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Improving volatile fatty acids production by exploiting the residual substrates in post-fermented sludge: Protease catalysis of refractory protein

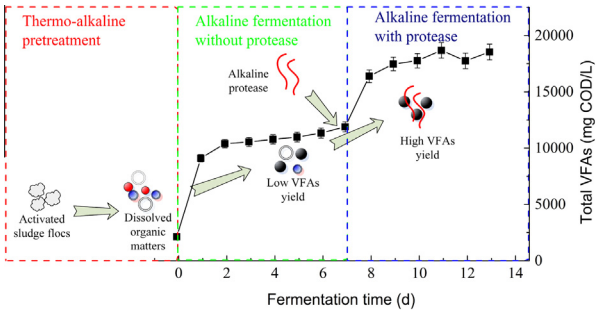
Bo Yin [a], Hongbo Liu [a,b,c], Yuanyuan Wang [a], Jie Bai [a], He Liu [a,b,c,*], Bo Fu [a,b,c]

[a] School of Environmental and Civil Engineering, Jiangnan University, 214122 Wuxi, Jiangsu Province, China
[b] Jiangsu Key Laboratory of Anaerobic Biotechnology, 214122 Wuxi, Jiangsu Province, China
[c] Jiangsu Coorperative Innovation Center of Technology and Material of Water Treatment, 215011 Suzhou, Jiangsu Province, China

HIGHLIGHTS

- Poor biodegradability decrease cause protein accumulation in post-fermented sludge.
- Biodegradability of residual refractory protein is greatly enhanced by protease.
- Protease addition improves VFAs yield significantly.
- Stable protease activity is maintained during the alkaline fermentation process.
- Secondary fermentation with protease addition in post-fermented sludge was proposed.

GRAPHICAL ABSTRACT



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ABSTRACT

The real cause to the low yield of volatile fatty acids (VFAs), from inhibition or low biodegradation, is uncertain in sludge anaerobic fermentation. In this study, poor biodegradability of proteins and fast decrease of the indigenous hydrolase activity in the residual post-fermented sludge were found to be the major reasons. With the addition of trypsin or alkaline protease in residual post-fermented sludge after primary alkaline fermentation, degradation efficiency of refractory protein increased by 33.6% and 34.8%, respectively. Accordingly, the VFAs yields were improved by 69.7% and 106.1%, respectively. Furthermore, the activities of added trypsin and alkaline protease could maintain at 13.52 U/mL and 19.11 U/mL in the alkaline fermentation process. This study demonstrated that exploiting the refractory proteins in residual post-fermented sludge by protease addition seems to be a very promising way for improving VFAs yield of conventional alkaline fermentations with waste activated sludge.

1. Introduction

With the improvement of wastewater treatment, the massive production of waste activated sludge (WAS) has become a big burden for wastewater treatment plants. Anaerobic digestion was considered to be a promising way for sludge treatment, by which the organic matters could be converted into valuable substances, such

* Corresponding author at: School of Environmental and Civil Engineering, Jiangnan University, 214122 Wuxi, Jiangsu Province, China. Tel./fax: +86 510 85326670.
E-mail address: liuhe@jiangnan.edu.cn (H. Liu).

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as biogas, volatile fatty acids (VFAs), etc., realizing sludge reduction and resource recovery (Lee et al., 2014). These VFAs attract intensive interests because of their wide range of applications such as in the bioenergy, bioplastics and the biological nutrients removal from wastewater (Lee et al., 2014). Unfortunately, low VFAs yield is one of the major bottlenecks that limit the industrial VFAs production by anaerobic fermentation with low cost.

It has been reported that many factors may result in low VFAs yield through anaerobic fermentation from WAS, such as inhibition (Chen et al., 2008), low biodegradability of the substrates (Maspolim et al., 2015), unfavorable C/N for the acidogenic microbes (Silvestre et al., 2015), etc. However, the deciding limiting factor for low VFAs yield is still not clear. Protein is the major organic component with the percentage as high as 50–60% of the total COD in the organic matters of pretreated WAS (Yang et al., 2015a). The hydrolysis of organic matters is the key step of anaerobic fermentation as previously reported (Guo et al., 2014). Some studies demonstrated that the hydrolysis of protein was very slow because protein was unsusceptible to protease cleavage in its native folded conformation (Herman et al., 2006). If the unfolding of protein could be achieved, the hydrolysis rate would be improved and the VFAs production could also be enhanced (Xiao et al., 2013).

In recent studies, thermal-alkaline pretreatment of sludge and alkaline fermentation (pH 10.0) were widely reported as effective measures to improving the VFAs yield from WAS (Carrere et al., 2016). During the anaerobic alkaline fermentation, the proteins in the sludge could be efficiently dissolved into the liquid phase and then hydrolyzed to peptides and amino acids, and finally converted to VFAs by acidogenic microorganisms. For example, the final removal efficiencies of protein by alkaline fermentation (pH 10.0) was higher than 32.2% which anaerobic fermentation at pH 7.0 (Zheng et al., 2013). And after anaerobic fermentation, the solubilization of organic matters and the VFAs production with thermal-alkaline pretreatment would be 60% and 75% higher than that untreated, respectively (Liu et al., 2009). Though thermal-alkaline pretreatment and alkaline fermentation (pH 10.0) are effective for improving VFAs production, the protein was found to accumulate in the residual sludge after fermentation. For instance, after thermal-alkaline pretreatment, only 40–50% of protein was converted into VFAs (Liu et al., 2012). In a study with the bovine serum albumin (BSA) as the unique substrate for alkaline fermentation, the residual BSA was as high as about 54%, suggesting that a high percentage of protein was not converted into VFAs in the acidogenic stage of fermentation (Yuan et al., 2006). The category and composition of proteins from the microbial cells in the sludge are very complicated and little is known about their characteristics. Wang et al. (2015) reported that protein could be fragmentation and the efficiency of protein denaturation could be increased after thermal-alkaline pretreatment. And a recent report from Tan et al. (2012) indicated the residual proteins were mainly outer membrane proteins and mainly came from Gram-positive bacteria and the proteins from Gram-negative bacteria were easily degraded. Therefore, how to significantly enhance the utilization of the residual refractory proteins in the sludge after primary fermentation is still a challenging problem for pursuing higher VFAs yield from sludge by anaerobic fermentation.

