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ABSTRACT

Anaerobic digestion (AD) is a sustainable pathway towards recovering chemical energy from excess sludge, and humic substances (HSs) contained in sludge can inhibit energy (methane/CH_4) conversion efficiency. This study aims to investigate the impact of humic acids (HA) on the various processes in a batch anaerobic digestion process. For this purpose, "clean" sludge was cultivated in a laboratory to avoid HSs presence. The cultivated sludge was used in a series of batch experiments, with humic acids added at different levels. A complete AD test, as well as three sub-phase tests (hydrolytic phase; acidogenic phase; methanogenic phase) was performed and analyzed with and without HA dosing. In the single-phase AD system, dosing with HA inhibited the methanogenic efficiency by 35.1% at HA:VSS = 15%. However, the effects of HA on the three sub-phases revealed something very different. HA inhibited hydrolytic efficiency by 38.2%, promoted acidogenic efficiency by 101.5%, and finally inhibited methanogenic efficiency by 52.2%. The combined efficiency of the three sub-phases without HA dosing is calculated at 15.7%; and with HA dosing (HA:VSS = 15%) at 10.2%. Overall, the combined inhibition efficiency of the three sub-phases is equal to 35.0%, which is almost identical (35.1%) to the result observed in the single-phase AD process. The possible mechanisms behind the phenomena were analyzed and summarized in the context.

1. Introduction

In today's world, moving wastewater treatment plants (WWTPs) toward carbon neutrality is a key issue around the world, and so means of capturing organic energy from excess sludge have made anaerobic digestion (AD) regain popularity again (Appels et al., 2011; van Loosdrecht and Brdjanovic, 2014; Hao et al., 2015 & 2017b). Although AD can convert excess sludge into biogas (CH_4) relatively easily (Appels et al., 2011), its conversion efficiency is not very great due to the stable structure of bacterial cells and refractory organics such as lignocellulose substances and humic substances (HSs) (Liu et al., 2016). Moreover, HSs contained in excess sludge account for about 6–20% of volatile suspended solids (VSS), which are produced during the decay and transformation of organic substances, and can inhibit the process of AD, and thus become an obstacle to the conversion of organic compounds into energy (Chen et al., 2008; Li et al., 2014; Azman et al., 2017).

As a major form of HSs, humic acids (HA) have very complicated chemical structures, and contain many active functional groups, such as carboxylic acid, phenolic, alcoholic hydroxyls, quinone and ketone groups (Yang et al., 2014), which have hydrophobic, surfactant properties and behave with a high degree of aromaticity (H/C < 1) (Dang et al., 2016). The complexity of HA makes it difficult to understand the effects and mechanisms of HA on an AD system (Kyzas et al., 2017; Long et al., 2017).

The inhibiting phenomena of HA on the hydrolysis of organic substances has been extensively observed with key hydrolytic enzymes (Qi et al., 2004; Fernandes et al., 2015; Liu et al., 2015; Ye et al., 2016; Azman et al., 2017) and the effect is often related to the carboxylic and phenolic groups. However, HA could enhance acidification, potentially due to quinone and ketone groups with the ability to shuttle electrons (electron transferring capacity, ETC) (Thauer et al., 1977; Piepenbrock and Kappler, 2012; Yuan et al., 2017). Finally, HA can also inhibit hydrogenotrophic methanogenesis and/or aceticlastic methanogenesis, supposedly due to its negative charge on its surface and/or the capacity as terminal electron acceptor (TEA) (Khadem et al., 2017; Liu et al., 2015).

There have been some studies about HA evolving in AD, but always with model substances or synthesized wastewater (Moura

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Nomenclature

AD	Anaerobic digestion
BLMDO	Biodegradable low-molecular dissolved organics
CH_4	Methane
CO_2	Carbon dioxide
COD	Chemical oxygen demand (mg/L)
ETC	electron transferring chains
FISH	Fluorescence in situ hybridization
FTIR	Fourier transform infrared
GC	Gas chromatographer
HA	Humic acid
HRT	Hydraulic retention time (d)
HSs	Humic substances
IC	Ion chromatographer
IOD	Integral optical density
MLSS	Mixed liquid suspended solids (mg/L)
MLVSS	Mixed liquid volatile suspended solids (mg/L)
ORP	Oxidation-reduction potential (mV)
SRT	Solids retention time (d)
TEA	terminal electron acceptors
TS	Total solids (mg/L)
TVFA	Total volatile fatty acid (mg/L, mg COD/L)
VFAs	Volatile fatty acids (mg/L, mg COD/L)
VSS	Volatile suspended solids (mg/L)
WWTPs	Wastewater treatment plants

et al., 2007; Feng et al., 2008; Azman et al., 2017). Individual studies focusing on single-phases of AD only reveal evidence of HA partially inhibiting AD, and there is still much to be done before full understanding is achieved. However, this study does demonstrate some progress towards a fuller understanding. In it, "clean" excess sludge, which was cultured in a SBR tank with a synthetic substrate containing no HSs, was used to ascertain the effects of different levels of HA on both the single and the sub-phases (hydrolytic phase, acidogenic phase, hydrolytic phase) of the processes of AD. The intention of the study was to ascertain the mechanisms of how HA can affect a lab-scale AD system, as well as the efficiency of inhibition or promotion.

2. Materials and methods

2.1. Cultured and inoculum sludge

There are both original HSs and metal irons like Ca^{2+}, Mg^{2+}, Al^{3+}, Fe^{3+}, etc. in excess sludge from WWTPs. For this reason, difficulties arise when studying the inhibiting effect of HSs on AD, due to the possible interactions between HSs and metal ions (Azman et al., 2015; Cruz-Zavala et al., 2016). Therefore, a synthetic substrate with trace metals and no HSs was used to culture "clean" excess sludge (the composition of this synthetic substrate is listed in Table 1). A SBR tank was used to culture the sludge, and was operated at V = 60 L, HRT = 3 d and SRT = 12 d, with aeration for 22 h and settlement for 2 h every day. The sludge was collected and concentrated by a 0.15-mm filter, sieved, and then stored in a refrigerator (4 °C) for use.

The inoculum sludge was taken from a 6-L fermenter (BIOSTAT B plus, German) with SRT = 32 d, which was originally used for feeding municipal excess sludge, and was later to be fed with the "clean" cultured sludge for half a year. In this way, HSs contained in the fermenter were fully washed out and the "clean" inoculum sludge was obtained. Prior to taking inoculum sludge for the experiments, the fermenter cease to both feed and discharge for a week, so that biodegradable low-molecular dissolved organics (BLMDO) could be totally consumed to avoid a sudden initial biogas peak in the experiments. The characteristics of both concentrated and inoculum sludge are listed in Table 2.

Table 1
Composition of the synthetic substrate.

Compounds	Unit (mg/L)	Trace elements	Unit (mg/L)
CH_3COONa	3200	$FeCl_3 \cdot 6H_2O$	1500
NH_4Cl	320	H_3BO_4	150
$NaHCO_3$	1500	$Na_2MoO_4 \cdot 2H_2O$	60
KH_2PO_4	80	$ZnSO_4 \cdot 7H_2O$	120
$CaCl_2$	200	KI	180
$MgSO_4 \cdot 7H_2O$	320	$CoCl_2 \cdot 6H_2O$	150
		$CuSO_4 \cdot 5H_2O$	30
		$MnCl_2 \cdot 4H_2O$	120
		EDTA	10,000

*1-L substrate solution contains 1-mL trace elements solution.

2.2. Characteristics of humic acids (HA)

In this study, a merchant HA was selected to replace the genuine HA contained in excess sludge. The similarity of the two types of HAs was first evaluated using a Thermo Fisher FTIR (Fourier transform infrared) spectrometer, as shown in Fig. 1, in which specific molecular structures and chemical functional groups can easily be compared. Fig. 1 shows there were no significant difference between two types of HAs at the wavenumber from 4, 000 to 1700 cm^{-1}. Two wide, strong absorption peaks occurred between 3500 and 3100 cm^{-1}, which are considered to have been caused by the absorption of associative −OH stretching vibration in the hydroxyl and alcoholic hydroxyl groups, respectively. The acromion peaks at 2925 and 2850 cm^{-1} represent the anti-symmetric and symmetrical stretching vibration of the groups $-CH_2-$ in the aliphatic alkane structures, respectively, which are the typical absorption peaks of alkane structures. In short, the results demonstrate that both types of HAs contain phenolic hydroxyl, alcoholic hydroxyl groups and alkane structures.

