Novel Stepwise pH Control Strategy to Improve Short Chain Fatty Acid Production from Sludge Anaerobic Fermentation

Jianwei Zhao [a,b,c], Dongbo Wang [b,c], Yiwen Liu [a], Huu Hao Ngo[a,*], Wenshan Guo[a], Qi Yang[b,c] Xiaoming Li [b,c]

[a] Joint Research Centre for Protective Infrastructure Technology and Environmental Green Bioprocess, School of Civil and Environmental Engineering, University of Technology Sydney, Ultimo, NSW 2007, Australia and Department of Environmental and Municipal Engineering, Tianjin Chengjian University, Tianjin 300384, China.

[b] College of Environmental Science and Engineering, Hunan University, Changsha 410082, P.R. China

[c] Key Laboratory of Environmental Biology and Pollution Control (Hunan University), Ministry of Education, Changsha 410082, P.R. China

* Corresponding author: E-mail: ngohuuhao121@gmail.com

Abstract: This study reports an innovative strategy known as stepwise pH fermentation, developed to enhance the production of short chain volatile fatty acids (SCFA) from waste activated sludge (WAS) anaerobic fermentation. Experimental results confirmed the optimal pH for WAS disruption and acidification was 11 and 9, respectively, and corresponding optimal time was, respectively, 5 d and 2 d. In this scenario, the optimal SCFA yield was 2356 mg chemical oxygen demand (COD)/L, which was much higher than that derived from alkaline fermentation system. Investigation of the mechanism indicated that pH11 could accelerate the disruption of WAS and inhibit the activities of methanogens; furthermore, pH9 was beneficial to the activity of acid-producing bacteria, resulting in more SCFA production. Stepwise pH fermentation integrated with sodium chloride (NaCl) present in WAS had synergistic impacts on WAS anaerobic fermentation.

Keywords: Waste activated sludge, Stepwise pH fermentation, Short chain volatile fatty acids, Sodium chloride

1. Introduction

Waste activated sludge (WAS), the main byproduct of the wastewater treatment plant (WWTP), is produced with substantial quantities daily. Studies reported recently that annual output of sludge in China was up to 34 million metric tonnes (80% water content), and with continuous development of urbanization process, the annual output of WAS will be greater in the future (Wang et al., 2015; Luo et al., 2016; Zhao et al., 2015a). On one hand, WAS contains a large number of pathogens, organic contaminants and heavy metals, which pose a serious risk to environmental ecology and human health if disposed improperly. On the other hand, WAS contains high levels of organic substance (for example, protein, polysaccharide and lipid, etc.), so consequently WAS is also a valuable renewable resource. However, around 80% of WAS is squandered in

many WWTPs via incineration or landfill (Luo et al., 2016). Therefore, how to effectively recover energy from WAS and achieve the reduction and harmless of WAS simultaneously have attracted growing scientific interest (Zhao et al., 2015b; Yang et al., 2015).

Anaerobic digestion technology is widely considered to be a promising and sustainable technology for sludge treatment because of its cost-effectiveness and energy recovery (Feng et al., 2014; Zhao et al., 2016ab; Zhao et al., 2017a). Anaerobic digestion can bioconvert the organic matter in WAS into valuable bioproducts such as short chain fatty acids (SCFA), hydrogen, methane, etc (Zhao et al., 2016c). SCFA production from WAS anaerobic fermentation has attracted increasing attention, because the value added SCFA can be used as sewage treatment plants' preferred carbon source directly to improve the efficiency of biological nutrient removal (Tong and Chen 2007, Zhao et al., 2016d). Normally, WAS is composed of a polymeric network formed by extracellular polymeric substances (EPS) and microbial cells that impede the release and further use of intracellular organic matter since cell walls and EPS present physical and chemical barriers (Zhang et al., 2016; Zhao et al., 2016c; Zhao et al., 2017c). Pretreatments are often employed to destroy sludge flocs to accelerate the sludge hydrolysis process (Zhen et al., 2017; Maspolim et al., 2015).

Widely applied pretreatment methods include mechanical, chemical, biological and their combination (Ariunbaatar et al., 2014; Alagöz et al., 2015; Ma et al., 2011). Of these alkaline fermentation has generated much interest because it can accelerate the hydrolysis of sludge while inhibit the activities of methanogens, resulting in the accumulation of SCFA (Zhang et al., 2010; Wu et al., 2010; Li et al., 2014). It is well known that at least three major classes of microorganisms (hydrolyzed acidified bacteria, homogeneous acetic acid bacteria, and methanogenic bacteria) participated synergistically in the process of sludge digestion, and their sensitivities to pH vary greatly with microbial species (Liang et al., 2014; Kwietniewska and Tys 2014). For example, an alkaline environment contributes to the destruction of sludge flocs and the release of extracellular

enzymes to further accelerate the cleavage of cell walls, while alkaline environments inhibit the growth of *Methanobacterium sp.* and *Methanobrevibacter sp.* (Zheng et al., 2013). A neutral environment benefits the growth and SCFA accumulation of a pure acetogenesis strain such as *Moorella thermoacetica*, which is the most metabolically diverse acetogen (Drake and Daniel 2004). As well, neutral pH favors enzyme catalysis and microbial activity.

Alkaline fermentation promoted the disintegration and hydrolysis of organic matter in WAS, resulting in sufficient biodegradable substrates for acidogenic microorganisms, and inhibited the activities of methanogenic *archaea*. Meanwhile, the alkaline pH greatly curtailed the growth and activities of acidogenic bacteria (Ma et al., 2016). It is evident that alkaline fermentation needs to maintain a high level of pH, resulting in substantial consumption of chemical reagents, which is not economically viable in small-scale WWTP. Considering the characteristics of different responses of anaerobic microorganisms to pH, stepwise pH fermentation may further improve the production of SCFA in comparison to alkaline fermentation. To date, however, this novel hypothesis has not been experimentally verified.

Operational conditions of the digester and characteristics of WAS are the two basic parameters affecting the production of SCFA from WAS fermentation (Wang et al., 2015; Luo et al., 2016). However, most research to date focused on the effect of operational conditions (such as temperature, sludge residence time, pretreatment) on sludge fermentation. For instance, Li et al. (2016) reported free nitrite acid serving as pretreatment could enhance the sludge hydrolysis process, thereby providing a more available digestive matrix for acid-producing bacteria. Li et al. (2014) investigated the influence of temperature on SCFA production from alkaline fermentation, and the experimental results showed that the optimal temperature for SCFA production was 35 ℃, and the acid production was 319.8 mg/g volatile suspended solid (VSS) L d. Apart from the external operating conditions of WAS fermentation, the characteristics of WAS are also one factor

affecting SCFA yield. However, currently little attention has been paid to this topic.