Protease is commonly used in waste matter pretreatment (Carrere et al., 2016). It can be inferred that part of protease would attack and broke the microbial cell wall and the residual protease acts to degrade the released bacterial proteins. This limits the efficiency of the protease for the subsequent fermentation (Liu et al., 2016). Considering the above facts, the addition of protease after the thermal-alkaline pretreatment will enhance the efficiency of protease by cleavage of released refractory proteins. Monlau et al. (2013) demonstrated that the enzymatic hydrolysis after thermal-alkaline pretreatment enhanced the biofuel production from agricultural residues.

Therefore, based on the above two facts that (a) large amount of proteins were released and accumulated in the residual sludge after primary fermentation, and (b) protease was effective to catalyze refractory protein degradation, we propose a novel idea of improving VFAs production by exploiting the residual substrates in sludge after primary fermentation, in which protease was added into the residual alkaline post-fermented sludge to improve the cleavage of refractory proteins, instead of the solubilization in raw sludge, to significantly improve the VFAs production from the residual sludge after primary fermentation. This study explored the real reason responsible for the low VFAs yield from WAS by anaerobic alkaline fermentation and verified that protease application in the residual post-fermented sludge could be a feasible method to significantly improve VFAs yield from post-fermented WAS.

2. Methods

2.1. Preparation of seeding sludge

Dewatered sludge samples were obtained from the dewatering process of a municipal wastewater treatment plant in Wuxi, China. The characteristics of dewatered sludge are shown in Table 1. For the preparation of seeding sludge, the dewatered sludge was adjusted to a concentration at 75 g/L and heated at 90 °C for 3 h, and then sludge was incubated in an upflow anaerobic sludge blanket reactor (UASB) for 10 days for acclimation (Liu et al., 2012). Then the seeding sludge was washed with distilled water 3 times for subsequent uses. The basic characteristics of the seeding sludge and raw sludge are shown in Table 1.

2.2. Pretreatment of WAS and preparation of residual substrate extract

The total solids (TS) of dewatered sludge were diluted to 75 g/L before thermo-alkaline pretreatment. The preparation of pretreated WAS supernatant was followed by the methods described elsewhere (Liu et al., 2012). The preparation of residual substrate extract was as the following steps: the fermented liquid was centrifuged at 7000*g* for 20 min to obtain the fermentation supernatant; then the protein was extracted from the supernatant by isoelectric point methods (Hwang et al., 2008); for the polysaccharide extraction, an equal volume of anhydrous ethanol was added into the remaining supernatant for precipitating the polysaccharide, then the supernatant was stirred at 30 rpm for 15 min and then centrifuged at 7000*g* for 30 min at 4 °C to obtain the polysaccharide (Yang et al., 2015b). The two kinds of precipitates (protein and polysaccharide) were dried at 50 °C for 16 h and then ground into powder and stored at 4 °C. The extracted protein and

Table 1
Basic characteristics of seeding sludge and sewage sludge.

Parameter	Seeding sludge	Sewage sludge	Dewatered sludge
pH	3.9 ± 0.1	6.4 ± 0.3	ND
Total solids (TS, g/L)	60.00 ± 1.30	75.17 ± 1.30	140 ± 1.30
Volatile solids (VS, g/L)	29.90 ± 2.20	33.72 ± 3.20	79.8 ± 2.80
SCOD (g/L)	0.63 ± 0.05	ND	ND
VFAs (g/L)	0.148 ± 0.04	ND	ND
Soluble protein (g/L)	0.025 ± 0.003	ND	ND
Soluble polysaccharide (g/L)	0.014 ± 0.008	ND	ND
Soluble ammonia nitrogen (g/L)	0.002 ± 0.0001	ND	ND

ND: not determined.

polysaccharide were mixed as residual substrate extract and used for later experiments.

2.3. Experimental design for identifying limiting factors

To identify the limiting factors for VFAs production, the fermentation experiment was divided into three stages: stage I was primary alkaline fermentation; stage II was the secondary alkaline fermentation following stage I during which the fresh pretreated WAS supernatant was added into the fermenter; and stage IIIa was the third fermentation which repeated the same conditions as stage II. The stage IIIb was the negative control fermentation with the addition of distilled water. The process is shown in Fig. 1.