Minor changes occur between 1700 and 1250 cm^{-1}. The typical absorption peaks of the HA used in this study (at 1583 and

Table 2
Characteristics of both concentrated raw sludge and inoculum sludge.

Parameters	Concentrated raw sludge	Inoculum sludge
pH	7.2 ± 0.2	6.8 ± 0.2
TS (g/L)	17.6 ± 0.122	16.3 ± 0.006
VS (g/L)	11.0 ± 0.034	9.5 ± 0.017
MLSS (g/L)	13.2 ± 0.083	12.2 ± 0.010
MLVSS (g/L)	9.0 ± 0.021	7.8 ± 0.005
VS/TS (MLVSS/MLSS)	0.63 (0.68)	0.58 (0.62)
TCOD (g/L)	19.84 ± 0.166	10.8 ± 0.274
SCOD (g/L)	0.02 ± 0.005	5.2 ± 0.434
Ca^{2+} (Supernatant, g/L)	0.011 ± 0.005	0.005 ± 0.002
Mg^{2+} (Supernatant, g/L)	0.009 ± 0.002	0.004 ± 0.001
Al^{3+} (Supernatant, g/L)	ND	ND

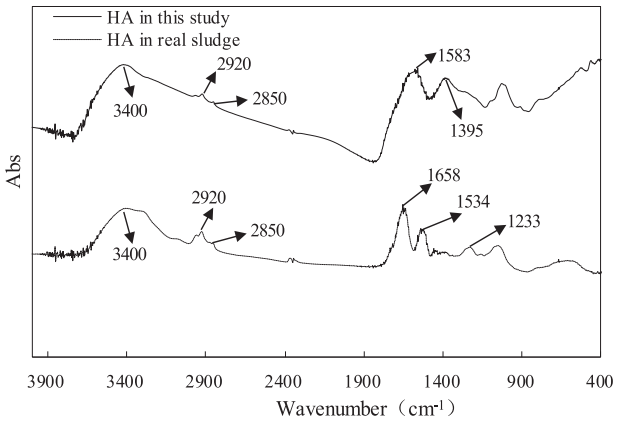

Fig. 1. Fourier transform infrared (FTIR) spectra of the humic acid (HA) used in this study and in real sludge.

1395 cm^{-1}) are caused by anti-symmetric shrinkage of functional groups carboxylate (−COO^-), phenolic carbonyl (−C−O−), phenolic carboxylate (−COO^-) and phenolic hydroxyl (−OH) vibration. However, the typical absorption peaks of HA in the real sludge occur at 1658 and 1534 cm^{-1}, representing highly conjugated carbonyl (C=O) and aromatic carbonyl (C=O) amide vibration in II-bending plane. Major differences occur between 1250 and 400 cm^{-1}. It is possible that these are caused by some impurities in HA, and have nothing to do with the structures of HA.

Finally, a quantitative analysis at the wavenumber from 4000 to 1250 cm^{-1} by FTIR spectrometer identification software reveals that the similarity between the two types of HA is as high as 87.3%, which demonstrates that the merchant HA used in this study functions as a suitable replacement for HA in real sludge.

2.3. Set-up of batch experiments

Batch experiments were conducted in 600-mL serum bottles (working volume = 400 mL, headspace volume = 200 mL, 20 bottle tests each time). Each bottle was fed with mixed sludge (concentrated and inoculum sludge) to 400 mL at a VSS ratio of 3:1. Designed dosages of HA were also added, followed by pH being adjusted to 7.0 ± 0.2 by NaOH (3 M) and/or HCl (3 M). The testing bottles were divided into four groups to analyze their hydrolytic, acidogenic and methanogenic phases.

i) Group A had the CH_4 inhibitor (BrES, the Methyl-CoM enzyme-like substance) added to it, to prevent methanogenic phase occurring. It was mainly used for analyzing the liquid samples in both the hydrolytic and acidogenic phases.
ii) Groups M_G (two sub-groups: M_{G1} and M_{G2} in parallel) were used to analyze biogas and CH_4 production, in which the liquid samples were held in to minimize experimental errors. Following this, the liquid samples were prepared for analysis of the methanogenic phase.
iii) Group W was used to analyze the whole AD process, including both liquid and gas samples.

In each of the group tests, five different concentrations of HA (HA/VSS) were added as follows: 0% (R0), 5% (R1), 10% (R2), 15% (R3) and 20% (R4). Filled testing bottles were all purged with N_2 for 3 min, and were then sealed with rubber stoppers and moved into an air-bath shaker (160 rpm and 35 ± 2 °C). The ending period of AD was determined by biogas volume produced: some 1–2% of the totally accumulated volume of biogas in a tested period. The operational parameters of the group tests are illustrated in Table 3.

2.4. Analytical methods

Liquid samples were separated from testing bottles and were immediately analyzed for pH and ORP (oxidation-reduction potential), following which they were centrifuged at 11,000 rpm and 4 °C for 5 min. Centrifuged supernatant samples were filtered through 0.45-μm membrane filters to analyze: i) SCOD by the standard method ($K_2Cr_2O_7$) (APHA et al., 1998); ii) VFAs using Ion Chromatographer (883 Basic IC Plus, Metrohm AG); iii) soluble polysaccharide and protein by Phenol-sulfuric acid and the Lorry method, respectively (APHA et al., 1998). Biogas production was measured by a gas-liquid device and the biogas composition was analyzed by a Gas Chromatographer (GC126, Shanghai-INESA).

Five strains of microorganisms associated with the acidogenic phase, and two stains of microorganisms associated with the methanogenic phase were respectively detected by FISH (Fluorescence In Situ Hybridization) with the help of a confocal laser scanning microscope (CLSM, Zeiss LSM700), according to the procedure of our previous studies (Hao et al., 2017b). Fluorescence labels of the oligonucleotide probes and their target microorganisms and corresponding gene sequences are listed in Table 4.

Other liquid samples were washed with 100-mM phosphate buffer (PBS, pH = 7.4). MLSS samples were sonicated at 4 °C for 30 min and then separated by centrifuging at 11, 000 rpm and 4 °C for 30 min. Treated supernatants were placed on ice-bags in order to analyze the activities of their enzymes. Different enzymes were analyzed by different methods, including amylase (DNS method), protease (L-lysine method) in the hydrolytic phase (based on the methods used by Liu et al., 2015), acetate kinase (AK), butyrate kinase (BK) (analyzed according to Allen et al., 1964), phosphotransacetylase (PTA), phosphotransbutyrylase (PTB) (used by Andersch et al., 1983), pyruvate-ferredoxin oxidoreductase (POR) (used by Yakunin and Hallenbeck, 1998) in the acidogenic phase, and F_{420}-reducing hydrogenase (used by Trevors, 1984) in the methanogenic phase, respectively.

TS (total solid), VS (volatile solids), MLSS (mixed liquor suspended solids), MLVSS (mixed liquor volatile suspended solids), pH and ORP were detected, according to the standard methods (APHA et al., 1998).

2.5. Evaluation methods

2.5.1. Calculation evaluation

A formula in AQUASIM 2.1d was applied to analyze the non-linear accumulated CH_4 (B_t) over time (t), as shown in Eq. (1); the first-order kinetic (K) in Eq. (1) was used to evaluate the hydrolytic phase (Koch and Drewes, 2014). Due to having almost no BLMDO in the mixed sludge, the lag time was not included in Eq. (1).

$$B_t = B_0(1 - e^{-Kt}) \quad (1)$$

Where, B_t represents CH_4 yield at time t, mL/g VSS; B_0 is the fitted maximum CH_4 yield, mL/g VSS; K is the first-order kinetic rate, d^{-1}.

2.5.2. Efficiency evaluation

Some efficiency equations were applied to quantitatively evaluate the AD systems:

$$AD\ efficiency = \frac{CH_4}{TCOD} \quad (2)$$

Table 3
Operational parameters of the group tests.

Group test	HA (HA/VSS, %)	BrES (mM)	Concentrated sludge (mL)	Inoculum (mL)	N_2 (min)	Studied phase
A	0 (R0) 5 (R1)	50	290	110	3	hydrolytic and acidogenic phase
M_G (M_{G1} and M_{G2})	10 (R2) 15 (R3)	0	290	110	3	methanogenic phase/biogas production
W	20 (R4)	0	290	110	3	whole AD process

Table 4
Probes and targets for Fluorescence In Situ Hybridization (FISH).