Sodium chloride (NaCl) is widely used in the chemical and food industries and fisheries, leading to high levels of salinity (1%~5%) wastewater being produced (Su et al., 2016). Some coastal cities use seawater to flush toilets due to the lack of fresh water sources, which constituted one important factor resulting in high concentrations of salt wastewater. The high level of salt wastewater can be discharged into the WWTP, and NaCl is further adsorbed or transferred into sludge. The presence of NaCl can affect the production of hydrogen and methane from sludge anaerobic fermentation and/or digestion (Zhao et al., 2016c; Cui et al., 2015). NaCl can severely inhibit the growth of methanogens, and stimulate the production of extracellular polymeric substances (Dimroth and Thomer 1989; Ma et al., 2012). It was documented that the presence of NaCl accelerates the release of soluble proteins and polysaccharides, yet seriously inhibited the activities of methanogens (Su et al., 2016; Jin et al., 2016). A floc disintegration characterized as the decreased floc size and increased porosity in the floc matrix was observed when NaCl was added into WAS. Additionally, the presence of NaCl caused a slight decrease in normalized capillary suction time (Cui et al., 2015). All of the above observations indicated that NaCl benefits the disintegration of sludge flocs and the release of organic matters. Whether NaCl's presence integration with stepwise pH fermentation cause positive synergies on WAS fermentation remains as yet unknown.

Therefore, the main objective of this study is to report a novel strategy for the enhanced production of SCFA from WAS anaerobic fermentation utilizing stepwise pH fermentation. Firstly, the optimal pH and time for WAS disruption and acidification are determined. Secondly, the mechanisms for the stepwise pH fermentation improving the production of SCFA were explored by analyzing the variations of soluble chemical oxygen demand (SCOD), protein, polysaccharide, and key enzyme activities. Finally, the synergistic effect of stepwise fermentation integrated with NaCl on each step in WAS anaerobic fermentation

was confirmed. This is the first study confirming that stepwise pH fermentation can greatly promote SCFA production from WAS fermentation. The findings achieved here have the potential to: firstly, help the recovery of SCFA from WAS anaerobic fermentation; and secondly, have significant implications for WAS operations in real situations.

2. Materials and methods

2.1 Characteristics of WAS

The WAS used in this study was obtained from the secondary sedimentation tank of a WWTP located in Changsha, China. WAS was first filtered through a 0.5 mm × 0.5 mm gauze to remove the insoluble materials and then stored in a refrigerator at 4℃. The main characteristics (average value plus standard deviation of three tests) of WAS are presented as follows: pH 6.8 ± 0.1, total suspended solids (TSS) 13320 ± 230 mg/L, volatile suspended solids (VSS) 8400 ± 210 mg/L, total chemical oxygen demand (TCOD) 13260 ± 230 mg/L, soluble COD 130 ± 20 mg/L, total protein 6512 ± 230 mg/L, total carbohydrate 1230 ± 210 mg/L, total lipids 358 ± 13 mg/L, NH_4^+-N 29 ± 2 mg/L, PO_4^{3-}-P 33 ± 2.3 mg/L.

2.2 Optimization of pH and time for WAS disruption

Batch experiments were carried out to optimize the pH and time for WAS disruption in six identical serum bottles with a working volume of 600 mL each. Each bottle first received 600 mL of the above-mentioned WAS. Because an alkaline condition benefits the accumulation of SCFA, the pH of each reactor was controlled at 7.0, 8.0, 9.0, 10.0, 11.0, and 12.0, respectively, by adding 4.0 M sodium hydroxide (NaOH) or hydrogen chloride (HCl) via an automatic titrator. Afterwards, all serum bottles were flushed with high purity nitrogen for 60 s to remove oxygen, and then sealed. It should be noted that no inoculum was added in this study, so the WAS served as both fermentation substrate and inoculum. Finally, all serum bottles were placed in an air-bath shaker (120 rpm) at 37 ± 1 ℃. The above experiment was repeated three

times, and the experimental results reported are the average of these three times.

2.3 Optimization of pH and time for sludge acidification

The batch experiments were conducted in twelve identical serum bottles, and the twelve serum bottles were first divided into two Groups (Group-□ and Group-□) with six in each group. Then each serum bottle of the two Groups received 600 mL of WAS, and the pH in Group-□ and Group-□ were, respectively, controlled at pH 11 and 12 for 5 d according to the results reported in Section 2.2. Afterwards, at the fermentation time of 5 d, the pH in the six serum bottles of each Group was adjusted to 7.0, 8.0, 9.0, 10.0, 11.0, and 12.0, respectively, by adding 4.0 M NaOH or HCl via an automatic titrator. Finally, all serum bottles were flushed with high purity nitrogen for 60 s to remove oxygen, sealed, and then placed in an air-bath shaker (120 rpm) at 37 ± 1 ℃. All other operating conditions are similar to those described above.

2.4 Comparison of the influence of stepwise pH fermentation and alkaline fermentation on hydrolysis acidification, and methonogenesis processes using model substrates

The rates of hydrolysis, acidification, and methonogenesis can be expressed by the degradation rates of the model compounds in synthetic wastewater. Therefore, it is necessary to compare the effect of stepwise pH fermentation and alkaline fermentation on of the processes of hydrolysis, acidification and methanogenesis using model substrates. Nine replicate serum bottles with a working volume of 1.0 L each containing synthetic wastewater were used for the experiment. Nine serum bottles were first divided into three Tests (Test-□, Test-II, and Test-□) with three bottles in each test. Test-□ was used to compare the influences of stepwise pH fermentation and alkaline fermentation on the process of hydrolysis, whereas Test-II and Test-□ were employed to compare the effects of stepwise pH fermentation and alkaline fermentation on acidification and methanation, respectively. Each serum bottle in each test received 100 mL of the inoculum and 900 mL of synthetic wastewater, and the information concerning the synthetic wastewater is described in more detail

below. The inoculum was taken from an anaerobic reactor for the treatment of sludge in the laboratory and the main characteristics of the inoculum are presented in supporting information. Then the pH of one serum bottle (defined as alkaline fermentation) was controlled at pH 10 by adding 4.0 M NaOH or HCl via an automatic titrator. The pH level in another serum bottle (defined as stepwise pH fermentation) was first maintained at 11 for 5 d and then controlled at 9 for the remaining fermentation time. The pH in the last serum bottle was not controlled throughout the fermentation process serving as a blank. Afterwards, all bottles were flushed with nitrogen gas for 60 s to ensure the anaerobic state was maintained. Following this, all those serum bottles were capped with rubber stoppers, sealed, and then placed in an air-bath shaker (120 rpm) at 37 ± 1 ℃

Test-□: The synthetic wastewater of Test-□ contains 4.34 g/L bovine serum albumin (BSA, average molecular weight 67000, model protein compound) and 1.16 g/L dextran (average molecular weight 23800, model polysaccharide compound). The contents of BSA and dextran were similar to the contents of protein and polysaccharide in the raw WAS. By analyzing the model compounds' degradation rates in synthetic wastewater, the effects of stepwise fermentation and alkaline fermentation on the hydrolysis can be evaluated.

Test-II: the model compound L-alanine (model amino acid compound) and glucose (model monosaccharide compound) were used to replace BSA and dextran.

Test-□: The synthetic wastewater of Test-□ contains 1.0 g/L acetate.

2.5 Synergistic effect of stepwise fermentation integrated with salinity on SCFA production

Recently, a large amount of salinity was detected in sludge due to the development and utilization of seawater. The level of salinity in the municipal sludge was 0.6~1.6% in terms of mass fraction (Chen and Leung. 2000; Jin et al., 2016). The initial concentration of NaCl in WAS was about 3.6 mg/L, and the WAS used in this experiment was washed three times with tap water to eliminate the inherent NaCl. The initial

concentration of NaCl selected was 1% of the WAS, and the NaCl concentration was not controlled throughout the process, so that the synergistic effect of stepwise pH fermentation integrated with NaCl on WAS anaerobic fermentation could be evaluated.