In the all three fermentation stages, the seeding sludge was 75 g/L, the fermenter volume is 500 mL and the pH was adjusted to 10.0. At the beginning of the fermentation and after each sampling, N_2 (99.9%) was flushed into the fermenter for 15 min to eliminate oxygen. Fermentation experiments were carried out at 37 ± 1 °C in an air-tight shaker (120 rpm). To keep the same concentration of VFAs in fermented liquid, the water was evaporated from the liquid as the following steps: the fermented mixed liquid from stage I was centrifuged at 8000*g* for 10 min, and then the supernatant was evaporated at 60 °C to the required volume. During the evaporation, most of ammonia was removed but the VFAs was kept in the liquid under alkaline condition (Trussell, 1972). Then, the precipitated sludge after centrifuge, remaining fermentation liquid after evaporation and pretreated WAS supernatant replacing the loss volume of the supernatant of the fermented sludge were added into the fermenter to continue the next stage of fermentation.

2.4. Residual substrates fermentation with protease addition

In order to test the feasibility of the protease addition for the improvement of the VFAs production from residual protein, two kinds of proteases, eg. trypsin and alkaline protease were used as catalysts for the protein degradation. Although there are many types of protease, the reason that trypsin and alkaline protease were selected as the catalysts in this study is that their optimal application conditions such as temperature and pH follow the fermentation conditions in this study, e.g. 37 °C and 8.0–10.0, respectively. In addition, these two proteases were also widely applied in the enzymatic pretreatment of the sludge. In the fermentation with residual substrate extract, 50 mg/L trypsin (5 million U/g, Amano Enzyme Manufacturing Co., Ltd., Japan) or alkaline protease (10 million U/g, Amano Enzyme Manufacturing Co., Ltd., Japan) was added into the fermenter and 5.0 g substrate extract was used as substrate for VFAs production. The other fermentation conditions and operation were the same as the above alkaline fermentation. The optimal pH of trypsin activity is 8.0 and under that pH the methanogenesis will stilly happen and exhaust the VFAs. However, the optimal pH of alkaline protease is 10.0 and under that pH almost most methanogenesis was inhibited. So, with the addition of trypsin, 0.1 mol/L 2-bromoethanesulfonic acid, sodium salt (BES. Nanjing Robiot Co., Ltd., China), which is an inhibitor for the methanogenesis (Conrad and Klose, 2000), was also added to inhibit the methane generation for the VFAs accumulation. In the fermentation with residual post-fermented sludge, 50 mg/L alkaline protease (10 million U/g) or 50 mg/L trypsin (5 million U/g) was added into the fermenter when the fermentation processes were stable. To deduct the VFAs production from the protease itself by anaerobic fermentation, a controlling fermentation was conducted with the same conditions above except the residual substrate extract or residual post-fermented sludge was absent.

2.5. Analytical methods

Liquid samples were collected every day to measure pH and VFAs. A pH meter (Mettler Toledo FE20, Germany) was used to monitor pH values. GC-2010 gas chromatography (Shimadzu Corporation, Tokyo, Japan) was employed to measure VFAs, namely acetic, propionic, iso-butyric, *n*-butyric, iso-valeric, and *n*-valeric acids, in all experiments. The method was followed the description elsewhere (Liu et al., 2012). Before each measurement, liquid samples were centrifuged at 10,000*g* for 10 min and filtered through a 0.45-μm membrane.

Soluble chemical oxygen demand (SCOD), ammonia, totals solids (TS), volatile solids (VS) were measured in accordance with the standard methods (APHA, 1998); soluble protein was determined by Lowry's method (Lowry et al., 1951); soluble polysaccharide was measured by the phenol–sulfuric method (Herbert et al., 1971); the five-day biochemical oxygen demand (BOD_5) was determined by a BOD tester (ET99724A, Lovibond Co., Ltd., Germany) with the dilution and seeding method reported by SEPA (HJ, 2009).

The conversion coefficients of protein and polysaccharide to COD were 1.5 and 1.07, respectively (Grady et al., 2012). To evaluate the biodegradability of the substrates in the residual post-fermented sludge, the BOD_{sol}/COD_{sol} (B/C) was introduced, in which the BOD_5 or COD from VFAs was deducted. The BOD_{sol} and COD_{sol} were calculated according to Eqs. (1) and (2).

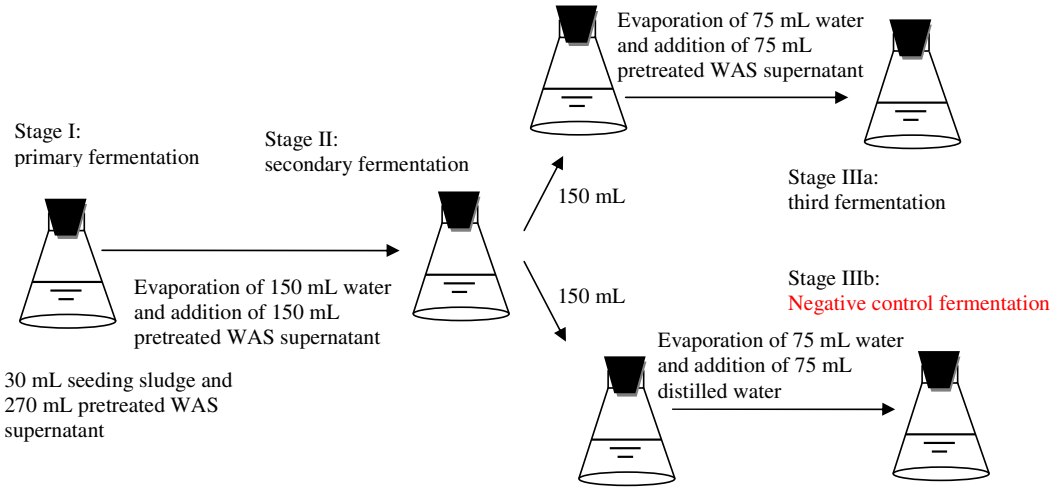

Fig. 1. The schematic of alkaline anaerobic fermentation for VFAs production.