Probes	Gene sequence (5′-3′)	Microbial community
S223 [a]	ACGCAGACTCATCCCCGTG	Propionate to acetate
Synm126 [a]	CGCTTATGGGTAGGTTGCC	Butyrate to acetate
AW [a]	GGCTATTCCTTTCCATAGGG	Homoacetogenesis
Chis150 [a]	TTATGCGGTATTAATCTYCCTTT	
MS821 [a,b]	CGCCATGCCTGACACCTAGCGAGC	Aceticlastic methanogenesis
MX825 [a,b]	TCGCACCGTG GCCGACACCTAGC	
MB1174 [a,b]	TACCGTCGTCCACTCCTTCCTC	Hydrogenotrophic methanogenesis
MC1109 [a,b]	GCAACATAGGGCACGGGTCT	
MG1200 [a,b]	CGGATAATTCGGGGCATGCTG	

[a] Probes used in the acidogenic phase.
[b] Probes used in the methanogenic phase.

$$Inhibition\ efficiency = \frac{V_{R0} - V_{Ri}}{V_{R0}} \quad (3)$$

$$Hydrolysis\ efficiency = \frac{\Delta SCOD}{TCOD} \quad (4)$$

$$Acidogenic\ efficiency = \frac{TVFA}{\Delta SCOD} \quad (5)$$

$$Methangenesisefficiency = \frac{CH_4}{VFA} \quad (6)$$

Where subscript i is the reactor No. (0–4); TCOD is the initial total COD in bottle tests, mg/L; CH_4 stands for total accumulated CH_4 production, mg COD/L (1 mg/L COD = 0.25 mg/L CH_4); ΔSCOD is the difference of SCOD between the initial and ending points, mg/L; TVFA is the maximal total VFA production in the acidogenic system, mg COD/L (VFAs (mg/L): COD (mg/L): acetate = 1.07, propionate = 1.57, butyrate/isobutyrate = 1.82 and valerate/isovalerate = 2.04).

3. Results and discussion

3.1. Effect of HA on the single-phase anaerobic digestion (AD)

3.1.1. Biogas production

The accumulated biogas produced in the Group-W tests is shown in Fig. 2. The biogas production tended to decrease with increasing dosages of HA, until above HA = 10% an inhibition level of approximately 35% in biogas production was reached. The results confirm that HA can indeed inhibit biogas production in the batch AD system. However, the CH_4 content in each group test did not change significantly, all being within the range of 66% ($P > 0.05$).

Daily (see the upper-left corner) and accumulated CH_4 productions are shown in Fig. 3. The daily CH_4 production in each test steadily increased until they reached their peak points, but the blank test reached its peak point two days earlier (Day 6) than other tests with HA dosed, again confirming HA inhibiting on the batch AD process. Thus, it can be seen that the organic conversion efficiency to energy (CH_4) was largely inhibited with increased HA dosage.

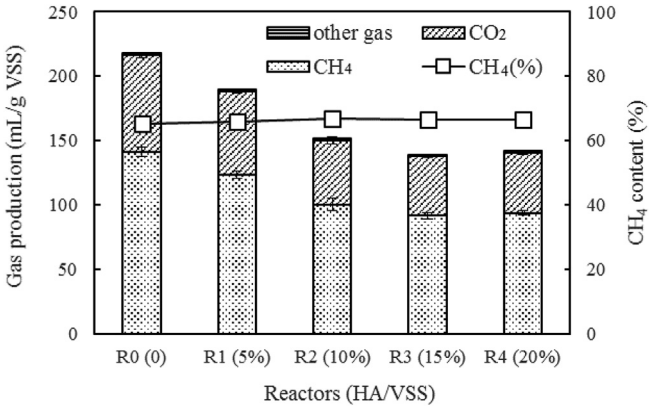

Fig. 2. Biogas production and composition in the Group-W tests (with no CH_4 inhibitor dosed).

3.1.2. Energy (CH_4) conversion efficiency

The AD efficiency calculated by Eq. (2) is respectively at 15.8% (R0), 13.7% (R1), 11.1% (R2), 10.1% (R3) and 10.4% (R4). Clearly, energy conversion efficiency decreased along with increasing HA dosage in the tests. According to Eq. (3), the CH_4 inhibition efficiency of R1, R2, R3 and R4 are respectively at 12.3%, 28.8%, 35.1% and 33.5%, as shown in Fig. 4. The strongest inhibition (35.1%) occurred at over HA:VSS = 5%.

The mechanisms of HA inhibiting the batch AD system should also be evaluated. Hereto, the sub-phases of the AD system (hydrolytic, acidogenic and methanogenic phase) was further studied, respectively.

3.2. Effect of HA on the hydrolytic phase

3.2.1. SCOD

In the Group-A tests, with the activity of hydrolytic and acidogenic bacteria being only due to the addition of inhibitor

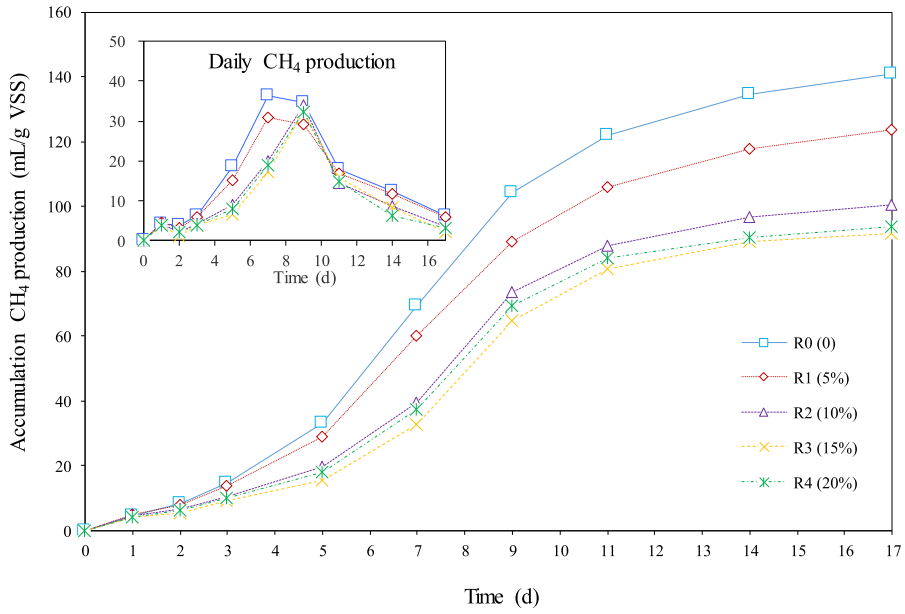

Fig. 3. Daily and accumulated CH_4 productions in the Group-W tests (without CH_4 inhibitor added).

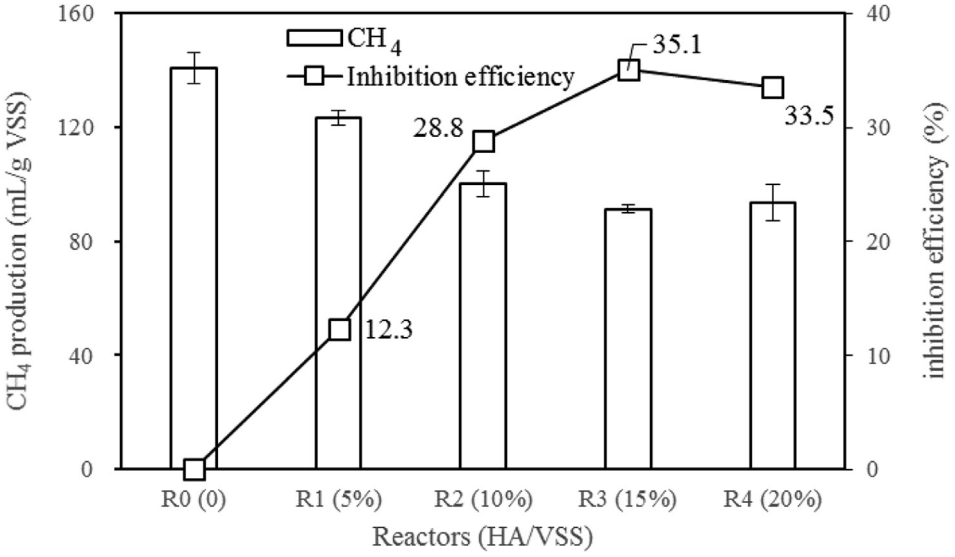

Fig. 4. CH_4 production and inhibited efficiency at different humic acid (HA) dosages.

