145 Where COD_{CH4} and COD_{H2} are methane and hydrogen production as COD
146 equivalents, COD_{VFAs} is VFAs production in the outlet as COD equivalents. $sCOD_o$
147 and $sCOD_i$ are soluble COD in the outlet and inlet, and $tCOD_i$ is the total COD in the
148 inlet.

$$\text{VSS destruction (\%)}=\frac{VSS_i - VSS_o}{VSS_i}\times 100\% \quad (3)$$

Where VSS_i and VSS_o are VSS concentrations (g/L) of feed sludge (inlet) and fermented sludge (outlet), respectively.

The volume of hydrogen (V_{H2}) was calculated with the ideal-gas state equation PV=nRT. The pressure of headspace was calculated as the sum of the partial pressure of nitrogen and hydrogen (Chen et al. 2016a). The volume of CH_4 (V_{CH4}) produced was calculated with the volume of biogas (V_{biogas}) multiplied by $P_{CH4.}$ Based on above results, the yields of CH_4, H_2 and VFAs were described in Eq. (4) and (5). All parameters were calculated based on the steady state at each steady pH level.

$$CH_4/H_2 \text{ yield (mL/g VSS)}=\frac{V_{CH4/H2}\times HRT}{VSS_i * V_{reactor}} \quad (4)$$

$$\text{VFAs yield (mg COD/g VSS)}=\frac{COD_{VFAs}}{VSS_i} \quad (5)$$

Where $V_{reactor}$ is the working volume of the reactor.

2.3 SEC-organic carbon detection organic nitrogen detection analysis

LC-OCD-OND system (DOC-LABOR, Karlsruhe, Germany) was used to characterize the DOM. These DOMs could be assigned to specific classes of compounds: biopolymers (high molecular weight of protein and carbohydrate), humic substances, building blocks, LMW acids, LMW neutrals, and hydrophobic organic carbon. The concentration of these DOMs was quantified by a software program

(ChromCALC, DOC-LABOR, Karlsruhe, Germany) (Huber et al. 2011) and the unit of these DOMs concentration is mg equivalent carbon per liter (mg-C/L). Based on the proposed chemical formulae of polysaccharide $(C_6H_{10}O_5)_n$ and protein $C_{52.5}H_{6.65}N_{16}O_{21.5}S_2$, 1 g of polysaccharide and protein equal to 0.444 and 0.497 g of equivalent carbon, respectively (Li et al. 2016). The concentrations of LMW protein and LMW carbohydrate were calculated by subtracting HMW protein and HMW carbohydrate from total protein and carbohydrate, respectively.

2.4 DNA extraction and Illumina high-throughput sequencing

The biomass for microbial community analysis were collected at Day 18, 51, 79 and 125, respectively, when system was operated at pH 7 (R11), pH 7.9 (R12), pH 8.9 (R13) and pH 9.9 (R14) at steady state. DNA was extracted from the collected biomass using the fast DNA extraction Kit (MP Biomedicals, Singapore) according to the manufacturer's instructions. The extracted DNA was then stored at -20^{o}C before further analysis. The primers used for high-throughput sequencing were modified 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3'), targeting the V3+V4 regions of both bacterial and archaeal 16S rRNA genes (Chen et al. 2016b). Sequencing was subsequently performed using Illumina MiSeq platform by the Beijing Genomic Institute (Hong Kong, China). Quality control (QC) assessment was done to remove low-quality sequences and some artificial replicate sequences. The QC passed sequences were clustered into operational taxonomic units (OTUs) at 97% similarity. All OTUs were analyzed using an RDP Bayesian classifier to cluster them into

192 relative species. In addition, rarefaction curves, Shannon, abundance based coverage
193 estimator (ACE), Chao1 estimators, and Venn diagram were calculated to illustrate
194 the similarity and difference among these samples. The sequencing data of the four
195 samples were archived in NCBI Sequence Read Archive (SRA) with the accession
196 number SRX2059048, SRX2114771, SRX2114776 and SRX2114778.
197

198 **3 Results and discussion**

199 **3.1 The performance of the mixed sludge fermentation**

200 The effect of stepwise increase of pH on sludge fermentation under thermophilic
201 conditions is shown in Figure 2. At pH 7 (0-19 d), the methane partial pressure (P_{CH4})
202 and carbon dioxide partial pressure (P_{CO2}) were maintained at 0.65 atm and 0.29 atm,
203 respectively. Hydrogen was undetected in the headspace (detection limit of 5×10^{-5} atm)
204 and the remaining gas was nitrogen (<0.06 atm). Correspondingly, the biogas
205 production rate was 1.66±0.04 L/d. Meantime, less than 2 mmol/L of acetate was
206 accumulated and propionate concentration was nearly 4 mmol/L in liquid phase.
207 Other VFAs (such as butyrate and valerate) were below 1 mmol/L during this period.