Three identical anaerobic reactors each with a working volume of 1.0 L were conducted. The operating mode of one reactor (defined as stepwise pH fermentation reactor) was carried out as stepwise pH fermentation as described above. One reactor (defined as NaCl reactor) with 1% NaCl (in terms of WAS mass fraction) was also conducted; the pH was not controlled during the entire process. Another reactor (defined as stepwise pH+NaCl reactor) was run in stepwise pH fermentation mode with 1% NaCl addition. The temperature in those reactors was controlled at 37 ± 1 ℃.

In order to explore the synergetic effect of stepwise pH fermentation and NaCl on each step of WAS anaerobic fermentation, batch tests with synthetic wastewater were carried out. First, nine replicated reactors were evenly divided into three Groups (Group-□, Group-□, and Group-□) with three each. Then, the reactors in each group first received 100 mL of inoculum and 900 mL of synthetic wastewater. The operating modes of the reactor in each group were as described above. Group-□ was applied to determine the synergistic effect of stepwise pH fermentation and salinity on the process of hydrolysis, while Group-□ and Group-□ assessed the synergistic impact of stepwise pH fermentation and salinity on the processes of acidification and methanation, respectively. Detailed information regarding the synthetic wastewater was presented in Section 2.4.

2.6 Analytical methods

The sample was first centrifuged at 12000 rpm for 15 minutes and then the supernatant was filtered through a 0.45 mm membrane filter to further analyze the sludge sample's soluble fraction. Total suspended solids (TSS), volatile suspended solids (VSS), COD, soluble COD, PO_4^{3-}-P, NH_4^+-N were determined in

accordance with the standard methods (APHA, 1998). Gas chromatography was used to detect the content and composition of SCFA, and the gas chromatographic model was Agilent 6890-DB-MAXETR. The detailed inspection processes are described in the supporting information. Protein and polysaccharides were measured using Lowry-Folin and phenol-sulfuric method with BSA and glucose as the standard substrates, respectively (Herbert et al., 1971; Lowry et al., 1951). The activities of key hydrolase (protease and α-glucosidase) and acetic acid production enzyme (acetate kinase (AK) and CoA transferase) are described in SI. The determination procedure of coenzyme F420 was the same as that reported in the literature (Mu and Chen 2011; Du et al., 2017; Sun et al., 2017).

2.6 Statistical analysis

All experiments were repeated three times. An analysis of variance was employed to evaluate the significance of the results, and $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1 Effect of pH on the disruption of WAS

During the process of disruption, the organic matter (mainly protein and polysaccharide) in solid suspended solids are disrupted into semi-solid or soluble organic matter through chemical and biological processes. Soluble organic matters after disruption can be divided into two major organic substances: soluble protein and soluble polysaccharide. Disruption is a physical process that does not require the involvement of microbes and key enzymes. Therefore, the effect of pH on the disruption of the WAS was first investigated, and the variations of SCOD, soluble protein and soluble polysaccharide helped to indicate the degree of sludge disruption (Li et al., 2016; Zhao et al., 2017b). From Fig. 1, it can be seen that when pH was 7, the variation in SCOD during the investigation was not obvious, which might be attributed to the fact that the neutral pH contributed little to sludge dissolution and SCOD could be quickly consumed by

methanogens *Archaea* for biogas production. When the pH was 8 and 9, the content of SCOD increased slowly with fermentation time. However, when pH was 10, 11 and 12, the level of SCOD first indicated a sharp rise during the initial 5 d and thereafter remained stable. The content of SCOD increased when pH also increased at the same fermentation time, indicating that higher pH benefited the disruption of the WAS, which was consistent with other studies (Chen et al., 2007; Zhao et al., 2015c). The effect of different pHs on the variations of soluble protein and polysaccharide was shown in supporting information. When pH rose from 7 to 12, the levels of soluble protein and polysaccharide also revealed an increasing trend, which was consistent with previous studies (Chen et al., 2007; Song et al., 2016; Yuan et al., 2006). These results suggested that the increase of pH benefited the release of soluble organic matter. At the fermentation time of 5 d, the contents of SCOD were 2485 and 2556 mg/L when the pH was 11 and 12, respectively. After 5d, the changes in SCOD at pH 11 and 12 were insignificant ($p > 0.05$). Thus, the optimal fermentation time for WAS disruption was 5 d while the optimal pHs were 11 and 12 because the contents of SCOD were similar at such pH levels.

3.2 Effect of pH on the process of sludge acidification

The acidification process is a biological process, which requires the microbial and key enzyme catalysis. The small molecule organics produced from the hydrolysis stage are further bio-converted into simpler SCFAs-containing end products and secreted into extracellular due to the actions of acidification bacteria, and this process is defined as the acidification process. The hydrolysis process and the acidification process cannot be thoroughly divided, so therefore a certain amount of SCFA will be produced during the sludge hydrolysis stage. Fig. 2 depicts the effect of pH on the accumulation of SCFA. From Fig. 2a, it can be seen that the content of SCFA revealed a tendency of first rising and then declining in each reactor during the fermentation time of 5 to 15 d. The maximal concentration of SCFA increased from 1124 to 2356 mg/L

when the pH increased from 7 to 9. However, a further increase in pH prevented the accumulation of SCFA. For instance, the highest concentration of SCFA fell from 2356 mg/L to 1151 mg/L when the fermentation pH further increased from 9 to 12. Consequently, the optimum pH for promoting SCFA production was pH 9.

The main reason for the increase in pH from 7 to 9 promoting the accumulation of SCFA may be attributed to the weak alkaline condition having no obvious impact on the activities of acidogenic bacteria. However, it seriously inhibited the activities of methanogens because the optimum pH for methanogens was around pH 7 (Wang et al., 2015; Latif et al., 2017). Although strong alkaline pH (i.e., 11) during the first 5 d decreased the relative abundances of *Methanobacterium sp.* and *Methanobrevibacter sp*., some species of methanogens can adapt to extreme environmental conditions (Zheng et al., 2013). The increase of pH from 9 to 12 led to a decrease in the amount of SCFA accumulation which was attributed to the strong alkaline toxicity in acidogenic bacteria. The SCFA yield can also be expressed as the mg COD per gram of VSS added. In this study, the maximal yield of SCFA by stage pH was 280.5 mg COD/g VSS added, which was around 1.2 times larger than that derived from sludge alkaline fermentation (Yuan et al., 2006).

Fig. 2b shows the impact of pH on the production of SCFA production followed by pH 12 for the first 5 d. It is evident in Fig. 2b that the pH in the 7 to 9 range promoted the accumulation of SCFA, whereas pH exceeding 9 proved to be detrimental to the accumulation of SCFA. The maximal SCFA accumulation of 612 mg/L occurred at pH 8 on 8 d . The maximal yield of SCFA followed by pH 12 for 5 d was significantly lower than that followed by pH 11 for 5 d ($p < 0.05$). The main reason for this phenomenon might be the strong toxicity of pH 12. It is documented that a strong alkaline state such as pH 12 can discourage the growth and activity of anaerobic fermentation microorganisms (Zhao et al., 2015c). From the above discussion, we can confidently state that stepwise pH fermentation, i.e., pH 11 for 5 d + pH 9 for 2 d, can improve the yield of SCFA from sludge. Furthermore the maximum production of SCFA was 2356 mg/L.