(BrES) for methanogens, biogas could not be produced, and SCOD remained in the liquid phase. Under these conditions, the SCOD level in the Group-A tests represented the maximum produced, and accumulated SCOD amounts in the batch AD system. As shown in Fig. 5, SCOD showed a waving mode (with the same changing trend in all three experiments): firstly increasing (due to hydrolytic bacteria) and then decreasing (due to consumption by acidogenic bacteria, and also being bound by HA, owing to the electrostatic force and sweep flocculation by its functional groups) (Fernandes et al., 2015; Liu et al., 2015), in which the hydrolytic rate of the substrate could not be judged as increasing or decreasing, and the differences of SCOD between the tests were caused by dosed HA (also SCOD).

3.2.2. First-order kinetic rate

Applying Eq. (1), the first-order kinetic rate can be estimated based on the data in Fig. 3, with the results being shown in Table 5. The calculated results reveal that HA decreased the hydrolytic rate of the substrate.

3.2.3. Activity of the hydrolytic enzymes

The first-order kinetic rate only represents the hydrolytic rate of the substrate, while hydrolytic efficiency depends on the activity of the associated hydrolytic enzymes (Liu et al., 2015). The concentrations of polysaccharide and protein, and the activities of α-amylase and protease on Day 3 are shown in Fig. 6; the activities of α-amylase and proteinase slightly decreased at the beginning and

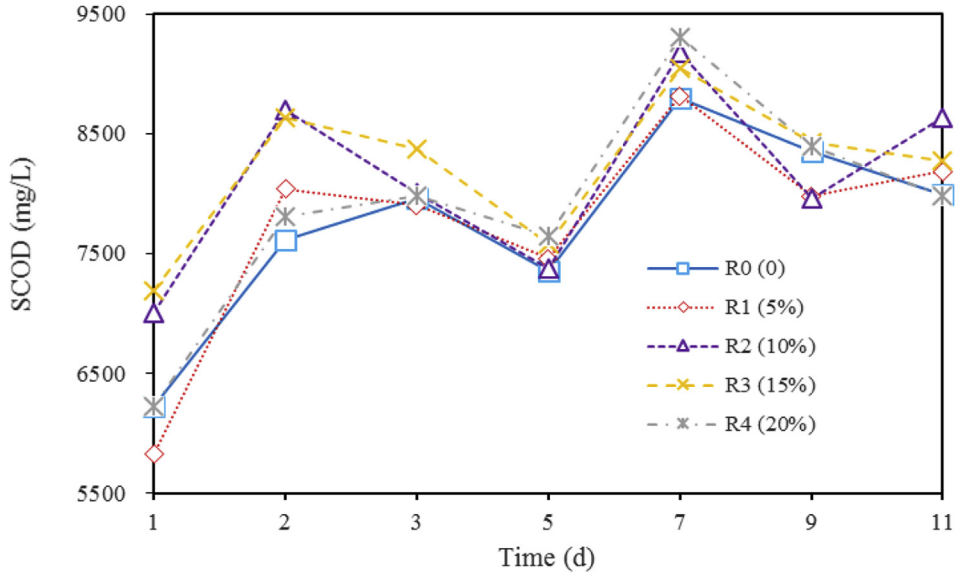

Fig. 5. Produced and accumulated soluble COD (SCOD) in the Group-A tests (with CH_4 inhibitor added).

Table 5
First-order kinetic rates calculated results by Eq. (1).

Tests	Parameters		R^2
	B_0 (mL/g VSS)	K (d^{-1})	
R0 (0)	288.1 ± 36.2	0.041 ± 0.002	0.942
R1 (5%)	261.3 ± 34.6	0.039 ± 0.003	0.947
R2 (10%)	257.8 ± 39.1	0.030 ± 0.001	0.930
R3 (15%)	230.0 ± 38.8	0.031 ± 0.040	0.923
R4 (20%)	227.7 ± 37.5	0.032 ± 0.002	0.927

then sharply decreased with increasing HA dosages. In the test of R4 (HA:VSS = 20%), the activities of the key enzymes were strongly inhibited, and the inhibited efficiencies of α-amylase and proteinase were at 59.0% and 87.6%, respectively. As shown in Fig. 6, the concentrations of both polysaccharide and protein in the R0 test are at the lowest values (almost fully hydrolyzed), while those in the R4 test are at the highest values (partly hydrolyzed).

Possible mechanisms behind the phenomena could be attributed to three pathways (as shown in Fig. 7): i) electrostatic force; ii) covalent bond; iii) sweep flocculation (Brons et al., 1985; Fernandes et al., 2015; Liu et al., 2015). In general, only a small dosage of HA would behave the impact on the enzymes by electrostatic force, which would inhibit hydrolysis (Brons et al., 1985; Fernandes et al., 2015; Liu et al., 2015). However, with increasing HA dosage, the rejection from negative charge of HA would become stronger than its hydrophobic interaction with the enzymes. HA would release the bound enzymes and mitigate the hydrolysis inhibition to some extent (Fernandes et al., 2015; Liu et al., 2015). Moreover, there are always interactions of covalent bond and sweep flocculation between HA and the enzymes (Fernandes et al., 2015; Liu et al., 2015). When HA dosage increases to a certain level, the dominant

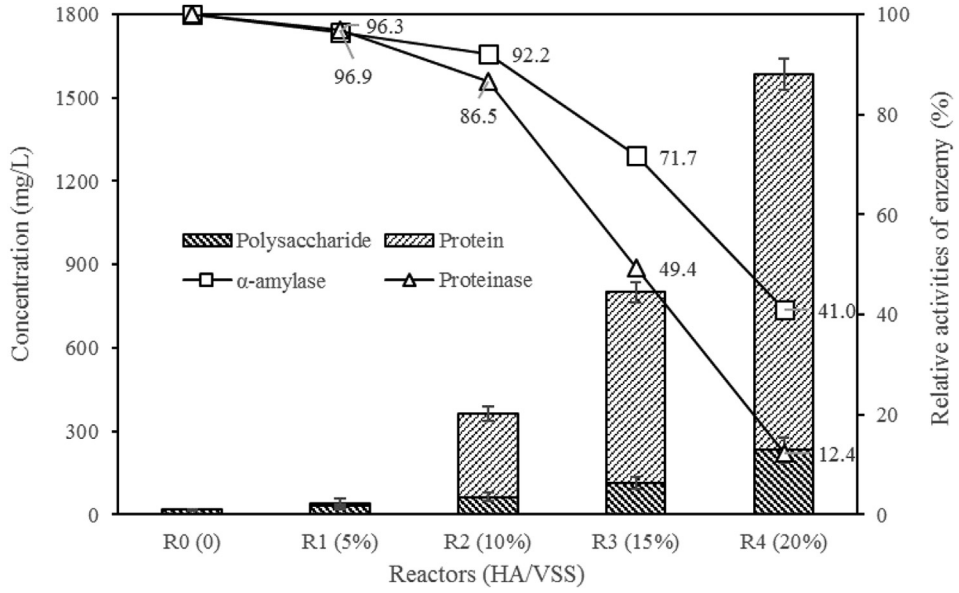

Fig. 6. Concentrations of polysaccharide and protein vs. activities of associated enzymes on Day 3.

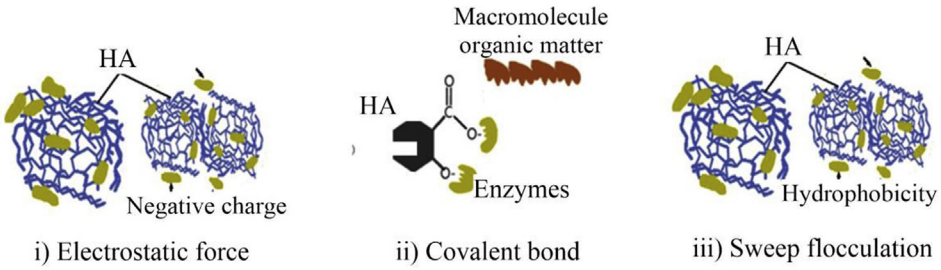

Fig. 7. Possible mechanisms of humic acid (HA) inhibiting the activities of associated enzymes.

interaction would become covalent bond, which would cause the strong inhibiting effect on the hydrolytic phase due to its irreversibility.