$$BOD_{sol} = BOD_5\ (mg/L) - VFAs\ (mgCOD/L) \quad (1)$$

$$COD_{sol} = SCOD\ (mg/L) - VFAs\ (mgCOD/L) \quad (2)$$

β-glucosidase (β-GLC) activity representing the capability of polysaccharides degradation was measured spectrophotometrically (Mapada UV-1600, China) in 5 cm cuvettes according to the procedure published by Li and Chróst (2006). The protease activity was determined followed a standard method (SB/T, 1988). Each determination was repeated three times.

3. Results and discussion

3.1. Composition of residual substrates in post-fermented sludge

The supernatant of WAS from thermal-alkaline pretreatment was used for the alkaline fermentation. The main components in raw sludge and residual post-fermented sludge supernatant (calculated as SCOD) on day 0 and day 7 are shown in Table 2. On day 0, the protein, polysaccharide, and VFAs accounted for 39.29%, 10.14%, and 3.32%, respectively. On day 7, the proportion of protein in post-fermented sludge residues decreased to 18.11% with a conversion rate of 53.9%. The proportion of polysaccharide in post-fermented sludge residues decreased to 4.49% with a conversion rate of 55.72%. Meanwhile, the percentage of VFAs (calculated as SCOD) increased from 3.32% to 33.41%, indicating that VFAs accumulation was consistent with the conversion of protein and polysaccharide. The results indicated that large amounts of unconverted substrates (66.59%) remained in the residual post-fermented sludge, including protein (18.11%) polysaccharide (4.49%), and other matters. So, the VFAs yield of sludge could be further improved by enhancing the utilization of those remained substrates in the residual post-fermented sludge.

3.2. VFAs production and substrate degradation at different fermentation stages

To explore the limiting factors for the large amount of protein accumulation and low yield of VFAs, a continuous anaerobic fermentation was divided into three stages by addition of fresh substrates (thermo-alkaline pretreated sludge supernatant) at different stages. Fig. 2A showed that the VFAs concentrations increased from 1600 to 6600 mg/L during the first 2 days and then remained stable at 8780 mg/L in stage I (primary fermentation). After the fresh substrate was added into the fermenter at day 8 in stage II (secondary fermentation), the VFAs concentration increased rapidly from 8780 to 12,440 mg/L at day 11 and remained stable in the next 3 days. The similar phenomenon was observed with the second time addition of fresh substrate at day 15 in which the VFAs concentration increased by 58.3% at the end of stage IIIa. In the fermentation of stage IIIb, the VFAs concentration maintained stable without increase in the next 6 days.

Fig. 2B showed that the average daily consumptions of protein in stages I, II and IIIa were 285.7, 257.1 and 271.4 mg/L, respectively. The protein concentration decreased from 4490 to 2715 mg/L in the first 2 days of stage I and gradually decreased

Table 2
Composition of polysaccharide, protein and VFAs in SCOD at the initial and final stages of pretreated sludge supernatant alkaline fermentation.

Stages	Protein in SCOD (%)	Polysaccharide in SCOD (%)	VFAs in SCOD (%)
Initial (day 0)	39.29	10.14	3.32
Final (day 7)	18.11	4.49	33.41

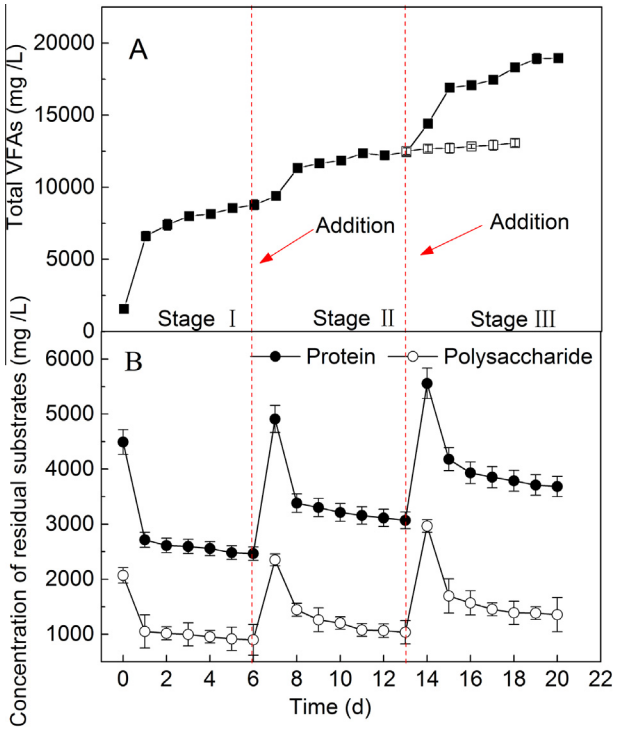

Fig. 2. Production of VFAs (A) and consumption of substrates (B) during different stages of sludge fermentation. Solid square: stage IIIa. Hollow square: stage IIIb. The arrows indicated the addition of fresh pretreated WAS supernatant.

to 2464 mg/L during the next 4 days. In stage II, when fresh substrate was supplied, the concentration of protein increased from 2464 to 4909 mg/L. And then it decreased to 3381 mg/L in the first 2 days and further decreased to 3068 mg/L at the end of stage II. There is a similar phenomenon in stage IIIa. Polysaccharide utilization occurred simultaneously with the protein consumption (Fig. 2B). In stage I, the polysaccharide concentration declined from 2069 to 1051 mg/L in the first 2 days. At day 8, the polysaccharide concentration increased to 2350 mg/L after replenishment with fresh substrate. However, it decreased by 1445 mg/L in 2 days and gradually declined to 1037 mg/L during the next 4 days. In stage IIIa, the polysaccharide concentration decreased from 2965 to 1696 mg/L in 2 days and further decreased to 1355 mg/L during the next 4 days.