208 At the beginning of pH 7.9 period (20-52 d), the reactor was purged with
209 nitrogen (99.99%) due to air leakage. Afterwards, the P_{CH4} increased sharply from 0 to
210 0.84 atm on Day 35 and stayed at 0.84 atm for the rest 17 days in this period. The
211 P_{CO2} decreased remarkably from 0.29 atm to 0.05 atm and the hydrogen was not
212 detectable. The biogas production rate decreased to 1.04±0.05 L/d. Meanwhile,
213 acetate and propionate were the dominant VFAs with concentration of 9 and 7

mmol/L respectively.

When pH was further increased to 8.9 (56-85 d), no CH_4 and CO_2 were produced. The hydrogen partial pressure (P_{H2}) was about 3×10^{-4} atm during this period. In contrast, acetate concentration increased rapidly from 9 to 40 mmol/L from Day 62 and was maintained at about 40 mmol/L during Days 63-85. Propionate also increased slightly from 7 to 8 mmol/L on Day 62 and decreased thereafter. Butyrate and valerate were detected with concentrations of 6 and 5 mmol/L, respectively.

At pH 9.9 (92-128 d), CH_4 and CO_2 remained undetectable in the gas phase. Hydrogen was present in the headspace with a partial pressure of 0.14 atm at Day 111. Reactor was purged with nitrogen (99.99%) due to air leakage on Day 112, the P_{H2} increased from 0 to 0.18 atm on Day 128. Acetate concentration decreased remarkably immediately after the pH raised to 9.9 and stayed at 25 mmol/L for 32 days. Others VFAs, such as propionate, butyrate and valerate decreased as well and the concentrations were 4.5, 4 and 3.5 mmol/L respectively. It was observed that ammonium concentration increased from pH 7 to 7.9 and remained fairly stable with the highest concentration observed at pH 8.9.

Table 2 summarized total VFAs, H_2 and CH_4 yields in Figure 2 under different pHs. CH_4 yield was 165.80±3.37 and 127.51±9.02 mL/g VSS at pH 7 and 7.9, respectively. No CH_4 was produced at pH above 7.9. H_2 yield at pH 9.9 was 14.73± 1.42 mL/g VSS. VFAs yield was enhanced significantly under alkaline conditions except at pH 9.9. The maximum VFAs yield of 423.22±25.49 mg COD/g VSS was obtained at pH 8.9. Apparently, pH 8.9 was the optimum pH condition for VFAs

236 production in this study.

237 **3.2 Sludge solubilisation and VSS destruction**

238 According to the methane, hydrogen and tVFAs profiles shown in Figure 2, the
239 extent of hydrolysis and acidification was calculated and shown in Figure 3. In
240 general, the extent of hydrolysis represents the percentage of larger organic matters
241 solubilized to smaller organic matters in sludge, and the extent of acidification is
242 defined as the biodegradability of the soluble organic matter, which is transformed
243 into VFAs, hydrogen and methane by the acidogens and methanogens.

244 As described in Figure 3, the value of hydrolysis (22%) was close to the value of
245 acidification (18.2%) at pH 7.0, suggesting that the hydrolysis was the rate-limiting
246 process. Furthermore, most of the COD produced from the hydrolysis was converted
247 to methane (16.7%). At pH 7.9, the extent of hydrolysis (25%) was slightly greater
248 than the value of acidification (18.7%). Methane and tVFAs consisted of 13.4% and
249 5.3% of the solubilized COD. No obvious changes of acidification degree were
250 observed compared to pH 7 (18.2%). At pH 8.9, the hydrolysis extent was further
251 increased to 29% while acidification efficiency was 21%. tVFAs contributed to the
252 100% of acidification. Compared to pH 7-7.9, the acidification was significant
253 enhanced at pH 8.9. Clearly, the most efficient hydrolysis occurred at pH 9.9 (42%)
254 compared to other pHs. This is reasonable that the alkaline pH improves the
255 hydrolysis of organic matters (Ma et al. 2016, Yang et al. 2014, Yu et al. 2008).

256 However, higher hydrolysis caused by the alkali action did not translate to higher
257 acidification at pH 9.9. The acidification extent was only 15.5% which consisted of

0.6% of hydrogen and 14.9% of tVFAs. The VFA content was the lowest compared to other pHs. This phenomenon might be attributed to the inhibitory effect of strong alkali conditions on acidogens (Huang et al. 2016, Ma et al. 2016, Yang et al. 2014). VSS destruction at different pH levels was also measured and presented in Figure 3. The increased VSS destruction with elevated pH was observed and the highest VSS reduction was at pH 9.9 (38%).

