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# Free nitrous acid serving as a pretreatment method for alkaline fermentation to enhance short-chain fatty acid production from waste activated sludge

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ABSTRACT

Alkaline condition (especially pH 10) has been demonstrated to be a promising method for short-chain fatty acid (SCFA) production from waste activated sludge anaerobic fermentation, because it can effectively inhibit the activities of methanogens. However, due to the limit of sludge solubilization rate, long fermentation time is required but SCFA yield is still limited. This paper reports a new pretreatment method for alkaline fermentation, i.e., using free nitrous acid (FNA) to pretreat sludge for 2 d, by which the fermentation time is remarkably shortened and meanwhile the SCFA production is significantly enhanced. Experimental results showed the highest SCFA production of 370.1 mg COD/g VSS (volatile suspended solids) was achieved at 1.54 mg FNA/L pretreatment integration with 2 d of pH 10 fermentation, which was 4.7- and 1.5-fold of that in the blank (uncontrolled) and sole pH 10 systems, respectively. The total time of this integration system was only 4 d, whereas the corresponding time was 15 d in the blank and 8 d in the sole pH 10 systems. The mechanism study showed that compared with pH 10, FNA pretreatment accelerated disruption of both extracellular polymeric substances and cell envelope. After FNA pretreatment, pH 10 treatment (1 d) caused 38.0% higher substrate solubilization than the sole FNA, which indicated that FNA integration with pH 10 could cause positive synergy on sludge solubilization. It was also observed that this integration method benefited hydrolysis and acidification processes. Therefore, more SCFA was produced, but less fermentation time was required in the integrated system.



## 1. Introduction

Short-chain fatty acids (SCFA) is a raw substrate for biodegradable plastic production and a preferred carbon source for biological nutrient removal (BNR) (Guo et al., 2007; Wang et al., 2012a, 2012b). Meanwhile, as a byproduct of wastewater treatment, waste activated sludge (WAS) is generated in huge quantities daily (Ni and Yu, 2008; Li and Yu, 2011; Zhao et al.,

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2015). WAS contains high levels of organic matter, which makes it a plentiful inexpensive source for SCFA production (Yuan et al., 2006; Wang et al., 2015). Thus, the production of SCFA from WAS has recently attracted growing attention (Jiang et al., 2009; Yuan et al., 2006; Zhang et al., 2010), by which WAS is reduced and reused, and the value added product SCFA is achieved.

The yield of SCFA from WAS was usually at low levels due to low rate of sludge solubilization and rapid consumption by methanogens (Yuan et al., 2006; Chen et al., 2013). To promote SCFA production, several pretreatment and/or fermentation control strategies such as acid, alkaline, thermal, Fenton, ozone, and ultrasonic have been tested (Carrère et al., 2010; Lee et al., 2014; Liu et al., 2012). Among them, alkaline (especially pH 10) fermentation has been verified to be a promising method, because this method can effectively inhibit the activities of methanogens (Yuan et al., 2006). It was reported that the number of total methanogenic archaea under pH 10 condition was much lower than that under pH uncontrolled condition (Zhang et al., 2010). Further investigation showed that pH 10 significantly decreased the relative abundances of *Methanobacterium* sp. and *Methanobrevibacter* sp. (Zheng et al., 2013). Additionally, alkaline fermentation of WAS, separation of the fermentation liquid, and application of the fermentation liquid to promote municipal nutrient removal have already been confirmed by a pilot-scale work carried out in a full-scale wastewater treatment plant (WWTP), exhibiting an excellent application perspective (Li et al., 2011).

pH 10 can promote the abundance of *Pseudomonas* sp., the microbe with the ability of excreting extracellular protease (Zheng et al., 2013), which thereby accelerating sludge solubilization to some extent. However, the sludge solubilization rate of this method is still limited. Previous studies showed that it would take approximately 8 d to achieve the maximal SCFA accumulation at pH 10 (Wang et al., 2013a; Yuan et al., 2006). Even under this fermentation time, more than 60% of volatile suspended solids (VSS) could not be degraded (Zhang et al., 2009). These results indicate that further improvement of SCFA production under pH 10 fermentation may be possible. Due to the huge quantity of WAS generated daily, any enhancement in current pH 10 fermentation should have important economic and ecological consequences.

Recently, free nitrous acid (FNA) has been demonstrated to cause a strong biocidal impact on microorganisms in WAS at mg/L levels (Pijuan et al., 2012). Pijuan et al. reported that WAS treatment with 1–2 mg/L of FNA for 24–48 h killed 50–80 % of sludge biomass, which substantially increased sludge biodegradability (Pijuan et al., 2012). Wang and his co-workers proved that both the hydrolysis rate and biochemical methane potential of WAS were significantly enhanced when WAS was pretreated with 2.13 mg/L of FNA for 24 h (Wang et al., 2014b, 2013b). According to the information documented in the literature, it is presumed that FNA-based technique might be beneficial to sludge solubilization. Since FNA is a renewable and cost-effective chemical that can be produced in situ in WWTPs by partial nitrification of the anaerobic digestion liquor (Law et al., 2015; Wang et al., 2013b), FNA seems to be a promising pretreatment technique for alkaline fermentation. To date, however, the effect of FNA pretreatment on SCFA production from sludge anaerobic alkaline fermentation has seldom been reported. Although some scientists pointed out that WAS with FNA treatment could increase sludge particulate release (Wang et al., 2014a), the underlying mechanisms are still needed to be elucidated. Moreover, whether FNA pretreatment integration with alkaline fermentation causes positive synergies on SCFA production also remains unknown.