It is well known that free ammonia and un-dissociated VFAs are two kinds of toxic substance to the acidogenic microorganisms during the anaerobic digestion (Chen et al., 2008). However, the actual inhibitory effects of these substances are depending on various factors, such as their concentrations, pH value, acclimated inoculum sludge, etc. The results in this study indicated that inhibitory effects of VFAs and ammonia on the sludge fermentation were not obvious. However, Pratt et al. (2012) reported that the inhibitory effect of VFAs was observed and the inhibiting level of VFAs was 17 g/L at a pH of 6.0. In this study, alkaline fermentation was conducted at pH 10.0 which was beneficial for the VFAs dissociation and this might be the main reason for the difference from the results of Pratt et al. (2012). We determined and calculated the concentrations of un-dissociated VFAs and free ammonia at the beginning and the end of each fermentation. The results showed that the concentrations of un-dissociated VFAs and free ammonia are very low at the end of each fermentation (see Supplementary data, Table A.1). Acidogenic bacteria are very sensitive to the concentration of free-state of VFAs, whereas the concentrations of VFAs at the un-dissociated state are very low even at

very high total VFAs concentration (see Supplementary data, Table A.1). Therefore, acidogenic bacteria were able to maintain their growth and metabolic activity, leading to the continuous production of VFAs. The ammonia-N evaporation was prior to the fermentation process to study the effect of ammonia-N on VFAs fermentation. As shown in the fermentation stage IIIb (Figs. 1 and 2), with the evaporation of 75 mL water, the ammonia-N was also evaporated and removed. However, during the subsequent fermentation, the VFAs concentration was not increased, indicating that it is not the ammonia-N inhibiting the degradation of proteins, but the refractory protein limits the VFAs production from protein degradation. And some ions, eg. Na^+, were found to be antagonistic to ammonia inhibition (Chen et al., 2008).

3.3. Biodegradability of the residual substrates

Protein and polysaccharide in the residual post-fermented sludge were extracted and dissolved in distilled water to test their biodegradability after the alkaline fermentation. As shown in Fig. 3, when the residual substrate extract was used for fermentation, the degradation rate of residual substrates was as low as 26.7% with the VFAs concentration increased from 0.3 to 1.8 g/L. BOD_{sol}/COD_{sol} (B/C) was usually used to evaluate the biodegradability of substrates in the biological fermentation. As shown in Fig. 3, substrates showed good biodegradability at the initial of each stage with the values at 0.42, 0.26 and 0.22, respectively. However, after fermentation, the B/C ratios decreased rapidly. In the end of stage I, II, and IIIa, they are 0.05, 0.02 and 0.03, respectively. These results indicated that low biodegradability of the residual substrates in the post-fermented sludge might be the limiting factor for the substrate accumulation after the alkaline fermentation. Therefore, considering the results in Fig. 2 that the VFAs production can be re-initiated after the addition of fresh substrate and the low undissociated VFAs and free ammonia concentrations in Table A.1. Since it is hard to identify what proteins and polysaccharides are refractory proteins and polysaccharides, because it depends on the treatment and fermentation conditions in different studies. In this study, the residual proteins and polysaccharides after fermentation were defined as refractory proteins and polysaccharides. We conclude that the poor biodegradability, instead of the inhibition from free VFAs and ammonia, resulted in the low VFAs yield for the VFAs production by anaerobic fermentation.

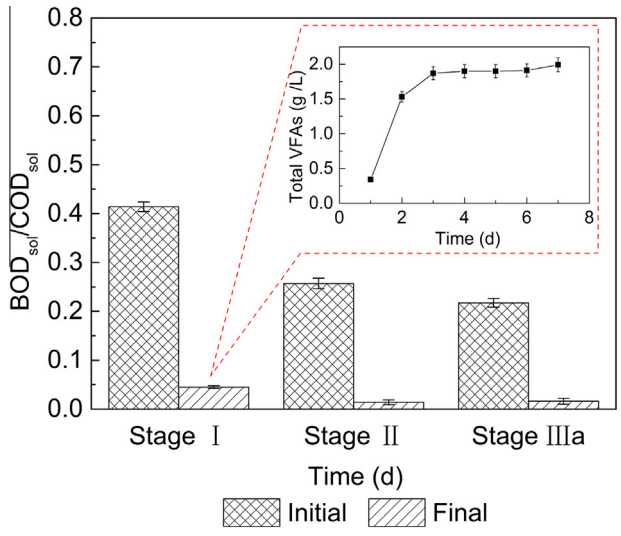

Fig. 3. Ratios of BOD_{sol}/COD_{sol} at the initial and the end of fermentation stage I, II and IIIa. The VFAs profile represented the VFAs concentration by using the residual substrate extract from the fermentation stage I.