3.3 Effect of stepwise pH increase on dissolved organic matters accumulation

It is well-known that protein and carbohydrate are key organic compounds accounted for 70–80% of wasted activated sludge (Wu et al. 2009). As shown in Figure 4, soluble protein was released during the whole operation period and its concentration was gradually increased from 0.49±0.03 at pH 7 to 2.63±0.14 g COD/L at pH 9.9. Soluble carbohydrate followed the similar trend that its concentrations increased from 0.12±0.01 at pH 7 to 1.03±0.07 g COD/L at pH 9.9. Apparently, the concentration of carbohydrate was always lower than protein, which confirmed that protein was the major substrate for acidogenesis (Feng et al. 2014, Liu et al. 2012a, Yuan et al. 2006).

More soluble protein and carbohydrate were released at higher pH condition and this was attributed to the dissociation of acidic groups in extracellular polymeric substances (EPS) and repulsions between the negatively charged EPS at alkaline pH (Feng et al. 2014, Yu et al. 2008, Yuan et al. 2015). The tVFAs concentrations increased when pH was changed from 7.0 to 8.9 and decreased at pH 9.9 and the percentage of tVFAs was accounted for 38.67%, 47.44%, 69.18%, and 35.18% at

different pHs. This further suggested that hydrolysis continued at higher pH while acidogenesis was slowed down.

To further analyze the detailed compounds of DOMs, LC-OCD-OND was applied. The concentrations of relevant compounds at different pHs were described in Table 3. Clearly, the concentrations of HOC and hydrophilic dissolved organic carbon (HI DOC; the sum of building blocks, LMW neutrals, LMW acids, HMW protein and HMW carbohydrate) increased with the increased pH and reached maximum at pH 8.9 (631.2 mg/L-C). In addition, more HI DOC was released than HOC at all pH conditions. Xiao et al. (2016) also concluded the HI DOC accounted for more than 80% of the dissolved organic matter measured as DOC in the pre-treatment sludge. Meanwhile, no observable humic acid was found, however, building blocks, which were the breakdown products of humic acid, were present and gradually increased from 102.01 to 642.74 mg/L-C with the increase of pH.

The concentration of LMW neutrals (including mono-oligosaccharides, alcohols, aldehydes, and ketones) increased by a factor of 7.21 from pH 7 to 9.9. Similarly, the maximum LMW acids concentration was also achieved at pH 8.9 (1040.99 mg/L-C, Figure 2) which coincides with VFA results. Soluble protein, LMW proteins in particular, increased with pH. The increase of LMW carbohydrate was more than that of HMW carbohydrate with elevated pH. These results implied that higher pH was in favor of hydrolysis of HMW DOMs to LMW DOMs, while it did not enhance the acidogenesis of LMW DOMs. This confirmed the results presented in Figure 4.

It is noteworthy that the LMW protein and carbohydrate, which should be the

substrates for VFA generation, were accumulated with the increased pH levels. It was firstly hypothesized that SRT may play a role in degradation of LMW protein and carbohydrate. However, a longer SRT of 10 days was not able to reduce the accumulation of both compounds (data not shown). Therefore, it is highly possible that acidogenesis was inhibited under high pH regardless of acclimation period. Secondly, it is also possible that the degradability of those LMW protein and carbohydrate was low. The detailed profiling of this range of protein and carbohydrate is required.

Table 4 shows a comparison of maximum VFAs yield in the literature with this study. Clearly, all the maximum VFAs yields reported were at the alkaline pH condition (>=8). Higher temperature also had positive effect on VFAs yield. For example, Zhang et al. (2009) reported that the optimum VFAs accumulation was 298 mg COD/g VSS with mesophilic fermentation, and 368 mg COD/g VSS with thermophilic fermentation, treating the same sludge (WAS) (Table 4). In addition, different sludge types and solids concentrations may result in the different maximum VFAs yield production. For example, Maspolim et al. (2015) showed that 410 mg COD/g VSS was obtained from mixed sludge which had higher VFAs yield than WAS only. Further, step-wise increased pH rather than shock pH may help to enrich certain hydrolytic and acidifying microbial communities which may assist hydrolysis and acidogenesis processes. Hence, combined factors may have a positive synergetic effect on VFA production, resulting in the maximum yield in this study (423.22 mg/VSS). SRT, on the other hand, did not show a strong correlation with VFA

production.

3.4 Microbial community and diversity at different pHs

Figure 5 described microbial community at different pH. At phylum level, *Euryarchaeota* (Archaea) was the most abundant (58%), followed by *Firmicutes* (19%), *Actinobacteria* (11%) and *Synergistetes (*8%) in R11. Similarly, *Euryarchaeota* also dominated the microbial community of R12 (60%), followed by *Firmicutes* (23%) and *Actinobacteria* (10%). In contrast, *Firmicutes* was the major phyla (60%) in the microbial community of R13, followed by *Actinobacteria* (24%) and *Proteobacteria* (6%). In R14, *Actinobacteria*, *Firmicutes* and *Proteobacteria* were abundant phyla and their relative abundance were 39%, 35% and 16%, respectively. It was reported that some species of *Actinobacteria* could produce abundant hydrolytic enzymes (Liu et al. 2016). *Proteobacteria* is the important microorganisms in anaerobic hydrolysis and acidification (Chen et al. 2016c). *Firmicutes* is well known to produce proteases, cellulases, lipases, and other extracellular enzymes and closely involved in organic compounds degradation (Chen et al. 2016b).