The purpose of this study is to assess the feasibility of FNA serving as a pretreatment method for alkaline fermentation to enhance SCFA production yield and rate from WAS. Firstly, the pretreatment time of FNA was optimized by analyzing the change of soluble chemical oxygen demand (COD) concentration with pretreatment time. Then, the effect of different levels of FNA pretreatment on SCFA production from sludge anaerobic alkaline (pH 10) fermentation was investigated. Finally, the mechanisms for FNA pretreatment integration with pH 10 fermentation enhancing SCFA were explored by analyzing the role of FNA on disruption of both extracellular polymeric substances (EPS) and cell envelope, the synergetic effect of FNA integration with pH 10 on the processes of solubilization, hydrolysis, and acidification, and the activities of key enzymes. To the best of our knowledge, this is the first report revealing the underlying mechanisms of FNA accelerating anaerobic sludge solubilization and applying FNA pretreatment to enhance SCFA production from WAS alkaline fermentation. The findings obtained in this work may help to develop a new promising solution to "nutrient removal-energy recovery" challenge faced by WWTPs.

## 2. Materials and methods

### 2.1. Source of WAS

The WAS used in this study was obtained from the secondary sedimentation tank of a municipal WWTP with sludge retention time of 20 d in Changsha, China. The collected sludge was concentrated for 24 h by settling at 4 °C before use, and the main characteristics of the concentrated WAS are as follows: pH 6.8 ± 0.1, total suspended solids (TSS) 11.8 ± 0.8 g/L, VSS 9.9 ± 0.4 g/L, total COD 12.1 ± 1.2 g/L, total protein 8.0 ± 0.4 g COD/L, total polysaccharide 1.3 ± 0.12 g COD/L (Error bars show 95% confidence intervals).

### 2.2. Optimization of FNA pretreatment time

This test was conducted in six identical serum bottles with a working volume of 1000 mL each. Each of serum bottles was fed with 600 mL WAS, and then different volumes of 2.0 M $NaNO_2$ stock solution were added into these bottles to gain 0, 200, 300, 600, 900, 1200 mg $NO_2^-$-N/L, respectively. All serum bottles were maintained at 20 ± 1 °C in a temperature-controlled room and stirred at a speed of 120 rpm. The pH was maintained at 6 by adding 2.0 M hydrochloric acid or 2.0 M sodium hydroxide. The temperature, pH and $NO_2^-$-N concentration applied here gave rise to initial FNA concentration of 0, 0.51, 0.77, 1.54, 2.31, and 3.08 mg/L in these reactors, respectively, which were determined by the formula $FNA = S_{NO2\text{-}N}^- / (K_a \times 10^{pH})$ with $K_a$ value determined by the formula

$K_a = e^{(-2300/(T+273))}$ for a given temperature T (°C) (Anthonisen et al., 1976).

### 2.3. Effect of FNA pretreatment concentration on SCFA production from WAS anaerobic alkaline fermentation

This batch test was performed in eight identical reactors (1000 mL). Each reactor was fed with 600 mL WAS. All reactors were mixed with stirrers at a speed of 120 rpm in a 20 ± 1 °C temperature-controlled room. pH adjustment was achieved by adding 2.0 M hydrochloric acid or 2.0 M sodium hydroxide through a programmable logic controller. Different volumes of $NaNO_2$ stock solution (2.0 M) were respectively added into reactor 1–5 to obtain 200, 300, 600, 900, and 1200 mg $NO_2^-$-N/L. The pH in reactor 1-5 during FNA pretreatment phase was controlled at 6, which resulted in 0.51, 0.77, 1.54, 2.31, and 3.08 mg/L of initial FNA concentration in these reactors, respectively. According to the results of above batch test, FNA pretreatment in these reactors was lasted for 2 d. After that, reactor 1–5 was moved to the step of alkaline fermentation by controlling pH at 10. For comparison, another three reactors (i.e., sole FNA treatment, sole pH 10 fermentation, and blank) were also performed. The pH and original concentration of nitrite in the sole FNA reactor was respectively controlled at 6 and 600 mg/L (1.54 mg/L of initial FNA concentration) since it was the optimal pretreatment concentration based on the preliminary result of this test. No nitrite stock was added into the sole pH 10 reactor, and the pH in this reactor was constantly controlled at 10. In the blank one, nitrite stock was not added and pH was also not adjusted.