SCFA production is an important parameter in assessing the anaerobic digestion efficiency of sludge, and a variety of methods are applied to improve the yield of SCFA. Luo et al.(2011) evaluated the effect of surfactant and mixed enzyme (sodium dodecyl sulfate (SDS) 0.10 g/g dry sludge (DS), mixed-enzyme, 0.06 g/g DS, protease: a-amylase = 3:1)on the yield of SCFA from sludge anaerobic digestion, and the maximum yield of SCFA was 240.8 mg COD/g VSS. Jiang et al. (2007) found that the addition of sodium dodecylbenzene sulfonate SDBS to the sludge significantly increased the acidification efficiency and when the dosage of SDBD was 0.02 g/g dry sludge, the optimal yield of SCFA was 240.5 mg COD/g VSS. Similarly, biosurfactants rhamnolipid also can improve the production of SCFA, and when the dosage of rhamnolipid was 0.02g/g dry sludge, the production of SCFA was 311 mg COD/ g VSS (Huang et al., 2015). Yan et al. (2010) investigated the effects of ultrasonic combined alkalinity on sludge anaerobic fermentation and the yield of SCFA was as high as 445 mg COD/g VSS. It emerged that the production of SCFA via stepwise pH fermentation was higher than that with some chemical surfactants. Although less SCFA from stepwise pH fermentation was produced compared to that from ultrasound combined with alkaline fermentation and biosurfactants, the ultrasound required much energy input, and the dosage of biosurfactant used in the literature was large (Huang et al., 2015). This, however, is not economical in practical engineering applications. For this reason stepwise pH fermentation to generate SCFA is a promising strategy from the perspective of SCFA production and energy inputs.

The composition of SCFA plays an important role in its subsequent utilization, so this study further explored the effect of pH on the composition of SCFA. The pH did not affect the composition of SCFA, and the contents of acetate and propionate at all pHs ranked in the top two, which accounted for about 52-65% of total SCFA. The content of valerate was the lowest among all the reactors, which accounted for only 5% of the total SCFA.

3.3 Comparison of sludge hydrolysis, acidification and methanation between stepwise pH fermentation and pH 10 fermentation

It is widely acknowledged that WAS anaerobic fermentation undergoes four successive steps: disruption, hydrolysis, acidification, and methanation. These processes are all closely related to the production of SCFA. The process of disruption can be expressed by variations of SCOD, soluble protein and soluble polysaccharides. The performance of the last three steps can be expressed by the degradation rate of the model compounds using synthetic wastewater. In this study, stepwise pH fermentation can give rise to a higher SCFA production than alkaline fermentation, so therefore one might compare the degradation of model compounds between stepwise pH fermentation and alkaline fermentation. As can be seen from Table 1, the degradation rates of model compounds in stepwise pH or alkaline fermentation were lower than those in the blank, suggesting stepwise pH or alkaline fermentation was detrimental to the model compounds' degradation. The processes of hydrolysis, acidification and methanation are biochemical reactions, which are related to the activities of anaerobic microorganisms and/or key biological enzymes, and according to the literature the neutral environment is conducive to the activities of microorganisms (Ma et al., 2016).

The degradation differences of model compounds between stepwise pH fermentation and alkaline fermentation on 3 d were insignificant ($p>0.05$) because the model compounds remained in a strong alkaline environment (pH 10 and pH 11) in the first three days. However, the degradation rate of the model compounds in the stepwise pH fermentation was significantly higher than that in alkaline fermentation on 7 d. For example, when the fermentation time was 7 d, the degradation rate of BSA was 61.5% in alkaline pH fermentation, whereas the degradation rate of BSA increased to 68.4% in stepwise pH fermentation. Similar experimental results were also documented for the degradation rates of dextran, L-alanine and glucose on 7 d. The above experimental results suggested that stepwise pH fermentation was more beneficial to the hydrolysis

and acidification of model compounds than those in alkaline fermentation after 5 d.

Compared with the blank, alkaline fermentation and stepwise pH fermentation can significantly decrease the degradation rate of acetate. For example, the degradation rate of acetate was 72.6% on 3 d, while the degradation rates of acetate in alkaline and stepwise pH fermentation were 10.3% and 10.5%, respectively, which were much lower than that in the blank. The lower degradation rate of acetate in alkaline or stepwise pH fermentation was mainly ascribed to the strong inhibition of alkaline environment on methanogens. It was documented that the optimal pH for methanogens was around 7.0, and an alkaline condition can kill methanogens such as *Methanobacterium sp.* and *Methanobrevibacter sp.* (Zheng et al., 2013). It should be noted that the differences between the degradation rates of acetate in alkaline fermentation and stepwise pH fermentation were insignificant ($p>0.05$), which was mainly attributed to the strong alkaline condition during the first 5 d of two kinds of fermentation systems. Stepwise pH fermentation can produce higher efficiencies of hydrolysis and acidification than those in alkaline fermentation. However, the effects of both stepwise pH and alkaline fermentation on methanation were insignificant, leading to more SCFA being produced in stepwise pH fermentation.

3.4 Effects of stepwise fermentation on the activities of key enzymes responsible for SCFA generation

SCFA production from WAS anaerobic fermentation is a biochemical process, which is mainly regulated by key enzymes. Detection of enzymatic activity was an alternative method to quantitatively describe microbial activity. Thus the activities of key enzymes associated with SCFA production were detected. Protease and α-glucosidase are respectively responsible for the protein and polysaccharide hydrolysis of protein and polysaccharide (Geol et al., 1998; Zhao et al., 2016c). Acetic and propionic acid are the predominant SCFAs in WAS fermentation, so consequently the activities of AK and CoA transferase which are respectively responsible for acetic and propionic acid generation are also determined. Additionally, F420 is

a unique coenzyme of methanogens and the activity of F420 can be used to reflect the activity of methane-producing microorganisms. As shown in Fig. 3, the activities of the enzymes involved in SCFA production in the two reaction systems were insignificant except F420 over 3 d The activity of F420 in the stepwise pH fermentation was lower than that in the alkaline fermentation, which was mainly ascribed to the stronger alkaline condition in the stepwise pH fermentation. However, the activities responsible for hydrolysis and acidification in the stepwise pH fermentation were more numerous compared to those in alkaline fermentation on 7 d. The activities of F420 in both fermentation systems were insignificant, and this proved to be consistent with the results documented in Table 1. The processes of hydrolysis and acidification are biochemical reactions that require enzyme catalysis.