At class level, the class *Clostridia* affiliated to phylum *Firmicutes* accounted for 18.55%, 19.74%, 59.16% and 31.10% in R11, R12, R13 and R14, respectively. As previous studies reported (Feng et al. 2009, Zhang et al. 2010b), many species of *Clostridia* could produce organic acids under anaerobic conditions. *Alphaproteobacteria* was the main class in the *Proteobacteria* phylum in all samples and its relative abundance was 1.14%, 2.51%, 6.37% and 15.65%, respectively. The

346 presence of *Alphaproteobacteria* had been reported to benefit the degradation of
347 carbohydrate (Ariesyady et al. 2007). The enrichment of the class *Actinobacteria* at
348 higher pH was observed which was similar to the study conducted by Wan et al.
349 (2016). *Actinobacteria* maximum relative abundance was 39.30% in this study.
350 *Synergistia* which could convert organic acids to acetate and hydrogen (Si et al. 2016),
351 decreased from 8.17% to 0.45% at the highest pH.

352 At genus level, *Methanosarcina* which can use acetate, as well as hydrogen and
353 carbon dioxide for methane production (Wong et al. 2013), was only present in R11
354 (7.8%). *Methanosaeta*, another acetoclastic methanogen (AM) was not found in all
355 samples. It is well known that the pH range for the growth of *Methanosarcina* is 5-8
356 (De Vrieze et al. 2012) and high temperature may inhibit *Methanosaeta*.
357 Hydrogenotrophic methanogens (HM, *Methanobrevibacter* and
358 *Methanothermobacter*), were the dominant archaea in R11, R12, R13 and R14.
359 However, the relative abundance of HM decreased more significantly with pH
360 increase. It indicated that the higher pH inhibited the methane production (Huang et al.
361 2016, Maspolim et al. 2016). *Coprothermobacter*, which can degrade proteins into
362 acetate, H_2 and CO_2 (Zamanzadeh et al. 2016), dominated in bacterial community of
363 R11 (12.54%) and R12 (9.39%). However, its relative abundance was barely detected
364 in R13 and R14. Meanwhile, the similar trend of *Anaerobaculum*, which are capable
365 of converting organics acids, peptides and a limited number of carbohydrates to
366 acetate, CO_2 and H_2 was observed. *Anaerobranca* was only detected in R14 and its
367 relative abundance was 13.42%. In consistent with Zhang et al. (2010b) study,

368 *Anaerobranca*, converting protein and carbohydrates to acetate, was a moderate
369 thermophilic microorganism and was predominant in alkaline condition.
370 *Tepidimicrobium* was dominant in the R13 (41.77%) and it was reported to grow on a
371 number of carbohydrates and a variety of proteinaceous compounds at pH 5.8-9.3 and
372 temperature 25-67^{o}C (Cibis et al. 2016, Dai et al. 2016). The relative abundance of
373 *Mycobacterium* increased from pH 7 to 9.9 with the maximum abundance of 17.95%.

374 The increase of relative abundance of phyla *Actinobacteria* and *Proteobacteria*
375 suggested that higher pH increased the hydrolytic microbial population which in turn
376 increased hydrolysis extent (Figure 3). In fact, the hydrolysis extent may also be
377 enhanced by the chemical reactions under alkaline condition. Hence, further studied is
378 recommended to differentiate the true contribution from both biological hydrolysis
379 and chemical hydrolysis at high pH. The *Euryarchaeota* phylum, which belongs to
380 archaea that is responsible for methane production, was completely inhibited when pH
381 exceeded 7.9. Even after a long acclimation phase with stepwise pH increase, the
382 archaea population was not recovered. The similar result was observed in Zhang et al.
383 (2009) and Yuan et al. (2015) that the activity of methanogens decreased at a higher
384 pH (>8) and the methane yield was marginal. Hence, in order to have higher VFA
385 yield, it is recommended to have high pH range and/or lower SRT to inhibit
386 methanogens activity. At pH 8.9 in this study, the highest relative abundance of the
387 genus *Tepidimicrobium* (41.77%) which belonged to the class *Clostridia* resulted in
388 the maximum VFAs production. At pH 9.9, *Anaerobranca*, who has a relatively lower
389 acidogenesis degradation activity than *Tepidimicrobium* was dominant (13.42%),

390 resulting in the decrease of VFAs production.