### 2.4. Differentiation of the contribution of FNA, $NO_2^-$-N, and weak acid (pH 6) to the increased SCFA production

By comparing the operations between the sole pH 10 and FNA integration with pH 10 reactors, it can be found that apart from FNA, $NO_2^-$-N and pH were also different. $NO_2^-$-N and pH may also make contributions to the increased SCFA production. To differentiate their contributions, a batch test with three identical serum bottles, which were respectively defined as FNA-reactor, $NO_2^-$-N-reactor, and pH 6-reactor, was carried out. The initial nitrite concentration in the FNA-reactor and $NO_2^-$-N-reactor was controlled at 600 mg/L while no nitrite was added into the pH 6-reactor. The pH in the FNA-reactor and pH 6-reactor was maintained at 6 in the first two days and then controlled at 10 in the remaining fermentation. To minimize the FNA influence, the pH in the $NO_2^-$-N-reactor was maintained at 10 all the time (initial FNA concentration: $1.54 \times 10^{-4}$ mg/L). All other operations were the same as described above.

### 2.5. Comparison of the effect of FNA and pH 10 on disruption of EPS and cell envelope

EPS and cell envelope are the key substances protecting cells against detrimental environments (Fig. S1, Supporting Information), thus their disruptions are directly relevant to the rate of sludge solubilization. The following batch experiments aim to compare the effect of FNA and pH 10 on disruptions of both EPS and cell envelope. Six serum bottles (each with a working volume of 250 mL) were divided into two groups with three in each. Group-I is used to compare the disruption rate of EPS caused by FNA and pH 10, while group-II is employed to evaluate the effect of FNA and pH 10 on the disruption of cell envelope.

Each bottle of group-I was fed with 100 mL of WAS. The operational conditions of these three bottles were the same as the sole FNA, sole pH 10, and blank reactors described in the Section 2.3, respectively. The carbohydrate, protein, and COD concentrations released in liquid phase were determined periodically during WAS treatment. After measuring the soluble COD, WAS was centrifuged (4000 rpm for 5 min) and re-suspended in tap water with the initial volume after decanting the supernatant. Then the sludge was undergone by a heat extraction process reported in the literature, which was widely used to extract both loosely and tightly bound EPS of sludge (Li and Yang, 2007). To judge whether cell envelope disruption occurs, COD mass balance was made according to the analysis of the total COD measured in the initial EPS, soluble COD content, and extracted COD by heating method. If the sum of the latter two does not show a significant increase as compared with the total COD in initial EPS, the disruption of cell envelope will not happen; or else, it will occur. The change of EPS structure was also measured with treatment time.

The sludge used in the group-II test should contain no/little EPS to eliminate the influence of EPS on disruption of cell envelope. To obtain this sludge, 100 mL WAS was centrifuged at 4000 rpm for 5 min and was re-suspended in tap water with a final volume of 300 mL. Then the extraction of both loosely and tightly bound EPS of sludge was performed by the heat extraction method (Li and Yang, 2007). Adenosine-triphosphate analysis showed that compared with the blank, insignificant release of adenosine-triphosphate was detected after this extraction process ($p > 0.05$), which confirmed that the cell envelope was not significantly destroyed by this EPS extraction method (Takahashi et al., 2009). After that, the sludge pellet was resuspended in tap water with a final volume of 300 mL before being divided equally into three serum bottles. The treatment conditions of these reactors were also the same as the sole FNA, sole pH 10, and blank reactors, respectively.

### 2.6. Effect of sole FNA, sole pH 10, and FNA integration with pH 10 on hydrolysis of solubilized sludge organic matter

This batch experiment was conducted using synthetic wastewater containing bovine serum albumin (BSA, average molecular weight 67000, model protein compound) and dextran (average molecular weight 23800, model polysaccharide compound). 1.8 L of synthetic wastewater with 5.35 g BSA/L and 1.26 g dextran/L (the mass ratio of protein and carbohydrate was almost the same as that in raw WAS) was divided equally into three serum bottles with a working volume of 1 L each. Then, 60 mL of WAS was added into each serum bottle as an inoculum. The initial FNA concentration in the sole FNA and FNA + pH 10 reactors was controlled at 1.54 mg/L. The pH in the FNA + pH 10 reactor was controlled at 6 during the initial 2 d and then maintained at 10 in the remainder treatment. The pH in the sole FNA and sole pH 10

reactors was respectively maintained at 6 and 10. All other operation conditions were the same as depicted above.

### 2.7. Effect of sole FNA, sole pH 10, and FNA integration with pH 10 on acidification of hydrolyzed products

This batch test was performed with the same method described in the Section 2.6 except that L-alanine (model amino acid compound) and glucose (model monosaccharide compound) were used to replace BSA and dextran, respectively.