3.4. VFAs production with protease addition from extracted residual substrates

Due to the low biodegradability of the residual protein in post-fermented sludge, protease was used to improve the biodegradability of protein. Trypsin and alkaline protease were used as models of protease for the protein biodegradation. As shown in Fig. 4A, both trypsin and alkaline protease could improve the degradation of refractory residual protein in post-fermented sludge. The concentration of residual protein (calculated as COD) was quickly reduced to 1754 from 4178 mg/L and finally remained at 1362 mg/L with the help of trypsin. Meanwhile, it reduced to 1804 from 3942 mg/L and finally reached 1255 mg/L with the addition of alkaline protease. Moreover, as shown in Table 3, further degradation of polysaccharide was also observed, from 3918 to 1368 mg/L, and 3417 to 1285 mg/L, upon addition of trypsin and alkaline protease, respectively. As shown in Fig. 4B, the VFAs yield was also greatly improved by the addition of trypsin and alkaline protease into the residual post-fermented sludge. VFAs concentrations reached 7120 mg/L with trypsin addition and 7480 mg/L with alkaline protease addition within 5 days. In the controlling experiment with protease but without residual substrates, VFAs concentration was about 1.0 g/L, suggesting that the VFAs produced from proteases and the organic matters in seeding sludge could be negligible.

During alkaline fermentation of sludge, substrate hydrolysis is mainly dependent upon extracellular enzymes, which hydrolyze macromolecular organic substrates into small molecules that are easily susceptible for acidogenic microbes (Confer and Logan,

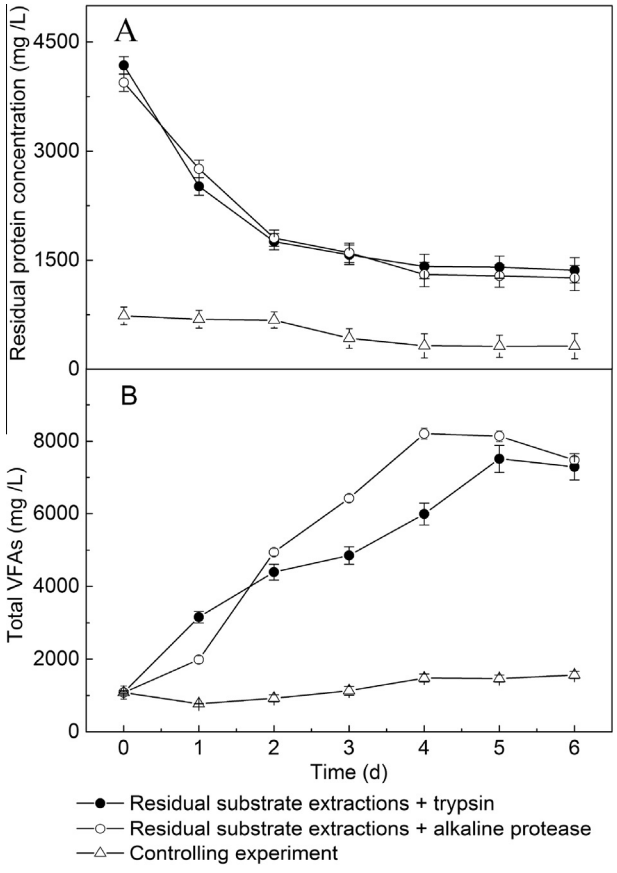

Fig. 4. Residual protein concentration (A) and VFAs productions (B) during the fermentation with residual substrate extract as substrate and trypsin or alkaline protease addition.

Table 3
The degradation rates of substrates and yield of VFAs.

Items	Stage I	Stage II	Stage IIIa	Trypsin addition	Alkaline protease addition
VFAs yield (g VFA-COD/g SCOD)	0.33	0.29	0.30	0.56	0.68
Protein degradation rate (%)	45.10	37.50	33.70	78.66	79.90
Polysaccharide degradation rate (%)	56.60	55.90	54.30	82.06	87.14

1997). According to Fig. 2, the protein and polysaccharide degradation mainly occurred in the first day of the fermentation process and accordingly that the VFAs production increased in the first day. With the fresh substrates addition, the VFAs production continued. The results of B/C ratios in Fig. 3 further explained the limited biodegradation of the substrates in the residual post-fermented sludge. Due to the low B/C ratio (<0.1) of the residual substrates, the biodegradation was limited and then the VFAs production was also limited at the end of each fermentation stage. Beside the poor biodegradability of the substrate, the low protease activity is another reason for the protein accumulation in the residual post-fermented sludge. Yuan et al. (2009) reported that the protein degradation rate and the activity of a protease are linearly correlated. According to Fig. 6, the protease activities decreased to their lowest level at the end of the alkaline fermentation, which caused the poor degradability of protein and its accumulation in the residual post-fermented sludge. It demonstrated the viewpoint of Maspolim et al. (2015) that organic matters with low biodegradability were unfavorable for subsequent biological processes during alkaline fermentation.

3.5. VFAs production with protease addition in residual post-fermented sludge

After the demonstration of VFAs improvement by protease addition in the fermentation with residual substrates extract as substrate, the effect of protease addition was further explored for the residual post-fermented sludge as substrate. As shown in Fig. 5A, further degradation of protein occurred and VFAs concentration increased again upon the protease addition. The results showed that the protein concentration gradually decreased from 3797 to 963 mg/L and from 3455 to 1114 mg/L upon the addition of trypsin and alkaline protease, respectively. Correspondingly, as shown in Fig. 5B, within 5 days, VFAs concentrations rose from 8990 to 13,950 mg/L and from 11,900 to 18,540 mg/L upon addition of trypsin and alkaline protease, respectively.