391 The microbial communities obtained in this study were different from other
392 alkaline fermentation studies. For example, Zheng et al. (2013) reported that
393 *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* in the reactor of pH 10 accounted for
394 83.6%, 13.3%, and 0.2% of total bacterial sequences. Yuan et al. (2015) described that
395 the main phylum in the pH 10 fermenter was *Chloroflexi* (30.1%). In this study,
396 *Actinobacteria*, *Firmicutes* and *Proteobacteria* with total abundance of 90% were
397 dominant at pH 9.9. These phyla are all related to hydrolysis and acidification
398 activities. Thus, step-wise increased pH rather than shock pH in this study may help to
399 enrich certain hydrolytic and acidifying microbial communities, and in turn improved
400 the hydrolysis and acidogenesis in sludge fermentation. The results suggested that pH
401 9.9 enriched large amount of hydrolytic microorganisms to favor hydrolysis and
402 release of organic matters from the sludge into the bulk liquid, while pH 8.9 induced
403 the population of acidification and enhanced VFAs production.

404

405 **4 Conclusions**

406 This study, for the first time, reported the long-term effect of stepwise pH
407 increase on dissolved organic matter transformation and microbial community change
408 in thermophilic sludge fermentation. The result showed protein, carbohydrate,
409 building blocks and LMW neutrals were effectively released and more LMW DOMs
410 were produced than HMW DOMs fractions with elevated pH. *Actinobacteria* and
411 *Proteobacteria* were enriched at pH 9.9, contributing to the maximum hydrolysis

412 extent (42%). While, the highest proportion of the class *Clostridia* (59.16%) was
413 found at pH 8.9 that resulted in the maximum acidification (21%). Thus, it can be
414 concluded that pH 9.9 was only in favor of hydrolysis and release of organic matters
415 from the sludge into the liquid while pH 8.9 was the best condition for acidogenesis
416 compared to pH 7, 7.9 and 9.9.

417

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573 **A list of Tables**

578

579 **Table 1.** Characteristics of mixed sludge used in the experiments.

Parameter	Units	Mixed sludge value
pH	1	5.7-6.0
Total suspend solids (TSS)	g/L	16.05±1.18
Volatile suspended solids (VSS)	g/L	12.69±0.79
Soluble chemical oxygen demand (sCOD)	g/L	2.55±0.34
Total chemical oxygen demand (tCOD)	g/L	21.43±1.30
Ammonium	mg/L	163.44±37.93

580

581

582 **Table 2.** Effect of stepwise pH increase on total VFAs, H_2 and CH_4 yield.

pH	CH_4 yield (mL/g VSS)	H_2 yield (mL/g VSS)	VFAs yield (mg COD/g VSS)
7	165.80±3.37	0	38.19±3.67
7.9	127.51±9.02	0	119.57±10.36
8.9	0	0	423.22±25.49
9.9	0	14.73±1.42	276.40±9.15

583
584

585 **Table 3.** Quantification of dissolved organic matters (mg/L –C) at different pHs.

pH	HOC	Building blocks	LMW neutrals	LMW acids	HMW protein	LMW protein	HMW carbohydrate	LMW carbohydrate
7	64.06	102.01	100.50	92.34	19.54	146.53	21.00	32.39
7.9	337.71	277.52	184.58	289.11	28.30	269.23	57.92	63.17
8.9	631.2	534.57	349.02	1040.99	79.16	455.27	75.87	214.64
9.9	481.69	642.74	721.61	726.94	137.73	698.00	70.38	331.79

586 "HOC" denotes hydrophobic dissolved organic carbon;

587

588 **Table 4.** Comparison of VFA yield with literature.

pH	Sludge type	SRT (d)	Temperature (^{o}C)	Maximum VFAs yield (mg COD/g VSS)	References
9 (shock)	WAS	5	35	298	(Zhang et al. 2009)
9 (shock)	thermal hydrolyzed sludge	6	35	348.63	(Dong et al. 2016)
10 (shock)	Excess sludge	10	28	302.40	(Jie et al. 2014)
8 (shock)	Mixed sludge	3	35	410	(Maspolim et al. 2015)
10 (shock)	WAS	8	30	260.67	(Yuan et al. 2015)
10 (shock)	Excess sludge	8	Room temperature	256.2	(Yuan et al. 2006)
10 (shock)	Primary sludge	5	Room temperature	301.61	(Wu et al. 2009)
8 (shock)	WAS	9	55	368	(Zhang et al.