### 2.8. Analytical methods

$NH_4^+$-N, $NO_2^-$-N, $PO_4^{3-}$-P, COD, TSS, VSS were determined according to standard methods (APHA, 1998). Soluble protein and carbohydrate were determined by the Lowry-Folin method with BSA as the standard and the phenol-sulfuric method with glucose as the standard, respectively (Lowry et al., 1951; Herbert et al., 1971). The COD conversion coefficients are 1.5 g COD/g protein and 1.06 g COD/g carbohydrate (Wang et al., 2015). The measurement of SCFA was the same as described in the literature (Yuan et al., 2006), and the composition in biogas was analyzed by gas chromatograph equipped with a thermal conductivity detector according to the method documented in the literature (Xie et al., 2012; Wang et al., 2015). L-alanine was determined according to the method documented in the literature (Bergmeyer and Horder, 1980). The activities of key hydrolytic enzymes (protease and α-glucosidase) were determined according to the method developed by Goel et al. (Geol et al., 1998), and the activities of key acid-forming enzymes, phosphotransacetylase (PTA), phosphotransbutyrylase (PTB), acetate kinase (AK), butyrate kinase (BK), oxaloacetate transcarboxylase (OAATC), CoA transferase, were measured according to the method reported in the literature (Feng et al., 2009). The measurement of adenosine-triphosphate was performed according to the method documented in the literature (Takahashi et al., 2009). Excitation emission matrix (EEM) fluorescence spectroscopy was applied to characterize the changes of EPS structure. EEM fluorescence spectroscopy was determined via a luminescence spectrometry (Fluoromax-4 Spectrofluorometer, HORIBA Scientific, France) with a 450 W Xe arc lamp, and the detailed method was reported previously (Luo et al., 2013).

### 2.9. Statistical analysis

All experiments were performed in triplicate. An analysis of variance was applied to evaluate the significance of results, and $p < 0.05$ was considered to be statistically significant.

## 3. Results and discussion

### 3.1. Effect of FNA pretreatment concentration on SCFA production

In this study, FNA was mainly used as a pretreatment tool for the enhancement of sludge solubilization. By analyzing the change of soluble COD with treatment time, the optimal pretreatment time can be achieved. It can be seen from Fig. S2 (Supporting Information) that soluble COD was increased with treatment time during the initial 2 d no matter what level of FNA selected in this study was tested, and further increase of treatment time did not cause significant increase of soluble COD concentration ($p > 0.05$). Thus, the optimal FNA pretreatment time was 2 d.

Fig. 1 shows the SCFA production under different operational conditions. The maximal SCFA production of 237.9 mg COD/g VSS was achieved at 8 d in the sole pH 10 reactor, which was 3.1-fold of that obtained in the blank one. All these observations were similar to those reported in the literature (Yuan et al., 2006; Li et al., 2011). Although the optimal fermentation time of sole FNA reactor was lower than that of sole pH 10 reactor (6 vs 8 d), the maximal SCFA yield in the former was also lower than that in the latter (195.7 vs 237.9 mg COD/g VSS). However, when FNA pretreatment and pH 10 fermentation were combined, the SCFA production was remarkably enhanced, but the required fermentation time was largely shortened. With the increase of FNA pretreatment level from 0.51 to 1.54 mg FNA/L the maximal SCFA production increased from 299.4 to 370.1 mg COD/g VSS. When FNA concentration further increased to 3.08 mg FNA/L, the SCFA generation was decreased to 285.3 mg COD/g VSS. Thus, the optimal FNA pretreatment concentration was 1.54 mg/L. It was emphasized that the optimal treatment time was 4 d (2 d pretreatment + 2 d fermentation) in all FNA pretreatment + pH 10 fermentation reactors whereas this value was 8 d in the sole pH 10 reactor. Clearly, FNA pretreatment not only enhanced the SCFA production yield but also accelerated the SCFA production rate.

Fig. S3 (Supporting Information) exhibits the percentage of individual SCFA in different reactors under their optimal fermentation conditions. The top two individual SCFAs in any investigated reactor were acetic acid and propionic acid, with a total percentage of 62.0–68.1%. The n-valeric acid was the

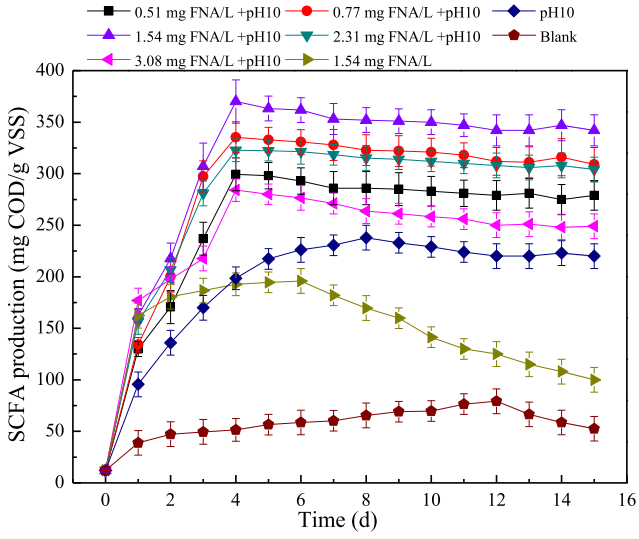


**Fig. 1 – The total SCFA production under different operational conditions during sludge anaerobic fermentation. Error bars show 95% confidence intervals.**

lowest SCFA production in all reactors, which was less than 5.0% of the total SCFA. Further analysis showed that the average order of individual SCFA in all reactors was in the sequence of acetic > propionic > iso-valeric > n-butyric > iso-butyric > n-valeric.