3.2.4. Hydrolytic efficiency

HA also contributed to SCOD (refractory) when added to the testing bottles, and so the different HA dosage in the tests contributed to the concentrations of SCOD in the supernatants. An accurate measurement of hydrolytic efficiency must exclude this part of SCOD, and thus the changed values of SCOD (ΔSCOD) from the starting point (30 min) to the ending point (Day 11) were taken, as shown in Fig. 8. Calculated by Eq. (3), the hydrolytic efficiency in the R0 test is at Top 1 (10.2%), followed by R4 (10.1%), R1 (9.7%), R2 (9.3%), and R3 (6.3%), which confirms that HA can indeed inhibit the hydrolytic process above a certain level of HA (Moura et al., 2007; Feng et al., 2008; Azman et al., 2017). Interestingly, the hydrolytic efficiency increased with HA:VSS = 20% in R4, which can be attributed to the increase in electrostatic repulsion by the ionization process from the functional groups of carboxylic acid and phenolic hydroxyl. As a result, the organics bound by HA were released into the liquid phase and the inhibition of HA on the hydrolytic phase was weakened (Feng et al., 2008; Long et al., 2017).

3.3. Effect of HA on the acidogenic phase

3.3.1. ORP

The monitored ORP and pH values in the Group-A tests are shown in Fig. 9. The initial ORP values were all in the region of −425 mV, which reveals that HA had no biochemical function (for changing ORP) at the start of the process (Polak et al., 2009). However, the ORP situation gradually changed as particular organics were oxidized to small molecule organics and VFAs, which led to ORP suddenly increasing on Day 2 and stabilizing on Day 5 (also with decrease in pH levels). The changing OPR values meant that acidification was occurring, and a high HA content corresponded to a low ORP (Fig. 9). In short, use of HA led to a decrease in ORP.

Clearly, HA influenced the biochemical conversion after the tests started. In fact, the functional groups of HA (quinone and other nitrogen & sulfur groups, etc.) make good electron transferring chains (ETC), and play a key role as electron transport agents in the acidogenic phase, similar to the function of the catalyst (HA as an electron transferring carrier) on accelerating the reaction rate. For this reason, HA can promote the electron transferring ability of organic conversion in the acidogenic phase, and also change the oxidation-reduction quality of the system, after which it causes a decrease in ORP as the HA dosage is increased.

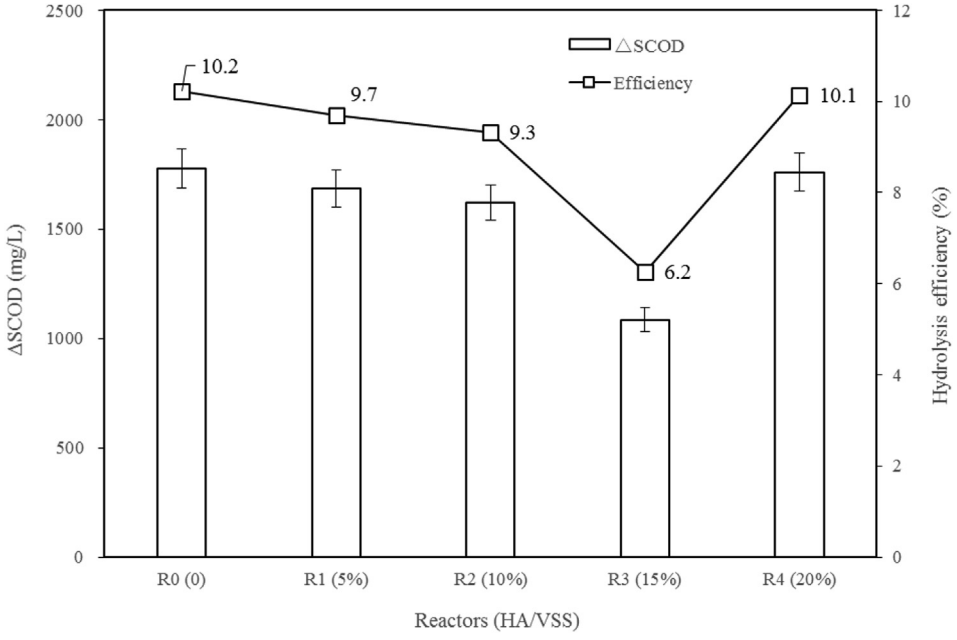

Fig. 8. ΔSCOD concentrations and the hydrolytic efficiency in the Group-A tests (with CH_4 inhibitor added).

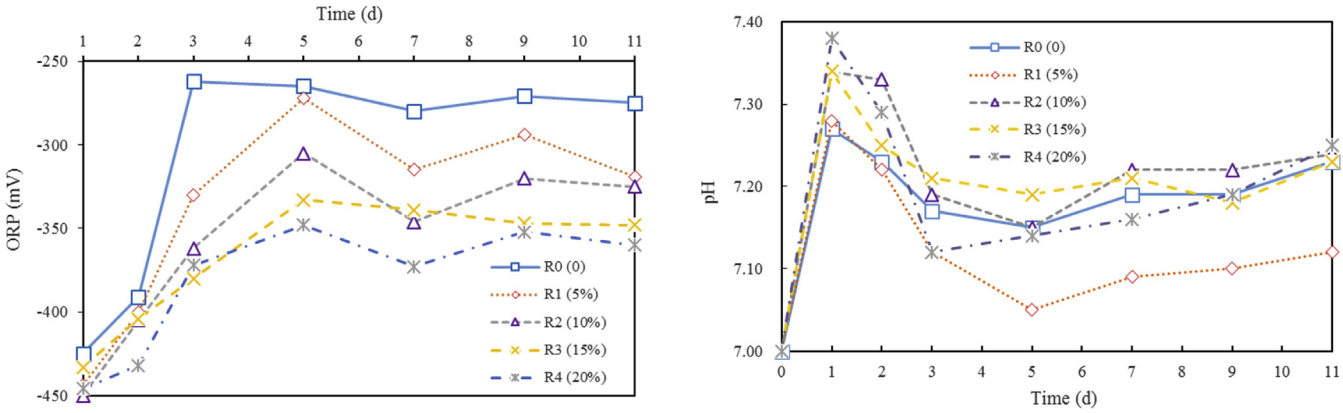

Fig. 9. Changing trend of ORP and pH in the Group-A tests (with CH_4 inhibitor added).

3.3.2. VFAs and activities of key enzymes

Variable ORP meant different acidogenic efficiencies. The changing concentrations of VFAs (acetate, propionate, butyrate, isobutyrate, valerate and isovalerate) are shown in Fig. 10. Due to the inhibition of BrES in the conversion of VFAs into CH_4, VFAs accumulated with increasing HA dosage. On Day 7 (Fig. 10), the total VFAs (TVFA) concentration in the R0-R4 tests reached up to 1145–1325 mg/L, with the increased percentages in the R1-R4 tests respectively at 8%, 11.3%, 13.4% and 15.7%, compared to the R0 test. HA existing in the batch AD system was able to stimulate the acidogenic phase.

On Day 11, the acidogenic process reached its maximum levels (1193–1386 mg/L). Compared to R0, the maximal acidification efficiency in R4 increased by 13.8%. Although there were no important differences in the accumulated VFA contents at different HA dosages, the time it took to reach peak levels was quite different – a high HA dosage corresponded to a fast acidification rate. As described above, the polysaccharides and proteins (the substrate of acidification) formed from the hydrolytic were also different in the Group-A tests. We can see, therefore, that there were both negative and positive different effects of HA dosage: i) inhibiting the hydrolytic phase; ii) stimulating the acidogenic phase. Finally, it is noteworthy that the two effects on TVFA increased along with HA dosing.