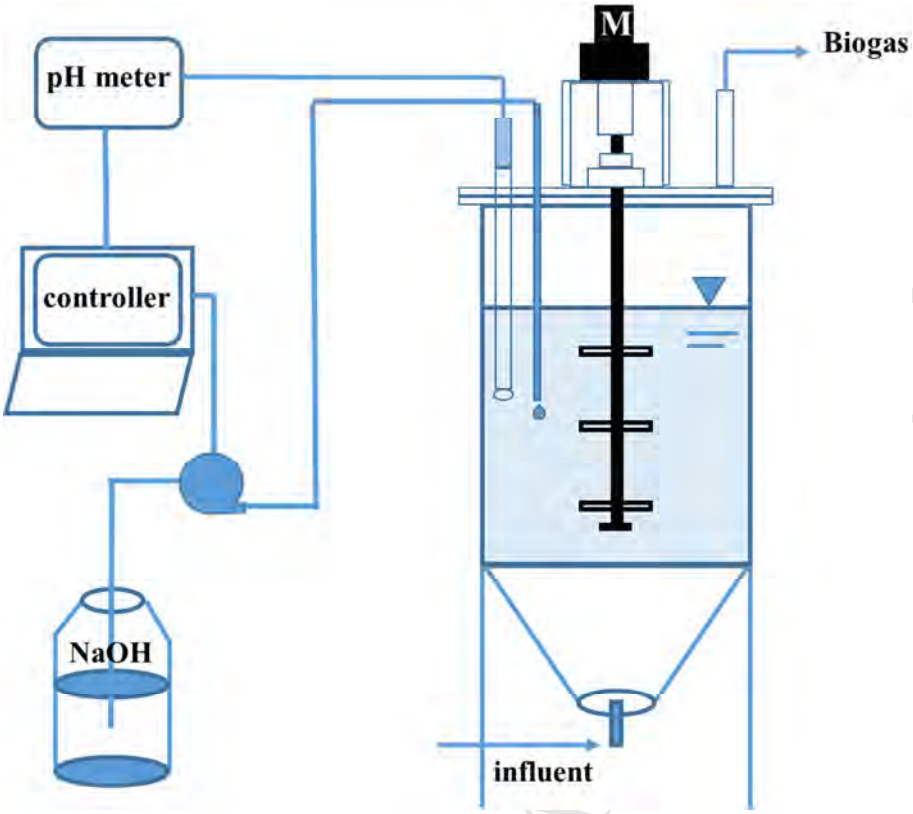
					2009)
9 (acclimated)	Mixed sludge semi	6	55	423.22	This study

589 Note: Shock denotes pH operation with one step, acclimated denotes pH operation

590 with step-wise.