### 3.2. Do nitrite addition and weak acid (pH 6) condition in the pretreatment step contribute the increased SCFA production?

From the "Materials and methods" section, it can be found that different FNA levels in the pretreatment step were achieved by controlling pH 6 and different concentrations of nitrite. Apart from FNA, it was obvious that nitrite and pH in these integrated reactors were also different from those in the sole pH 10 reactor. It was reported that nitrite can be removed via denitrification in both acidic and alkaline fermentation systems (Wang et al., 2014a), and nitrite reduction was also observed in all nitrite added reactors in this study (Table S1, Supporting Information). Thus, it is necessary to confirm whether nitrite and pH 6 in the pretreatment step make contributions to the increased SCFA production. Experimental results showed that both soluble COD (2 d) and SCFA production (4 d) in the $NO_2^-$-N-reactor and pH 6-reactor were much lower than those in the FNA-reactor (Table S2, Supporting Information). Compared with the sole pH 10-reactor, the pH 6-reactor showed lower soluble COD and SCFA production. Though soluble COD and SCFA production measured in the $NO_2^-$-N-reactor were slightly higher than those detected in the sole pH 10-reactor, further analysis found that their increases were both insignificant ($p > 0.05$). The results revealed that nitrite and pH 6 did not significantly contribute the increased SCFA production and further confirmed that FNA was the contributor.

### 3.3. Is FNA more beneficial to sludge solubilization than pH 10?

EPS and cell envelope are the key substances protecting cells against hostile environments, thus their disruptions are closely relevant to cell lysis (Fig. S1, Supporting Information). To understand how FNA pretreatment enhanced SCFA production, comparison of the effect of FNA and pH 10 on the disruption of EPS and cell envelop was first made. Fig. 2a shows the changes of soluble protein, carbohydrate, and COD during the initial 12 h of treatment. According to the results of COD mass balance analysis (Table S3, Supporting Information), cell envelop did not disrupt during the initial 10 h of treatment in all investigated reactors, because the total amount of soluble COD and extracted COD by heating method showed an insignificant increase as compared with the total COD measured in initial EPS ($p > 0.05$). When the sludge was treated at 1.54 mg FNA/L for 12 h, the sum of soluble COD and extracted COD by heating method was significantly higher than the total COD measured in initial EPS ($p < 0.05$), which indicated that the disruption of cell envelope happened. Thus, the soluble COD and protein (carbohydrate) during the initial 10 h of treatment can be applied to indicate EPS disruption. As seen from Fig. 2a, all the soluble protein, carbohydrate, and COD in the sole FNA reactor were higher than those in the sole pH 10 one, which revealed that FNA was more beneficial to EPS disruption, as compared with pH 10.