Generally, a neutral environment is good for the catalysis of enzymes and the growth of most microorganisms (Ma et al., 2016). Drake and Steven (2004) reported that neutral pH favored the growth and SCFA accumulation of a pure acetogenesis strain such as *Moorella thermoacetica*. Feng et al. (2009) also demonstrated that the activities of AK and CoA transferase at pH 9 were higher than those at pH 10 (AK 4.08 VS 2.82; CoA transferase 1.01 VS 0.48 U/mg VSS). Similar experimental results were also obtained in both systems after 5 d. Regarding the activity of F420, there was no significant difference between the two fermentation systems. A strong alkaline environment might completely wash out the methanogens in both systems. The above results clearly indicated that the activities of key enzymes responsible for hydrolysis and acidification in stepwise fermentation were significantly higher than that in the alkaline fermentation group after 5 d ($p < 0.05$), which was one reason for achieving greater SCFA production in the stepwise fermentation reactor.

3.5 Synergistic effect of stepwise fermentation integrated with NaCl on WAS fermentation

Previous studies have reported the presence of NaCl in WAS can promote the disintegration of sludge

flocs and release more soluble organic matter for subsequent microbial utilization (Su et al., 2016; Cui et al., 2015). However, whether stepwise pH fermentation integrated with NaCl had a synergistic effect on the production of SCFA or not still remains unknown. Fig. 4 shows the effect of stepwise pH fermentation integrated with NaCl on the variation of SCOD during WAS anaerobic fermentation. As shown in Fig. 4, the integration of stepwise pH fermentation and NaCl greatly promoted the release of SCOD from WAS anaerobic fermentation. The highest level of soluble COD in stepwise fermentation reactor was 2485 mg/L. When NaCl was added into stepwise fermentation system, the highest concentration of soluble COD increased to 2845 mg/L. Those data indicated that stepwise pH fermentation integrated with NaCl posed a synergistic effect on the disruption of WAS. A similar synergistic effect of stepwise fermentation combined with NaCl on the maximal SCFA production was also obtained. The maximum SCFA yield from stepwise pH fermentation combined with NaCl was 3623 mg/L, which was significantly higher than that of stepwise fermentation or NaCl fermentation. The impact of stepwise fermentation integrated with NaCl on the degradation rates of model compounds is shown in Fig. 5. As illustrated in Fig. 5, stepwise pH fermentation and NaCl synergistically inhibited the degradation rates of model compounds using synthetic wastewater. For example, the degradation rates of BSA in stepwise pH and NaCl fermentation reactor were 41.7% and 56.9% on 3 d, respectively. However, when stepwise pH fermentation and NaCl were combined, the degradation rate of BSA decreased to 35.6%, which was much lower than that in the stepwise pH or NaCl fermentation reactor. Similar experimental results were also obtained in other model compounds (i.e., dextran, L-alanine, glucose) degradation. As for the degradation of acetate, stepwise pH fermentation combined with NaCl also caused synergistic inhibition of acetate degradation. For example, the degradation rate of acetate was 42.6% and 10.5% in the stepwise pH fermentation and NaCl fermentation on 3d, respectively. However, the degradation rate of acetate decreased to 6.5% when the stepwise pH fermentation

combined with NaCl. Those results showed that the combination of stepwise pH fermentation and NaCl could create synergistic outcomes for the anaerobic digestion of sludge. It can be seen from Fig. 4 that stepwise pH fermentation combined with NaCl can enhance the disintegration of WAS, thereby releasing more intracellular organic matter, and providing sufficient material for acidogenic bacteria. In addition, the stepwise pH fermentation combined NaCl can further inhibit the activities of methanogens and reduce the consumption of SCFA. Although the stepwise pH fermentation combined with NaCl resulted in a certain inhibition on the processes of hydrolysis and acidification, the sufficient material and the least consumption of SCFA gave rise to the largest accumulation of SCFA.

4. Conclusion

This study reported a new method referred to as stepwise pH fermentation, which was devised to enhance the production of SCFA from WAS anaerobic fermentation. Results showed the optimal stepwise pH and time were pH 11 for 5 d+ pH 9 for 2 d. In this scenario, 2356 mg/L was obtained and this was higher than that from 1.2-fold of that from alkaline fermentation or blank. Finally, stepwise pH fermentation combined with NaCl present in the WAS wielded synergistic effects on WAS anaerobic fermentation, resulting in more SCFA production.

Acknowledgements

This research was financially supported by the project of National Natural Science Foundation of China (NSFC) (51478170, 51521006, and 51508178), and Planned Science and Technology Project of Hunan Province, China (No. 2015SF2067). The research collaboration between Hu Nan University and University of Technology Sydney is grateful.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version.

References

1. Alagöz, B. A., Yenigün, O., Erdinçler, A. 2015. Enhancement of anaerobic digestion efficiency of wastewater sludge and olive waste: synergistic effect of co-digestion and ultrasonic/microwave sludge pre-treatment. Waste Manag. 46, 182-188.

2. APHA (American Public Health Association), 1998. Standard Methods for the Examination of Water and Wastewater, twentieth ed. Washington, DC, USA.

3. Ariunbaatar, J., Panico, A., Esposito, G., Pirozzi, F., Lens, P. N. 2014. Pretreatment methods to enhance anaerobic digestion of organic solid waste. Appl. Energy 123, 143-156.

4. Chen, G. H., Leung, D. H. W. 2000. Utilization of oxygen in a sanitary gravity sewer. Water Res. 34(15), 3813-3821.

5. Chen, Y., Jiang, S., Yuan, H., Zhou, Q., Gu, G. 2007. Hydrolysis and acidification of waste activated sludge at different pHs. Water Res. 41(3), 683-689.

6. Cui, Y., Su, H., Chen, Y., Chen, Y., Peng, Y. 2015. Mechanism of activated sludge floc disintegration induced by excess addition of NaCl. Clean – Soil Air, Water 43(8), 1197-1206.

7. Dimroth, P., Thomer, A. 1989. A primary respiratory Na^+ pump of an anaerobic bacterium: the Na^+-dependent NADH: quinone oxidoreductase of Klebsiella pneumoniae. Arch. Microbiol. 151(5), 439-444.

8. Drake, H. L., Daniel, S. L. 2004. Physiology of the thermophilic acetogen Moorella thermoacetica. Res. Microbiol. 155(10), 869-883.

9.Du, X., Shi, Z., Peng, Z., Zhao, C., Zhang, Y., Wang, Z., Li, X., Liu, G., Li, X. 2017. Acetoacetate induces hepatocytes apoptosis by the ROS□mediated MAPKs pathway in ketotic cows. J. Cell Physiol. 232:3296-3308.

10. Feng, Y., Zhang, Y., Quan, X., Chen, S. 2014. Enhanced anaerobic digestion of waste activated sludge digestion

by the addition of zero valent iron. Water Res. 52, 242-250.

11. Goel, R., Mino, T., Satoh, H., Matsuo, T. 1998. Enzyme activities under anaerobic and aerobic conditions in activated sludge sequencing batch reactor. Water Res. 32(7), 2081-2088.

12. Herbert, D., Philipps, P.J., Strange, R.E., 1971. Carbohydrate analysis. Methods Enzymol. 5B, 265-277.

13. Huang, X., Shen, C., Liu, J., Lu, L., 2015. Improved volatile fatty acid production during waste activated sludge anaerobic fermentation by different bio-surfactants. Chem. Eng. J. 264, 280-290.

14. Jiang, S., Chen, Y., Zhou, Q., Gu, G., 2007. Biological short-chain fatty acids (SCFAs) production from waste-activated sludge affected by surfactant. Water Res. 41(14), 3112-3120.