Various VFA concentrations steadily increased along with HA dosing, as shown in Fig. 11. Higher acetate and propionate concentrations in the dosed HA tests demonstrate that HA can indeed stimulate the acidogenic phase. From Day 9 to Day 11, surprisingly, propionate in R2, R3 and R4 gradually decreased and acetate correspondingly increased. For the stimulation mechanism of HA on the acidogenic phase, there are two possible explanations:

1) HA directly changed the activities of enzymes or microorganisms and then stimulated the acidogenic phase. In fact, as shown in Table 6, the associated key enzymes (POR, AK, PTA, BK, PTB, etc.) underwent no significant changes in any of the tests ($P > 0.05$). Moreover, as shown in Fig. 12, FISH was applied to analyze the impact of HA on the microbial community. The sum of integral optical density (IOD) was used to represent the abundance of microorganisms instead of average IOD. The

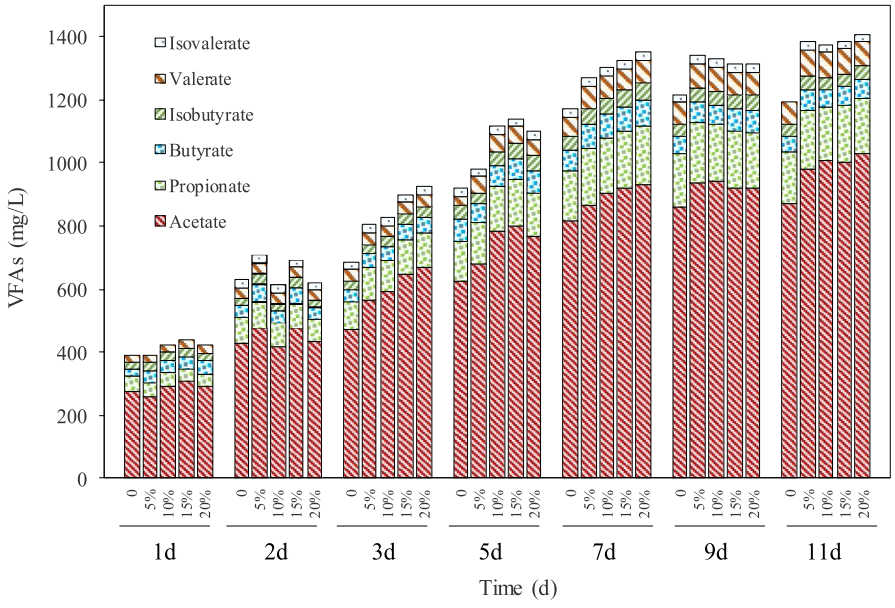

Fig. 10. Changes of VFAs concentrations in the Group-A tests (with CH_4 inhibitor added).

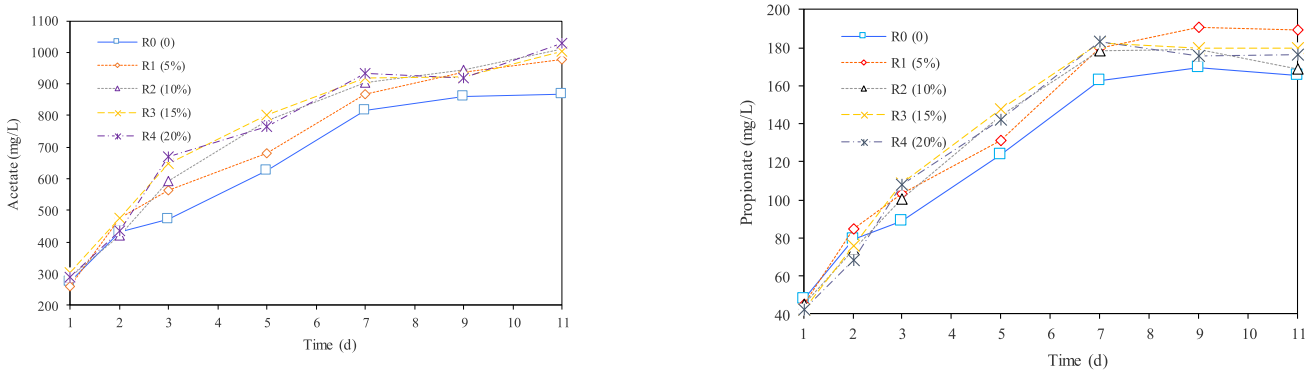

Fig. 11. Changing concentrations of acetate and propionate in the Group-A tests (with CH_4 inhibitor added).

Table 6
Relative activities of the associated enzymes in the acidogenic phase.

Enzymes	Tests (relative activities, %)					P
	R0 (0)	R1 (5%)	R2 (10%)	R3 (15%)	R4 (20%)	
POR	100	97.1 ± 1.2	96.0 ± 3.4	96.1 ± 5.5	103.7 ± 3.2	0.47
AK	100	101.0 ± 1.4	92.9 ± 3.5	100.6 ± 5.3	90.9 ± 0.4	0.15
PTA	100	85.0 ± 4.7	95.8 ± 2.6	88.0 ± 2.4	92.5 ± 3.15	0.08
BK	100	85.6 ± 5.0	86.0 ± 1.4	87.7 ± 1.8	92.1 ± 3.5	0.07
PTB	100	80.1 ± 9.5	90.1 ± 4.0	110.3 ± 5.0	96.9 ± 3.7	0.12

results illustrate that the microorganisms involved in the acidogenic phase (FISH) also behaved no significances. For this reason, HA seemed not to simulate acidification by changing the activities of enzymes.

2) HA could stimulate acidification as an active terminal electron acceptor (TEA) or the electron transferring chains (ETC) from the functional groups (quinone, etc.). In the normal acidogenic phase, there are two obstacles which inhibit acidification: i) non-methanogens, like fermenting bacteria, lack the electron transport system and have to release "surplus electrons" produced by the fermenting process to other substrates, otherwise the acetate produced process would be hindered; ii) the thermodynamics of bio-conversions indicates that the Gibbs free energy ($\triangle G0'$) of different VFAs converted into acetate is over zero ($\triangle G_0'>0$), as shown in Table 7 (Thauer et al., 1977; Cervantes et al., 2000), which means that the bio-conversions occur spontaneously by controlling the hydrogen pressure, otherwise they would consume outer energy and hinder their metabolisms. When HA was added in the tests, for example, anthraquinone-2,6-disulfonnat (AQDS, a HA quinone analogue) would not only act as TEA for accepting the "surplus electrons" from propionate; the bio-conversion could also lead to a spontaneous reaction ($\triangle G_0'<0$), after which the hydrogen pressure in the tests would no longer be a decisive factor. Moreover, decreased ORPs could indirectly stimulate the conversion of VFAs. So we can see that HA, therefore, had the positive effect on the acidogenic phase of improving the acidogenic efficiency.

3.3.3. *Acidogenic efficiency*

The acidogenic efficiency was calculated by Eq. (4) and the results are shown in Fig. 13. The acidogenic efficiencies (78%–124%) of R1, R2, R3 and R4 are all higher than those of R0 (61.6%). Specifically, the acidogenic efficiency (124.1%) of R3 is two times higher that of R0. The acidogenic efficiency over 100% implies that not only ΔSOCD was all converted into VFAs, but some initial SCOD was also converted into VFAs.

As TEA, the capacity of HA for accepting electrons in the system could increase along with the HA dosage, which would cause

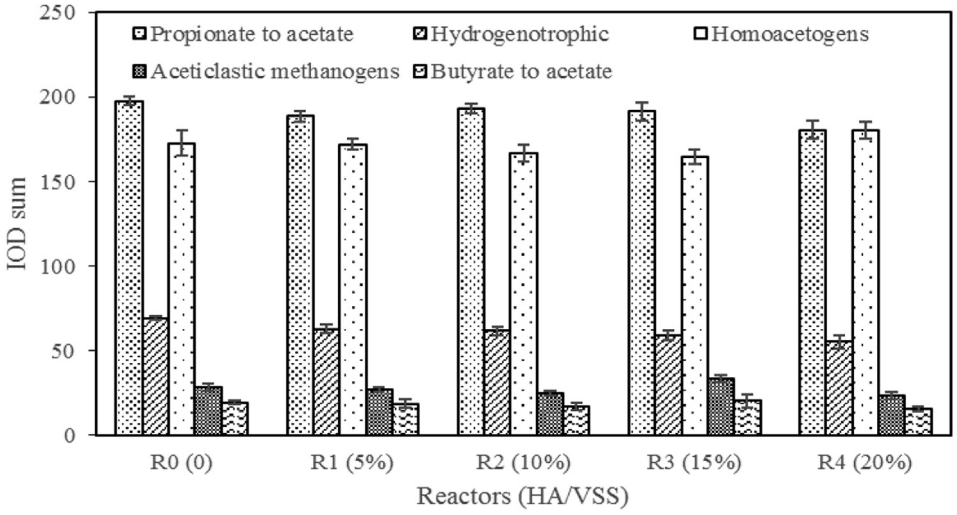

Fig. 12. Abundance of microorganisms in the acidogenic phase (FISH) in the Group-A tests (with CH_4 inhibitor added).

Table 7
Gibbs free energy of different VFAs converted into acetate in the acidogenic phase.