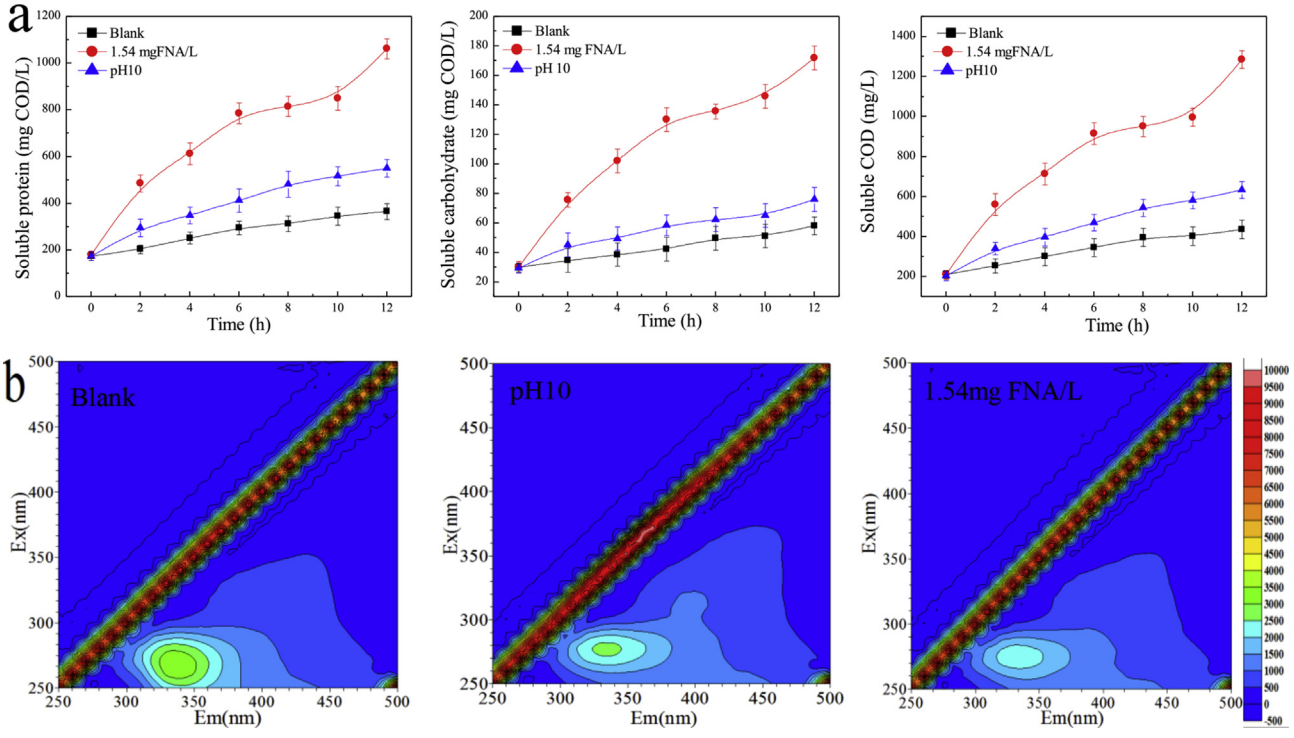


**Fig. 2 – Variation of soluble EPS with time (a) and EEM profile of sludge EPS (b) at 10 h of treatment in the sole FNA (1.54 mg FNA/L), sole pH10, and blank reactors.**

**Table 1 – Comparison of soluble protein and carbohydrate concentrations and VSS reduction among the sole FNA-, sole pH10-, and blank reactors on 2 d.[a]**

| Item | 1.54 mg FNA/L-reactor | pH 10-reactor | Blank |
|---|---|---|---|
| Soluble protein (mg COD/L) | 1524.3 ± 91.2 | 924.6 ± 83.0 | 432.7 ± 49.2 |
| Soluble carbohydrate (mg COD/L) | 263.8 ± 37.1 | 187.6 ± 44.7 | 84.6 ± 16.2 |
| VSS reduction rate (%) | 38.4 ± 9.0 | 25.6 ± 5.2 | 17.2 ± 3.4 |

[a] Error bars show 95% confidence intervals.

The above result can be further supported by EEM profiles (Fig. 2b). The EEM fluorescence spectroscopy is widely applied to characterize the changes of EPS structure (Chen et al., 2013; Sheng and Yu, 2006). It was reported that the main peak of tryptophan protein-like substances was located at the Ex/Em of 275/340 nm, and blue-shift in EEM was related to the structure changes of EPS (Sheng and Yu, 2006; Chen et al., 2013). It was observed that pH 10 and 1.54 mg/L of FNA decreased the fluorescence intensity to 89.7 and 61.3% of the blank, respectively. In addition, pH 10 caused 5 nm of emission wavelength blue shift (from 345 nm to 340 nm) as compared with the blank, while 1.54 mg/L of FNA resulted in 10 nm of emission wavelength blue shift (from 345 nm to 335 nm). All the facts revealed that FNA caused severer disruption of EPS than pH 10, which was consistent with the data presented in Fig. 2a. Previous publication also reported that FNA itself and its derivatives could damage proteins in cells/EPS by reacting with them (Yoon et al., 2006).

To assess the potential different impacts of FNA and pH 10 on the disruption of cell envelope, another batch experiment using sludge with EPS extraction was carried out, and the results are displayed in Table 1. Since the sludge with EPS extraction applied in this batch study was the same, protein (carbohydrate) release and VSS reduction could be used to indicate the disruption of cell envelope. From Table 1, it can be found that both pH 10 and FNA treatments caused much higher disruption of cell envelop than uncontrolled condition. Compared with pH 10, 1.54 mg FNA/L gave rise to 64.9% (40.6%) higher of protein (carbohydrate) release and 50.0% higher of VSS reduction, respectively.

Based on the above results, it can be concluded that FNA was more beneficial to the disruption of both EPS and cell envelope, as compared with pH 10. Therefore, more VSS was degraded, and more sludge organic matters such as protein and carbohydrate were released, which thereby provided more soluble substrates for subsequent hydrolysis and acidification (Fig. 3).

### 3.4. *Does FNA integration with pH 10 cause synergetic effect on the processes of solubilization, hydrolysis, and acidification?*

In the integration systems, the reactors were maintained at pH 10 after FNA pretreatment. Both FNA and pH 10 are extremely hostile environments to cells, thus whether the combination of these two strategies resulted in any synergetic impact was further investigated. The following four steps: solubilization, hydrolysis, acidification, and methanation are usually included during sludge anaerobic digestion. Since pH 10 has already been demonstrated to effectively inhibit the activities of methanogens (Yuan et al., 2006; Zhang et al., 2010), the evaluation of potential synergy of FNA integration with pH 10 was mainly focused on the three former steps.