15. Jin, B., Wang, S., Xing, L., Li, B., Peng, Y. 2016. Effect of Salinity on Enhancing Waste Activated Sludge Alkaline Fermentation at Different Temperatures. Clean-Soil Air Water 44(12), 1750-1758.

16. Kwietniewska, E., Tys, J. 2014. Process characteristics, inhibition factors and methane yields of anaerobic digestion process, with particular focus on microalgal biomass fermentation. Renew. Sustain. Energy Rev. 34, 491-500.

17. Latif, M. A., Mehta, C. M., Batstone, D. J. 2017. Influence of low pH on continuous anaerobic digestion of waste activated sludge. Water Res. 113, 42-49.

18. Li, X., Peng, Y., Ren, N., Li, B., Chai, T., Zhang, L. 2014. Effect of temperature on short chain fatty acids (SCFAs) accumulation and microbiological transformation in sludge alkaline fermentation with Ca $(OH)_2$ adjustment. Water Res. 61, 34-45.

19. Li, X., Zhao, J., Wang, D., Yang, Q., Xu, Q., Deng, Y., Yang, W., Zeng, G. 2016. An efficient and green pretreatment to stimulate short-chain fatty acids production from waste activated sludge anaerobic fermentation using free nitrous acid. Chemosphere, 144, 160-167.

20. Liang, B., Cheng, H., Van Nostrand, J. D., Ma, J., Yu, H., Kong, D., Liu, W., Ren, N., Wu, L., Wang, A.,. Lee, D.

J. 2014. Microbial community structure and function of nitrobenzene reduction biocathode in response to carbon source switchover. Water Res. 54, 137-148.

21. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem . 193(1), 265-275.

22. Luo, J., Chen, Y., Feng, L. 2016. Polycyclic Aromatic Hydrocarbon Affects Acetic Acid Production during Anaerobic Fermentation of Waste Activated Sludge by Altering Activity and Viability of Acetogen. Environ. Sci. Technol. 50(13), 6921-6929.

23. Luo, K., Yang, Q., Yu, J., Li, X. M., Yang, G. J., Xie, B. X., Zeng, G. M., 2011. Combined effect of sodium dodecyl sulfate and enzyme on waste activated sludge hydrolysis and acidification. Bioresour. Technol. 102(14), 7103-7110.

24. Ma, C., Jin, R. C., Yang, G. F., Yu, J. J., Xing, B. S., Zhang, Q. Q. 2012. Impacts of transient salinity shock loads on Anammox process performance. Bioresour. Technol.112, 124-130.

25. Ma, H., Chen, X., Liu, H., Liu, H., Fu, B. 2016. Improved volatile fatty acids anaerobic production from waste activated sludge by pH regulation: Alkaline or neutral pH? Waste Manag. 48, 397-403.

26. Ma, J., Duong, T. H., Smits, M., Verstraete, W., Carballa, M. 2011. Enhanced biomethanation of kitchen waste by different pre-treatments. Bioresour. Technol. 102(2), 592-599.

27. Maspolim, Y., Zhou, Y., Guo, C., Xiao, K., Ng, W. J. 2015. The effect of pH on solubilization of organic matter and microbial community structures in sludge fermentation. Bioresour. Technol. 190, 289-298.

28. Mu, H., Chen, Y. 2011. Long-term effect of ZnO nanoparticles on waste activated sludge anaerobic digestion. Water Res. 45(17), 5612-5620.

29. Su, G., Wang, S., Yuan, Z., Peng, Y. 2016. Enhanced volatile fatty acids production of waste activated sludge under salinity conditions: Performance and mechanisms. J. Biosci. Bioeng. 121(3), 293-298.

30. Sun, X., Yuan, X., Chen, L., Wang, T., Wang, Z., Sun, G., Li, X. Li, X., Liu, G. 2017. Histamine induces bovine rumen epithelial cell inflammatory response via NF-κB pathway. Cell. Physiol. Biochem. 42(3), 1109-1119.

31. Song, Y., Li, N., Gu, J., Fu, S., Peng, Z., Zhao, C., Zhang, Y., Li, X., Wang, Z., Li, X., Liu, G. 2016. β-Hydroxybutyrate induces bovine hepatocyte apoptosis via an ROS-p38 signaling pathway.J Dairy Sci. 99(11), 9184-9198.

32. Tong, J., Chen, Y. 2007. Enhanced biological phosphorus removal driven by short-chain fatty acids produced from waste activated sludge alkaline fermentation. Environ. Sci. Technol. 41(20), 7126-7130.

33. Wang, D., Zhao, J., Zeng, G., Chen, Y., Bond, P. L., Li, X. 2015. How does poly (hydroxyalkanoate) affect methane production from the anaerobic digestion of waste-activated sludge? Environ. Sci. Technol. 49(20), 12253-12262.

34. Wu, H., Gao, J., Yang, D., Zhou, Q., Liu, W. 2010. Alkaline fermentation of primary sludge for short-chain fatty acids accumulation and mechanism. Chem. Eng. J. 160(1), 1-7.

35. Yan, Y., Feng, L., Zhang, C., Wisniewski, C., Zhou, Q. 2010. Ultrasonic enhancement of waste activated sludge hydrolysis and volatile fatty acids accumulation at pH 10.0. Water Res. 44(11), 3329-3336.

36. Yang, G., Zhang, G., Wang, H. 2015. Current state of sludge production, management, treatment and disposal in China. Water Res. 78, 60-73.

37. Yuan, H., Chen, Y., Zhang, H., Jiang, S., Zhou, Q., Gu, G. 2006. Improved bioproduction of short-chain fatty acids (SCFAs) from excess sludge under alkaline conditions. Environ. Sci. Technol. 40(6), 2025-2029.

38. Zhang, J., Lv, C., Tong, J., Liu, J., Liu, J., Yu, D., Wang, Y., Chen, M., Wei, Y. 2016. Optimization and microbial community analysis of anaerobic co-digestion of food waste and sewage sludge based on microwave pretreatment. Bioresour. Technol. 200, 253-261.

39. Zhang, P., Chen, Y., Zhou, Q., Zheng, X., Zhu, X., Zhao, Y. 2010. Understanding short-chain fatty acids

accumulation enhanced in waste activated sludge alkaline fermentation: kinetics and microbiology. Environ. Sci. Technol. 44(24), 9343-9348.

40. Zhao, C., Ma, Z., Shao, Q., Li, B., Ye, J., Peng, H. 2016a. Enzymatic hydrolysis and physiochemical characterization of corn leaf after H-AFEX pretreatment. Energy Fuels, 30(2), 1154-1161.

41. Zhao, C., Shao, Q., Ma, Z., Li, B., Zhao, X. 2016b. Physical and chemical characterizations of corn stalk resulting from hydrogen peroxide presoaking prior to ammonia fiber expansion pretreatment. Ind. Crops Prod. 83, 86-93.

42. Zhao, C., Qiao, X., Cao, Y., Shao, Q. 2017a. Application of hydrogen peroxide presoaking prior to ammonia fiber expansion pretreatment of energy crops. Fuel. 205, 184-191.

43. Zhao, J., Wang, D., Li, X., Yang, Q., Chen, H., Zhong, Y., Zeng, G. 2015a. Free nitrous acid serving as a pretreatment method for alkaline fermentation to enhance short-chain fatty acid production from waste activated sludge. Water Res. 78, 111-120.