Substances	Bioreactions/Equations	$\triangle G^{0'}$(kJ/mol)	No.
Propionate	$CH_3CH_2COOH+2H_2O \rightarrow CH_3COOH+3H_2+CO_2$	+76.1	(7)
Butyrate	$CH_3CH_2CH_2COOH+2H_2O \rightarrow 2CH_3COOH+2H_2$	+48.1	(8)
Valerate	$CH_3CH_2CH_2CH_2COOH+2H_2O \rightarrow CH_3COOH + CH_3CH_2COOH+2H_2$	+69.8	(9)
Propionate + HA	$CH_3CH_2COOH+3AQDS+2H_2O \rightarrow CH_3COOH+3AH_2QDS + CO_2$	−57.1	(10)

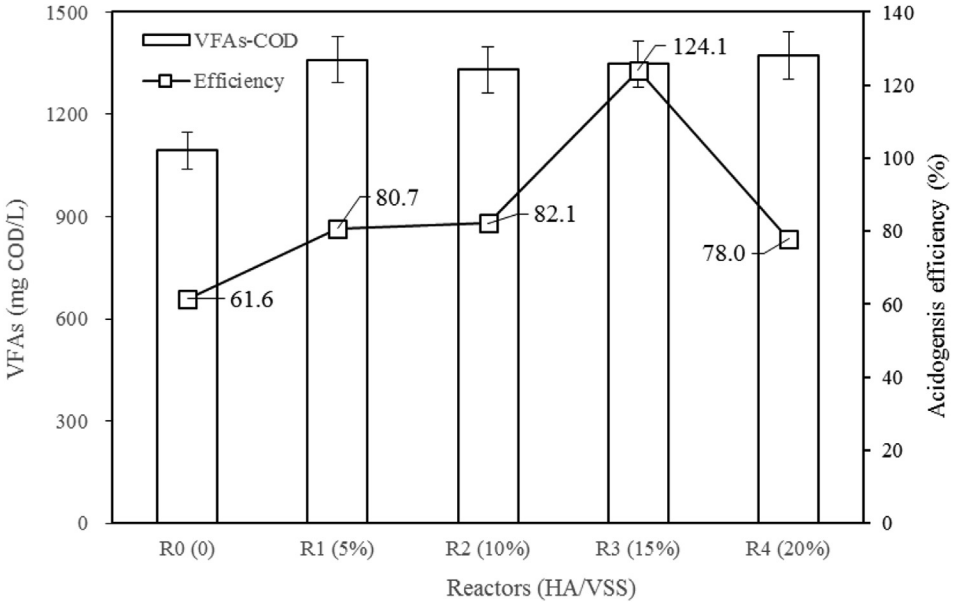

Fig. 13. Maximal VFAs production and the acidogenic efficiency in the Group-A tests (with CH_4 inhibitor added).

higher levels of acidogenic efficiency. As shown in Fig. 13, however, the acidogenic efficiency at the highest HA dosage (R4; HA:VSS = 20%) did not reach the highest level (below 124% in R3). Table 7 indicates that 1 mol of propionate can directly be converted into 2 mol of acetate, at least in theory. With HA involved in the tests, however, only 1 mol of acetate could be acquired. In other words, the highest HA dosage did not correspond to the highest acidogenic efficiency, as most of hydrolytic organics are likely to be converted into CO_2, not into acetate.

3.4. Effect of HA on the methanogenic phase

3.4.1. Activities of key enzymes and microorganisms

The methanogenic phase was analyzed in a whole batch AD system (Group M_G). The activities of a key enzyme (F_{420}-reducing hydrogenase) are shown in Table 8. The relative activities of F_{420} are at 100% (R0), 87.5% (R1), 81.0% (R2), 70.8% (R3) and 63.2% (R4), respectively. Clearly, HA is able to lower the activities of the key enzyme and inhibit the methanogenic phase. The abundance of the involved fermentative bacteria (measured by FISH) is illustrated in Fig. 14; there are minor differences among different tests, revealing almost no impact of HA on the involved microorganisms.

Table 8
Impact of humic acids (HA) on the activity of co-enzyme F_{420} (key factor in methanogenesis) in the batch AD system.

	Relative Activity (%)					P
	R0 (0)	R1 (5%)	R2 (10%)	R3 (15%)	R4 (20%)	
F_{420} - activity	100	87.5 ± 2.2	81.0 ± 0.7	70.8 ± 0.6	63.2 ± 1.9	0.00

HA could inhibit the methanogenic phase via two pathways: i) as a terminal electron acceptor (TEA), HA directly accepts the electrons from acetate and prevents them from being converted to CH_4 (Eq. 12 in Table 9). Although $\triangle G_0'$ for the direct oxidation of HA with acetate (Eq. 12) and for the conversion of acetate to CH4 (Eq. 11) are all less than zero (spontaneous reactions), Eq. 11 is more successful with the help of methanogenic bacteria in the system, while Eq. 12 is based on the respiration of special functional bacteria (humic substance reducing bacteria, etc.(Lovley et al., 1996)). Therefore, this inhibiting pathway was probably not relevant in our systems, since the cultures were grown in absence of HA (Cervantes et al., 2000); ii) HA can resemble a metabolic intermediate substance and hinder the successful transformation of acetate to CH_4, and HA might compete for metabolites, which could lead to the normal methane pathway being blocked (Liu et al., 2016).

As for the hydrogenotrophic methanogenesis, the reaction rate of H_2 with CO_2 (Eq. 13 in Table 9) could be much faster than that of H_2 with HA (Eq. 14). The impact of HA on this bioreaction is not as yet clear. Moreover, other studies (Smith et al., 2015; Dang et al., 2016) conclude that HA might promote the metabolisms of other syntrophic bacteria and cause the failure of methanogens competition in the AD system, instead of decreasing CH_4 production (Li et al., 2015; Azman, 2016). Despite progress made it is clear that a further study is still needed to address this question.

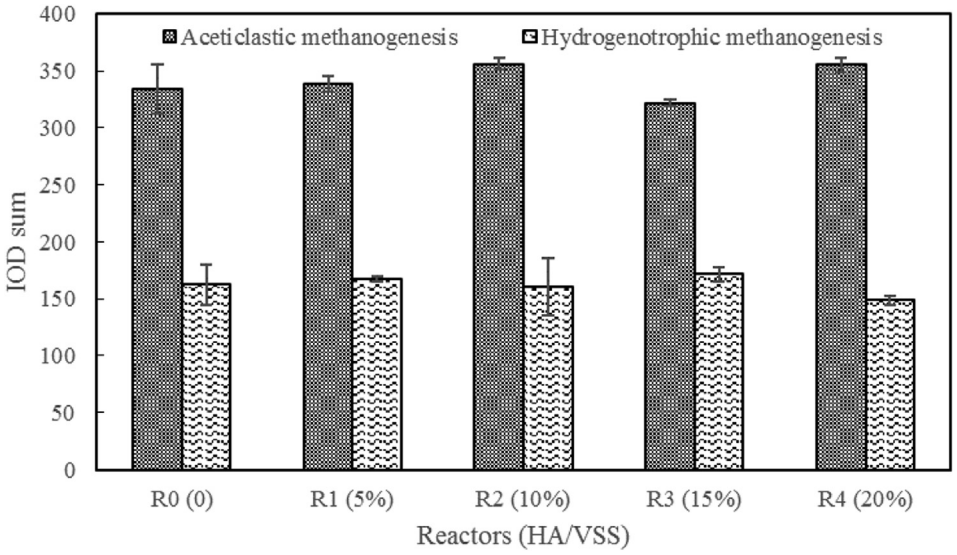

Fig. 14. Abundance of the aceticlastic and hydrogenotrophic methanogenesis (measured by FISH) in the methanogenic phase in the Group-M_G tests (with no CH_4 inhibitor dosed).

Table 9
Associated reactions of different substrates in the methanogenic phase (Thauer et al., 1977; Cervantes et al., 2000).