From Fig. 3b, it can be observed that with the increase of treatment time from 48 h to 72 h soluble COD was increased by 16.7% (from 2103 to 2454 mg/L) in the sole FNA reactor and 41.5% (from 1247 to 1765 mg/L) in the sole pH 10 reactor, respectively. In the 1.54 mg FNA/L + pH 10 reactor, however, soluble COD was increased by 54.7% (from 2164 to 3348 mg/L) during this period. Clearly, not only the concentration but also the increase ratio of soluble COD in the 1.54 mg FNA/L + pH 10 reactor was higher than that in the sole FNA and sole pH 10 ones. Similar observation was also made in other FNA + pH 10 reactors. It seems that alternating FNA and pH 10 treatments give rise to a positive synergy on sludge solubilization.

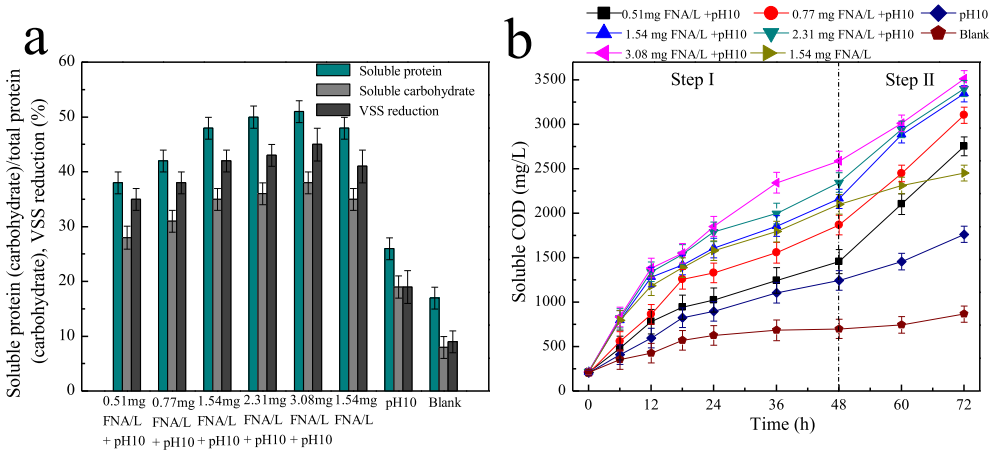


**Fig. 3 – Comparison of the soluble protein (carbohydrate) release ratios and VSS reduction at 2 d of treatment (a) and soluble COD concentration during the initial 3 d treatment (b). Error bars show 95% confidence intervals. Step I: pH was controlled at 6 in all the FNA + pH 10 reactors. Step II: pH was controlled at 10 in all the FNA + pH 10 reactors.**

After sludge solubilization, the solubilized substrates will undergo hydrolysis and acidification processes before they are finally converted to SCFA. The yield and fermentation time of SCFA production are closely related to the rates of hydrolysis and acidification. To evaluate the potential different effects of sole pH 10, sole FNA, and FNA + pH 10 on hydrolysis and acidification processes, batch experiments were conducted using synthetic wastewaters containing BSA and dextran, and L-alanine and glucose, respectively. Sole FNA (1.54 mg/L) caused higher degradation rates of all compounds than sole pH 10 no matter what treatment time was (Table 2). The treatment condition of 1.54 mg FNA/L + pH 10 reactor was the same as that of sole FNA (1.54 mg/L) reactor during the initial 2 d, thus the degradation rates of all compounds between the two reactors were approximately the same during this time. Nevertheless, when pH value in the 1.54 mg FNA/L + pH 10 reactor was controlled at 10 on 3 d, the degradation rates of BSA, dextran, L-alanine, and glucose were respectively increased to 79.8, 88.4, 78.2, and 81.5%, which were higher than those in the sole FNA reactor. The results showed that combining FNA with pH 10 enhanced both hydrolysis and acidification processes, it can be therefore understood that these integration systems produced greater SCFA but required lower treatment time.

SCFA production from WAS at pH 10 was mainly dominated by biological effects (Yuan et al., 2006), and determination of enzymes activities is an alternative method to evaluate microbial activity (Nybroe et al., 1992). Thus, the activities of key enzymes relevant to hydrolytic and acid-forming microbes were also assayed. Protease and α-glucosidase are respectively responsible for protein and polysaccharide hydrolysis (Goel et al., 1998), while PTA, AK, PTB, BK, OAATC, and CoA transferase are some key enzymes relevant to SCFA production (Feng et al., 2009). Accordingly, these enzymes are selected to be measured in this study, and the results are shown in Table S4 (Supporting Information). It can be observed that although pH 10 resulted in much higher increase of key enzyme activities than uncontrolled condition, FNA pretreatment could cause further enhancement. For instance, when 1.54 mg FNA/L pretreatment was applied for pH 10 fermentation, the relative activities of protease, α-glucosidase, AK, PTA, BK, PTB, OAATC, and CoA transferase increased by 35.9, 5.9, 12.6, 15.3, 38.3, 81.8, 56.3, and 73.8% than those in the sole pH 10 reactor, respectively. Similar observation was also observed in other FNA pretreatment reactors. In addition, COD mass balance analyses revealed that the VSS reduction in these reactors was in the order of FNA pretreatment + pH 10 > pH 10 > blank while methane production showed the opposite sequence (Fig. S4, Supporting Information). All these measurements were in agreement with their results of SCFA production.