44. Zhao, J., Yang, Q., Li, X., Wang, D., Luo, K., Zhong, Y., Xu, Q., Zeng, G. 2015b. Enhanced production of short-chain fatty acid from food waste stimulated by alkyl polyglycosides and its mechanism. Waste Manag. 46, 133-139.

45. Zhao, J., Liu, Y., Wang, D., Chen, F., Li, X., Zeng, G., Yang, Q. 2017b. Potential impact of salinity on methane production from food waste anaerobic digestion. Waste Manag. 67, 308-314.

46. Zhao, J., Zhang, C., Wang, D., Li, X., An, H., Xie, T., Chen, F., Xu, Q., Sun, Y., Zeng, G., Yang, Q. 2016c. Revealing the Underlying Mechanisms of How Sodium Chloride Affects Short-Chain Fatty Acid Production from the Cofermentation of Waste Activated Sludge and Food Waste. ACS Sustainable Chem. Eng. 4(9), 4675-4684.

47 Zhao, J., Wang, D., Li, X., Zeng, G., Yang, Q. 2016 d. Improved biological phosphorus removal induced by an

oxic/extended-idle process using glycerol and acetate at equal fractions. Rsc Adv. 6(89), 86165-86173.

48. Zhao, J., Gui, L., Wang, Q., Liu, Y., Wang, D., Ni, B. J., Li,X., Xu, R., Zeng, G., Yang, Q. 2017c. Aged refuse enhances anaerobic digestion of waste activated sludge. Water Res.123, 724-733.

49. Zhen, G., Lu, X., Kato, H., Zhao, Y., Li, Y. Y. 2017. Overview of pretreatment strategies for enhancing sewage sludge disintegration and subsequent anaerobic digestion: Current advances, full-scale application and future perspectives. Renew. Sustain. Energy Rev. 69, 559-577.

50. Zheng, X., Su, Y., Li, X., Xiao, N., Wang, D., Chen, Y. 2013. Pyrosequencing reveals the key microorganisms involved in sludge alkaline fermentation for efficient short-chain fatty acids production. Environ. Sci. Technol. 47(9), 4262-4268.

Figure captions

Fig. 1 Effect of pH on the variation of SCOD during WAS anaerobic fermentation. Error bars represent standard deviations of triplicate measurements.

Fig. 2 Effects of different pH on the accumulation of SCFA (a: the pH was controlled at 11 during the initial 5 d, b: the pH was controlled at 12 during the initial 5 d). Error bars represent standard deviations of triplicate measurements.

Fig. 3 Effect of alkaline fermentation and stepwise fermentation on the activities of key enzymes responsible for SCFA generation (a: 3 d; b:7d). Error bars represent standard deviations of triplicate measurements.

Fig. 4 Synergistic effect of stepwise pH fermentation and NaCl on soluble COD from WAS anaerobic fermentation. Error bars represent standard deviations of triplicate measurements.

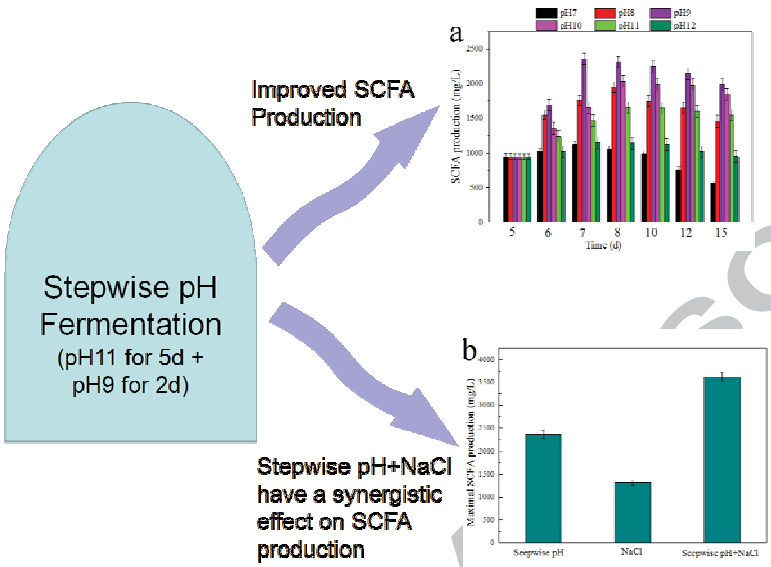
Fig.5 Synergistic effect stepwise pH fermentation with NaCl on the degradation rate of model compounds using synthetic wastewater on 3 d. Error bars represent standard deviations of triplicate measurements.

Table captions

Table 1. Comparison of degradation rate of model compounds using different fermentation types with time.

Highlights

- Stepwise pH fermentation improved the production of SCFA from WAS anaerobic fermentation.
- The mechanisms of enhanced SCFA production via stepwise pH fermentation were explored.
- Stepwise pH fermentation integrated with NaCl caused synergistic effect on WAS anaerobic fermentation.



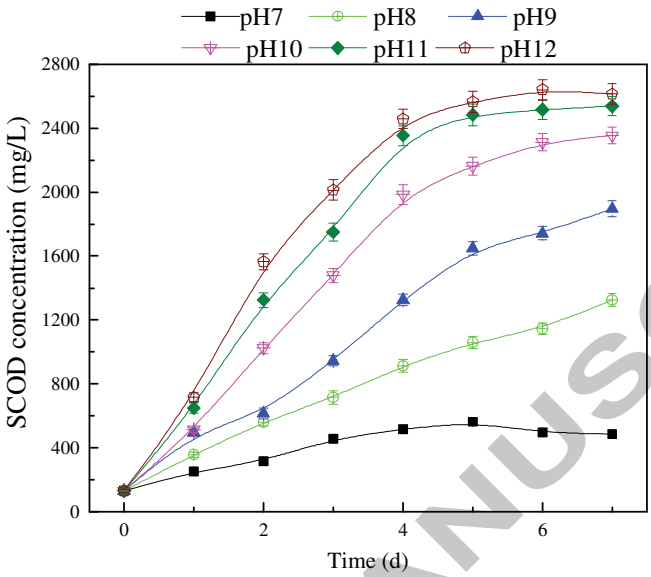


Fig. 1 Effect of pH on the variation of SCOD during WAS anaerobic fermentation. Error bars represent standard deviations of triplicate measurements.