Substances	Bioreactions/Equations	$\triangle G^{0'}$(kJ/mol)	No.
Acetate	$CH_3COOH \rightarrow CH_4+CO_2$	−31	(11)
Acetate + HA	$CH_3COOH+4AQDS+2H_2O \rightarrow 4AH_2QDS+2CO_2$	−73	(12)
Hydrogen	$4H_2+CO_2 \rightarrow CH_4+2H_2O$	−135.6	(13)
Hydrogen + HA	$H_2+AQDS \rightarrow AH_2QDS$	−44.4	(14)

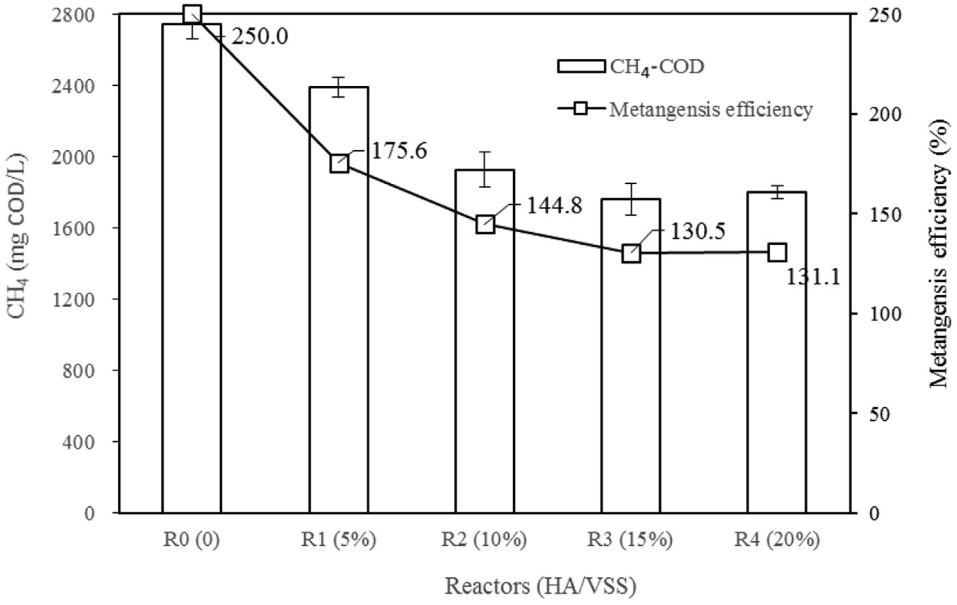

Fig. 15. CH_4 production and methanogenic efficiency (based on Eq. (6)).

3.4.2. Methanogenic efficiency

The methanogenic efficiency (calculated by Eq. (6)) is shown in Fig. 15. In the R0 test this reached up to 250.0%, for which not only all VFAs but also other organics were involved (accounting for about 150%). In addition, the methanogenic efficiency in R1, R2, R3 and R4 reached 131%–176%, but they are noticeably lower than in R0, revealing that HA had indeed inhibited the CH_4 production, with a maximal reduced efficiency of up to 120% (R3). In other words, the methanogenic efficiency was suppressed by approximately half (52.2%, calculated by Eq. (3)).

3.5. Summary of the possible mechanisms

The possible mechanisms of HA inhibition in the batch AD

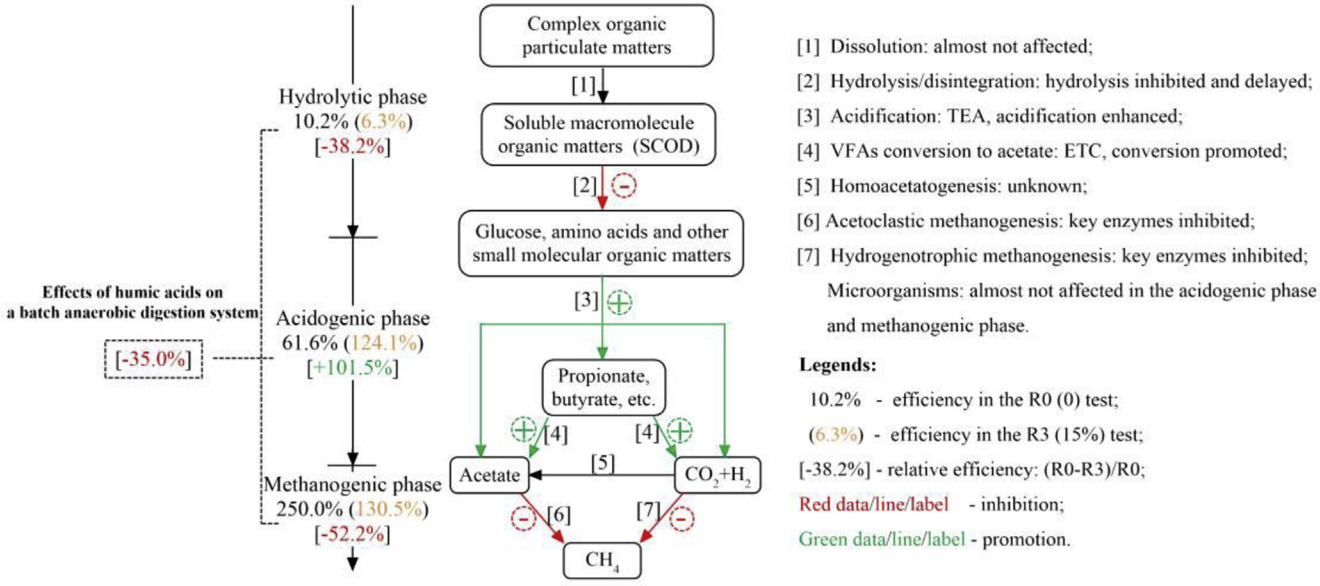

Fig. 16. Possible mechanisms of humic acids (HA) inhibiting the batch anaerobic digestion (AD) system.

system are shown in Fig. 16; the red data/line/label represents the negative effects of HA on the AD process and the green data/line/label stands for the positive effects. HA behaved differently in the three phases. During the hydrolysis phase, the activities of the associated key enzymes were lower and the hydrolytic process was inhibited. The hydrolytic efficiency in the test without HA dosage was at 10.2%, while the test with HA dosed (HA:VSS = 15%) decreased to 6.3%, accounting for an inhibition efficiency up to 38.2%. In the acidogenic phase, HA acted as terminal electron acceptors (TEA) or electron transferring chains (ETC) to promote the acidogenic process, and the acidogenic efficiency increased to 124.1% (HA:VSS = 15%) from 61.6% (HA:VSS = 0), elucidating a promotion efficiency up to 101.5%. In the methanogenic phase, however, HA decreased the methanogenic efficiency by 52.2% (250% at HA:VSS = 0 was lowered to 130.5% at HA:VSS = 15%) due to inhibiting the activities of the involved key enzymes. The FISH results demonstrate that HA had almost no effect on the involved microorganisms (by FISH) in both acidogenic and methanogenic phase with HA dosed.

Overall, the combined efficiency of the three sub-phases without HA dosing is calculated at 10.2 % × 61.6 % × 250% = 15.7%, which is in accordance with the calculated result by Eq. (2). As for the test with HA:VSS = 15%, the combined efficiency of the three sub-phases is at 6.3 % × 124.1 % × 130.5% = 10.2%. Finally, the whole inhibition efficiency of the three sub-phases can be calculated by Eq. (3) at (15.7%-10.2)/15.7% = 35.0%, which is almost identical (35.1%) to the result observed in the single-phase AD process (Section 3.1).

4. Conclusions

Anaerobic digestion (AD) of excess sludge can convert contained organics into energy (CH_4), but the existence of humic acid (HA) contained in sludge acts to create a negative effect on the energy efficiency. In this study, a single-phase and three sub-phases (hydrolytic phase, acidogenic phase and methanogenic phase) of AD were respectively tested and analyzed without and with HA dosing in the batch AD systems. In the single-phase AD, dosed HA did indeed inhibit the methanogenic efficiency (by 35.1% at HA:VSS = 15%).

Due to the complex chemical structural characteristics, however, the effects of HA on the three sub-phases was totally different. HA resulted in lowering the activities of the associated enzymes and also the first-order kinetic rates in the hydrolytic phase, acting as an inhibiting agent (by 38.2% at HA:VSS = 15%). In the acidogenic phase, HA acted in the role of ETC or TEA, stimulating acidification and resulting in a promoting function (by 101.5% at HA:VSS = 15%). Finally, in the methanogenic phase, HA inhibited a key enzyme (F_{420}) and reduced the CH_4 production (by 52.2% at HA:VSS = 15%).

Overall, the combined efficiency of the three sub-phases without HA dosing is calculated at 15.7%, and that of the three sub-phases at HA:VSS = 15% at 10.2%. Finally, the whole inhibition efficiency of the three sub-phases is equal to 35.0%, which is almost identical (35.1%) to the result observed in the single-phase AD process.

Agreement

All authors have seen and approved the final version of the manuscript. They warrant that the article is the authors' original work, has not been published prior and is not under consideration for publication elsewhere.

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