Table 3 summarized the comparison of VFAs yield and protein degradation rates between the alkaline fermentations with and without protease addition. It can be seen that in the alkaline fermentation the VFAs yield were 0.33, 0.29 and 0.30 g VFA-COD/g SCOD in the fermentation stage I, II and IIIa, respectively. However, with trypsin and alkaline protease addition, the VFAs yield increased to 0.56 and 0.68 g VFA-COD/g SCOD, which was 69.7% and 106.1% higher than the fermentation stage I, respectively. For the protein degradation rates, they increased by 33.6% and 34.8% higher than those in stage I with the addition of trypsin and alkaline protease, respectively.

It is well known that the protease pretreatment is an effective method to improve the VFAs yield from WAS (Liu et al., 2016). However, the improvement of the VFAs is mainly dependent on the cell lysis and damage, then enhancing the solubilization of proteins in the liquid phase. In the present study, the reason of protease addition in the post-fermented sludge instead of the raw sludge is to enhance the breakdown of the refractory protein, and then increasing the biodegradability of the accumulated protein in the post-fermented sludge. With the addition of trypsin and alkaline protease into the residual post-fermented sludge, the degradation of protein and the VFAs production from the residual protein were improved significantly (Figs. 4 and 5). During alkaline fermentation, the degradation rates of protein in liquid and solid fraction were 38% and 13%, respectively. However, the degradation rate of proteins was over 50% in both liquid and solid fraction after alkaline protease addition. According to Fig. 5C, it indicated that the increased VFAs were mainly from soluble substrates during the alkaline fermentation. The result was consistently with the report of Liu et al. (2012). And with addition of trypsin and alkaline protease, the degradation efficiency increased by 33.6% and 34.8%, respectively (Table 3). It should be noted that the trypsin showed a higher selectivity than the alkaline protease, only catalyzing the degradation reactions of lysine and arginine (Ohshima et al., 2008). In addition, alkaline protease can tolerate the alkaline condition at pH 10.0. Therefore, it is a better protein catalyst for the improvement of VFAs production from WAS.

3.6. Protease activity in the fermentation process

The enzyme activities of protease and β-glucosidase (β-GLC) were shown in Fig. 6. Usually, there is low protease activity in the initial stage of the fermentation, resulting from the adaptation of the seeding microorganisms to the conditions of the alkaline fermentation conditions (Yu et al., 2008). In the alkaline fermentation without protease addition, the indigenous protease decreased greatly in the first day and then increased gradually with the fermentation proceeded. The activity of β-GLC showed an obvious increase from 18.09 to 174.09 μmol/L/h and then decreased to 21.21 μmol/L/h in the first 2 days, and then maintained at about 20 μmol/L/h. With the addition of alkaline protease and trypsin at day 5, the protease activity increased significantly to 19.11 U/mL and 13.52 U/mL, respectively, and maintained at a high level in the later fermentation process. However, the activity of β-GLC only showed a slight increase with the addition of protease in the residual post-fermented sludge.

At the end of fermentation, refractory proteins accumulated because of its poor biodegradability and low protease activities. In this study, the addition of the protease into the residual post-fermented sludge improved the degradation of the refractory protein and provided more biodegradable substrates for the acidogenic microorganisms. It was reported that there was a positive correlation between the amounts of biodegradable substrates and enzyme activities (Goel et al., 1998; Li and Chróst, 2006). Interestingly, with the increase of the protease activity, the activity of β-GLC which was responsible for the degradation of the polysaccharide was also increased. The reason might be attributed to the complex type and structure of the proteins in sludge (De Kruif and Tuinier, 2001). For example, with the degradation of the complicated glycoprotein, not only the small molecular proteins and amino acids will be generated, but also the various small molecular polysaccharides or monosaccharide generated. The increase of biodegradable sugar compounds stimulated the activity of the β-glucosidase, which is also consistent with the theory of (Goel et al., 1998). We observed the significantly improvement of the polysaccharide products with the addition of protease (Table 3).

In Fig. 6 the hydrolase activities (protease and β-GLC) can last at least for 7 days in the alkaline fermentation, which are different from the indigenous hydrolase activities from the seeding sludge. Currently, the cost of protease addition is about 0.3 USD/kg TS