591

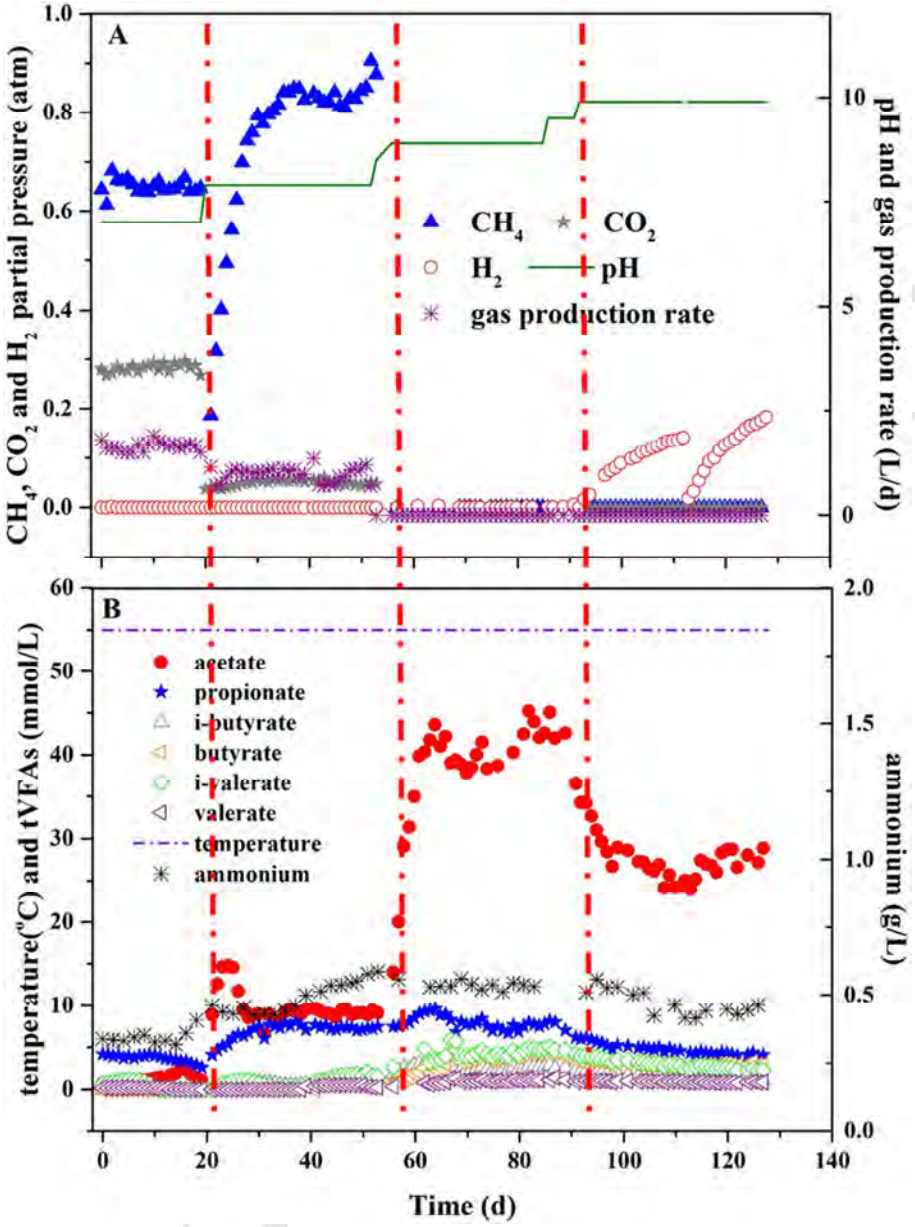
A list of Figures



610

611 **Figure 1.** Schematic diagram of fermenter treating mixed sludge.

612

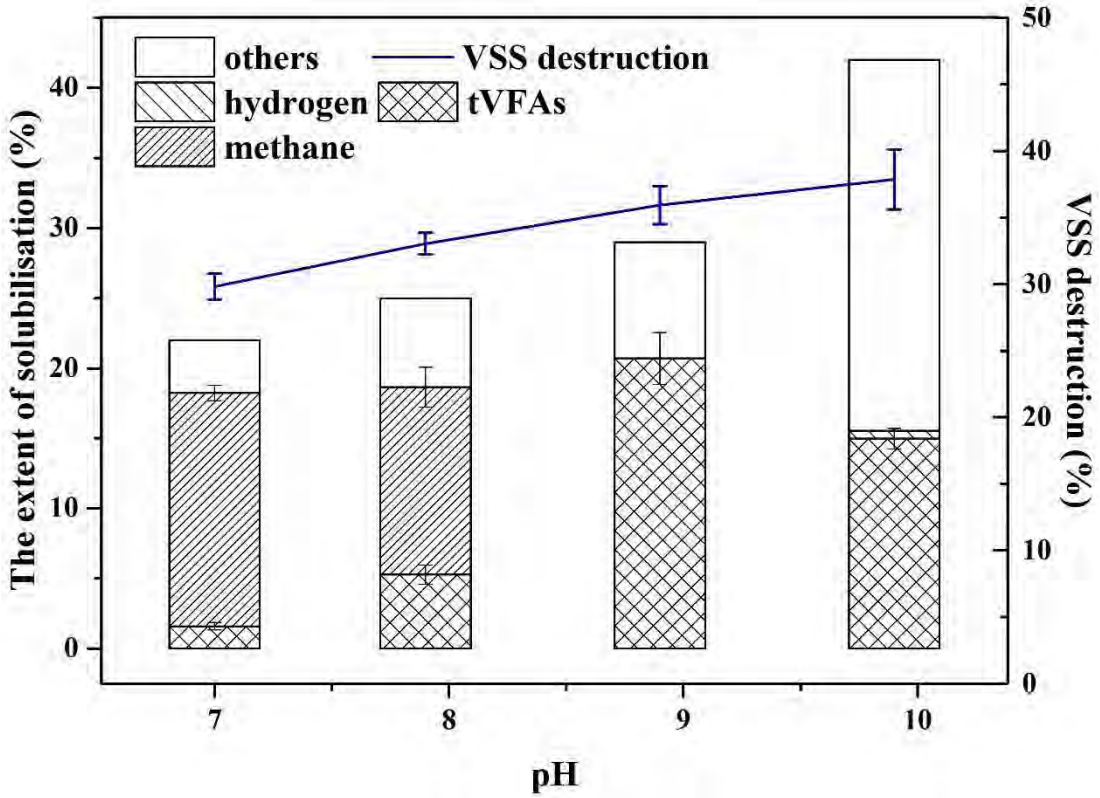


613

614 **Figure 2.** The performance of mixed sludge fermentation in semi-continuous mode by

615 the stepwise increase of pH operation: **(A)** gas phase; **(B)** liquid phase.