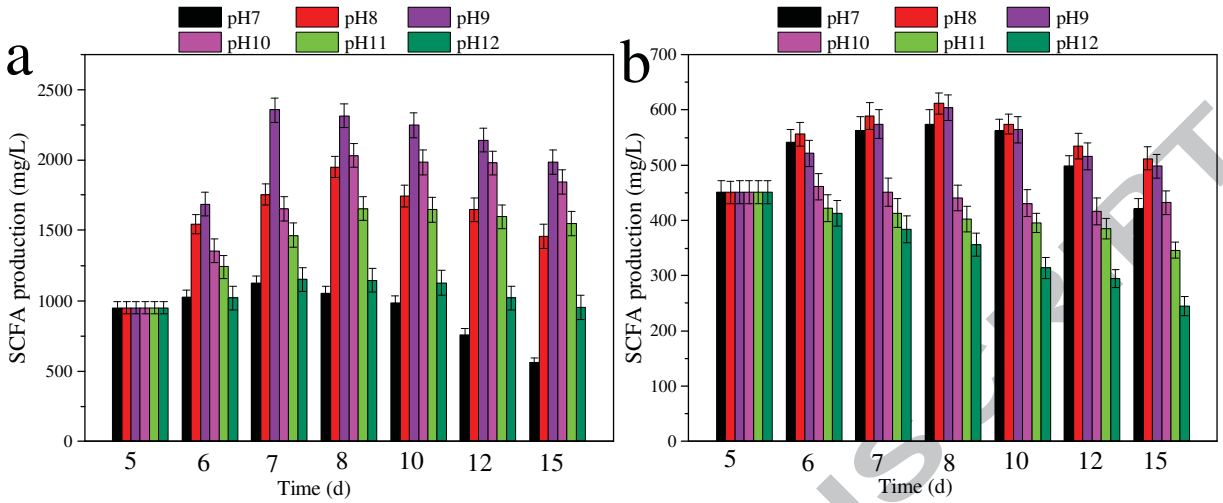


Fig. 2 Effects of different pH on the accumulation of SCFA(a: the pH was controlled at 11 during the initial 5 d, b: the pH was controlled at 12 during the initial 5 d). Error bars represent standard deviations of triplicate measurements.

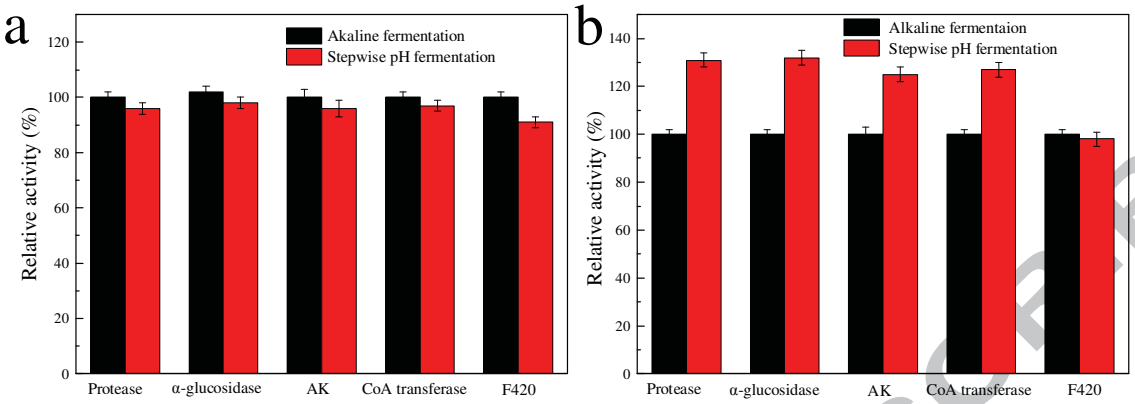


Fig.3 Effect of alkaline fermentation and stepwise fermentation on the activities of key enzymes responsible for SCFA generation (a: 3 d; b:7d). Error bars represent standard deviations of triplicate measurements.

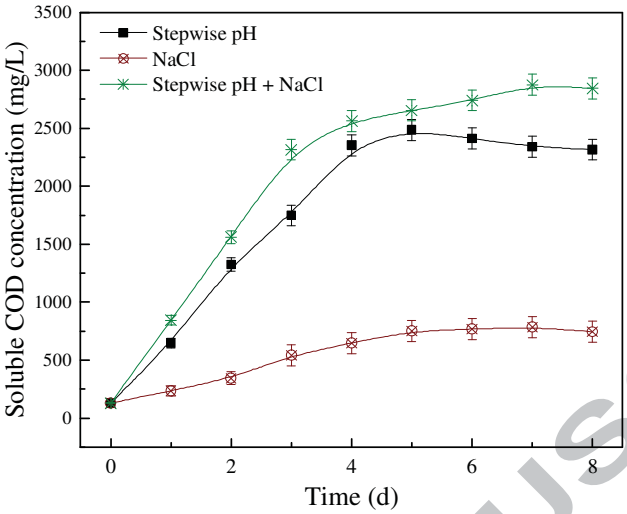


Fig. 4 Synergistic effect of stepwise pH fermentation and NaCl on soluble COD from WAS anaerobic fermentation. Error bars represent standard deviations of triplicate measurements.

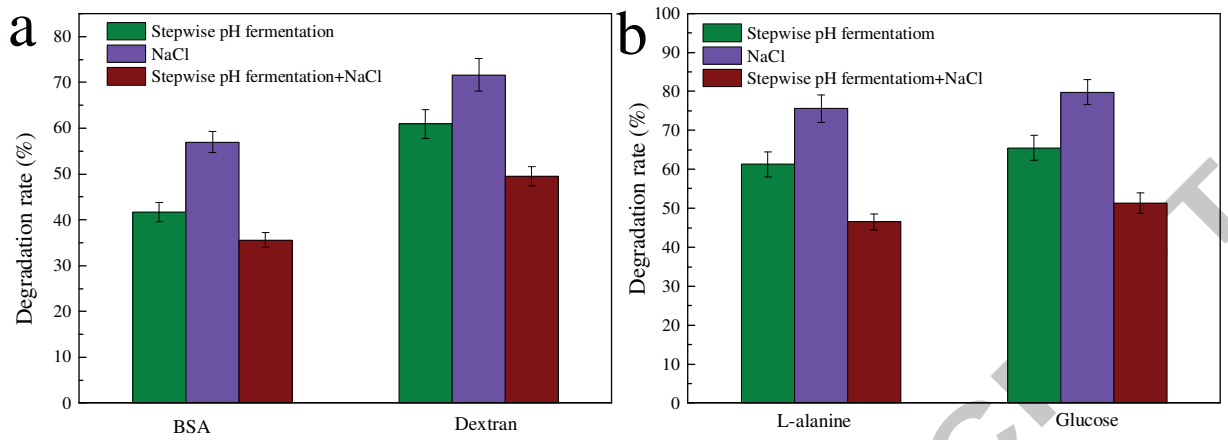


Fig.5 Synergistic effect stepwise pH fermentation with NaCl on the degradation rate of model compounds using synthetic wastewater on 3 d. Error bars represent standard deviations of triplicate measurements.

Fermentation types	Time (d)	Model Compounds Degradation rate (%)				
		BSA	Dextran	L-alanine	Glucose	Acetate
Blank	3	49.8	68.9	69.9	72.3	72.6
	6	62.5	75.6	73.4	85.4	84.2
	7	70.2	85.6	86.5	90.1	95.6
Alkaline fermentation	3	42.3	61.2	61.3	65.9	10.3
	6	49.8	65.8	69.4	72.3	26.5
	7	61.5	70.1	73.2	79.5	34.8
Stepwise pH fermentation	3	41.7	60.9	61.1	65.5	10.5
	6	54.9	69.4	73.2	76.8	25.9
	7	68.4	76.5	78.2	82.8	35.2

Table 1 Comparison of degradation rate of model compounds by different fermentation types with time.[a]

[a]:The data reported are the averages and their standard deviations in triplicate tests.