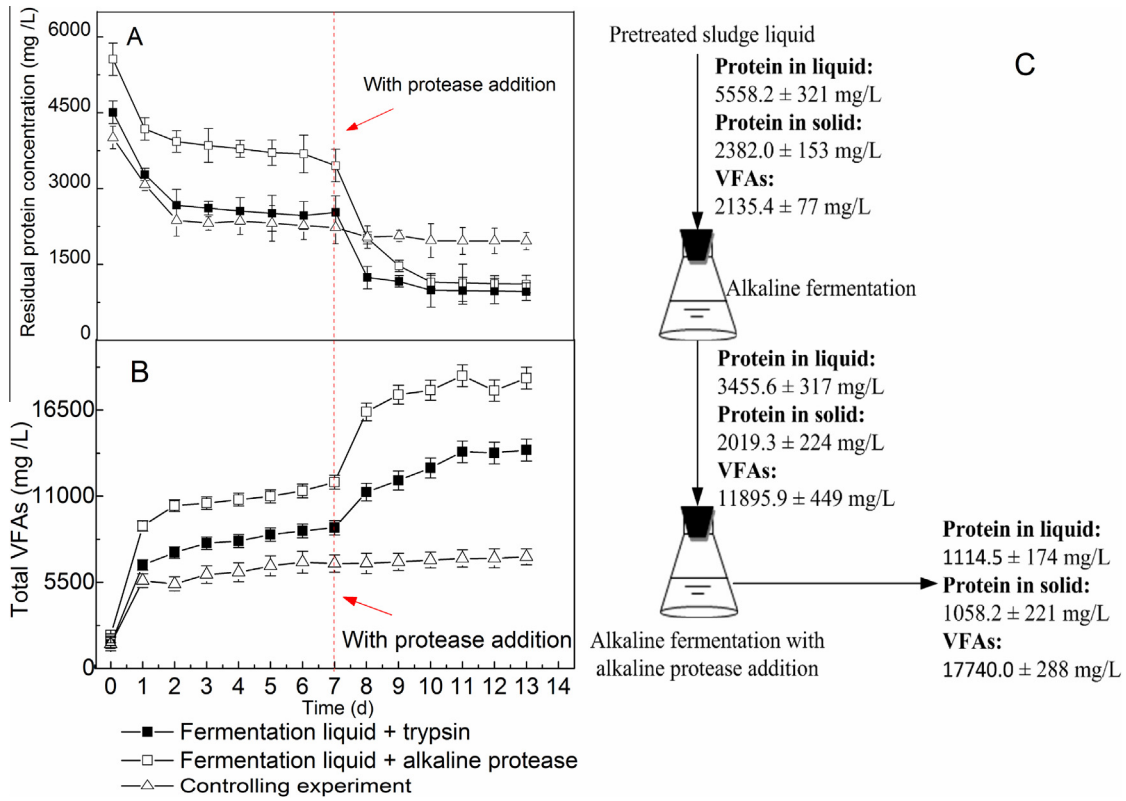

Fig. 5. Residual soluble protein concentrations (A) and VFAs productions (B) during the fermentation with residual fermented sludge as substrate and trypsin or alkaline protease addition. And the protein distribution in the supernatant vs. the solids fraction in the pretreatment sludge, fermentative sludge and fermentative sludge with alkaline protease treatment (C).

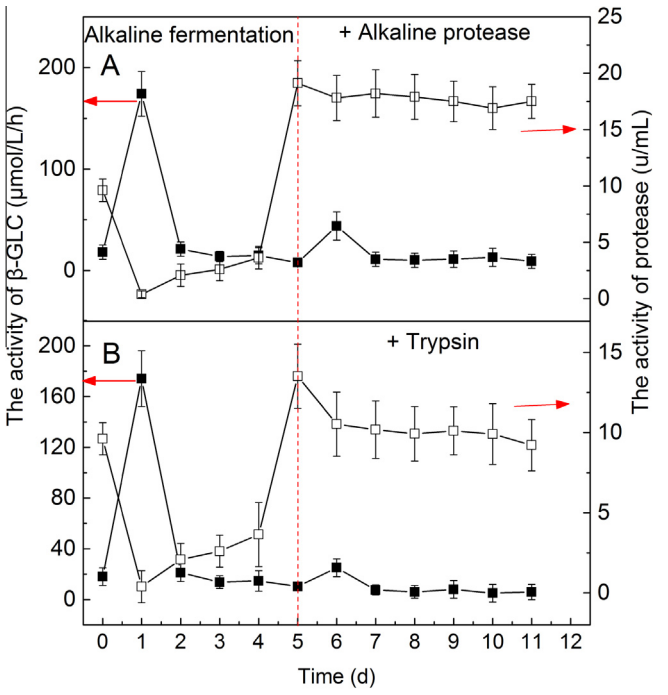

Fig. 6. Enzyme activity of alkaline protease (A) and trypsin (B) during the alkaline fermentation process.

sludge. Although the cost of protease addition is high, many studies reported that protease can be extracted and enriched from activated sludge (Karn and Kumar, 2015). For this reason, the protease is not necessary commercially purchased but can be produced from the sludge itself so that a sustainable alkaline fermentation technology of thermo-alkaline pretreatment combined with protease hydrolysis for enhancing VFAs production from WAS could be realized and become economically feasible in future as described in Fig. S1. Therefore, the VFAs production can be continuously carried out and improved with the presence of the hydrolase activities (Fig. 6). Consistent with the improvement of the protein degradation and increase of protease activity, the VFAs yield were increase by 69.7% and 106.1% with the addition of trypsin and alkaline protease, respectively (Table 3). Therefore, by addition of the protease into the residual post-fermented sludge, instead of the raw sludge, a novel process was successfully developed to increase VFAs production from the WAS.

4. Conclusions

A novel enhanced VFAs production process by anaerobic fermentation was successfully developed with protease addition into the residual post-fermented sludge. Although effective disintegration and solubilization in the alkaline fermentation for VFAs production, large amount of refractory proteins still accumulated in the residual post-fermented sludge. The results show that poor biodegradability of residual protein and the fast decrease of indigenous hydrolase activity are the main reasons for low yield of VFAs from WAS. VFAs yield was significantly improved by adding trypsin and alkaline protease at the end of sludge fermentation processes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.12.029.

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