616

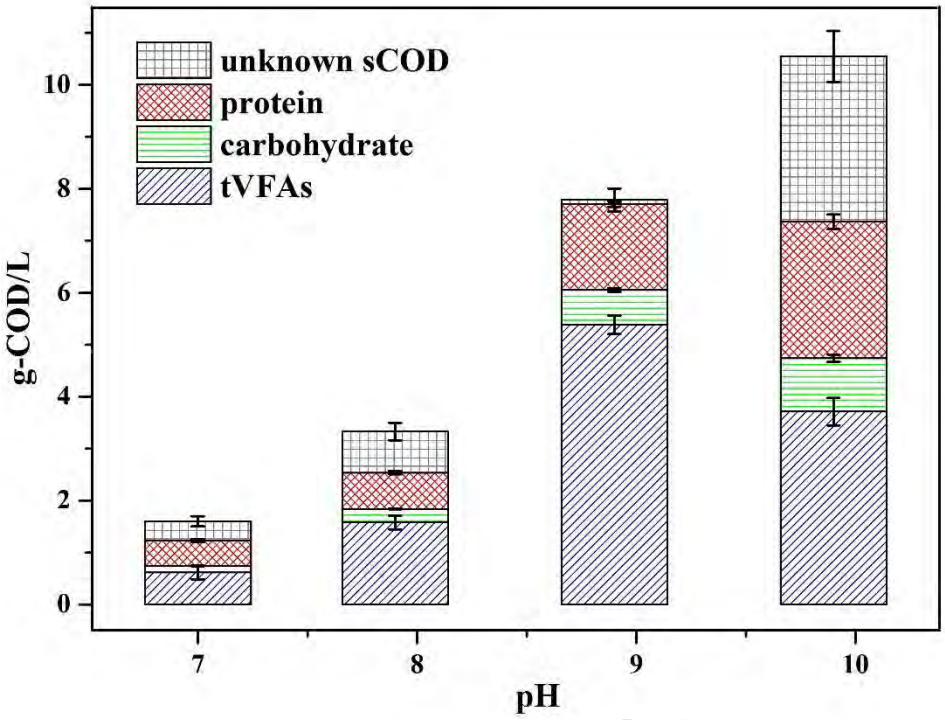


617

618 **Figure 3.** The extent of sludge hydrolysis and acidification as well as VSS destruction

619 at different pHs.

620



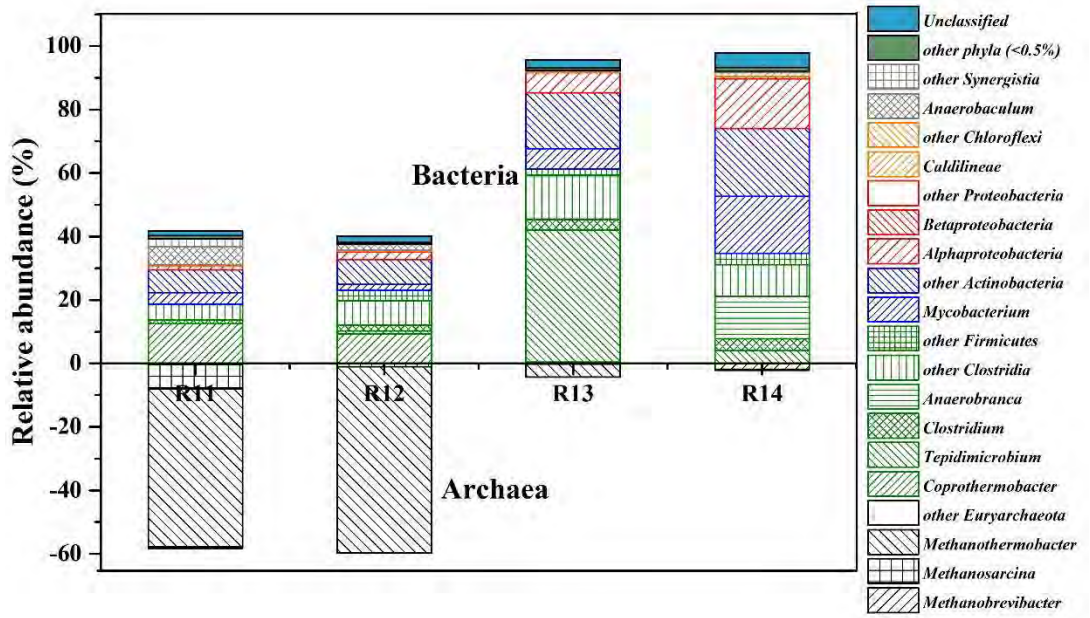
621

622 **Figure 4.** VFAs, protein, carbohydrate and soluble COD concentrations. The COD of

623 unknown soluble compounds was calculated by subtracting the COD of VFAs,

624 soluble carbohydrate, and soluble protein from sCOD.

625



626

627 **Figure 5.** Relative abundances of microbial communities which presented in
628 taxonomic phylum, class, and genus levels at different pHs. Phylogenetic groups
629 accounting for <0.5% of all the classified sequences are summarized in the artificial
630 group "others." (the grey column represents the phylum *Synergistete*; the orange
631 column represents the phylum *Chloroflexi*; the red column represents the phylum
632 *Proteobacteria*; the blue column represents phylum *Actinobacteria*; the olive column
633 represents phylum *Firmicutes*; the black column represents phylum *Euryarchaeota*).

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635

636

Highlights

- Effect of stepwise pH increase on thermophilic sludge fermentation was studied;
- The elevated pH was effective at releasing DOMs;
- The highest proportion of *Clostridia* resulted in the maximum VFAs yield at pH 8.9;
- The maximum hydrolysis efficiency was observed at pH 9.9;
- VFAs yield was enhanced in a thermophilic fermenter with step-wise pH operation.