From Fig. S2 (Supporting Information), it can be seen that soluble COD increased with the increase of FNA concentration, which implies that the higher FNA level is applied, the more substrates will be solubilized and provided for subsequent SCFA production. Nevertheless, the data of SCFA production presented in Fig. 1a clearly showed that the increase of FNA level from 1.54 to 3.08 mg/L caused SCFA accumulation not increase but decrease. As thus, the question as to why the increase of FNA concentration from 1.54 to 3.08 mg FNA/L posed a negative impact on SCFA accumulation needed to be discussed. Although 2.31 and 3.08 mg/L of FNA pretreatment resulted in higher sludge solubilization than 1.54 mg/L one at the end of pretreatment time (2 d), no significant increase of soluble COD was observed when 12 h of pH 10 fermentation was followed ($p > 0.05$, Table S5, Supporting Information). Similar observation was also made when 24 h of pH 10 fermentation was followed (Fig. 3b). Furthermore, the activities of key enzymes relevant to hydrolytic and acid-forming microbes in 2.31 and 3.08 mg FNA/L pretreatment reactors were found to be lower than those in the 1.54 mg/L one (Table S4, Supporting Information). It was reported that FNA had a strong biocidal impact on microorganisms in WWTPs (Zhou et al., 2011). Since WAS was employed for both substrate and inoculum in this study, it seems that 2.31 and 3.08 mg/L of FNA pretreatment caused severer inhibition to hydrolytic and acid-forming microbes than 1.54 mg/L one, which thereby resulted in lower SCFA production.

### 3.5. *FNA pretreatment + pH 10 fermentation as a potential strategy for enhancing SCFA production*

Several methods have been applied to enhance SCFA production from WAS, Table S6 outlines some representative methods documented in the literature. Among these available methods, pH 10 has been verified to be the most efficient way to inhibit the activities of methanogens. Compared with the sole pH 10, SCFA production could be enhanced by

**Table 2 – Effect of sole FNA, sole pH 10, and FNA + pH10 on degradation rate of model compounds with time.[a]**

| Treatment condition | Time (d) | Degradation rate (%) | | | |
|---|---|---|---|---|---|
| | | BSA | Dextran | L-alanine | Glucose |
| 1.54 mg FNA/L | 1 | 28.1 ± 6.5 | 42.4 ± 6.5 | 40.1 ± 5.2 | 32.1 ± 6.5 |
| | 2 | 52.4 ± 9.5 | 68.4 ± 7.7 | 63.3 ± 7.3 | 65.2 ± 5.2 |
| | 3 | 60.2 ± 5.2 | 72.6 ± 9.5 | 67.2 ± 9.0 | 76.4 ± 6.9 |
| pH 10 | 1 | 19.4 ± 6.5 | 34.3 ± 4.7 | 32.6 ± 6.0 | 28.6 ± 4.7 |
| | 2 | 29.5 ± 9.9 | 51.4 ± 5.6 | 50.7 ± 4.7 | 42.1 ± 5.2 |
| | 3 | 45.3 ± 9.0 | 67.2 ± 7.7 | 61.3 ± 5.2 | 67.4 ± 6.0 |
| 1.54 mg FNA/L + pH 10 | 1 | 30.1 ± 7.7 | 42.6 ± 6.5 | 41.8 ± 8.2 | 33.6 ± 5.6 |
| | 2 | 56.8 ± 9.0 | 68.2 ± 9.0 | 63.4 ± 6.5 | 62.1 ± 4.7 |
| | 3 | 79.8 ± 10.3 | 88.4 ± 6.0 | 78.2 ± 6.9 | 81.5 ± 8.2 |

[a] Error bars show 95% confidence intervals.

approximately 50.5% by applying 1.54 mg/L FNA pretreatment before pH 10 fermentation. Similar to other technologies applied in industries, in a WAS producing SCFA concept the yield of SCFA should be maximized, while the input should be minimized. Based on this principle, it seems that FNA pretreatment + pH 10 fermentation, as proposed in this study, is a promising and practical technology. Unlike other pretreatment methods such as ultrasonic, FNA is a waste-produced, renewable chemical that can be in situ generated in WWTPs as a byproduct of wastewater treatment by nitritation of the anaerobic digestion liquor (Law et al., 2015; Wang et al., 2013b), which otherwise needs to extra treatment. It should be noted that this FNA based fermentation strategy can also integrate with other methods. Thus, SCFA production will be further enhanced if this integration strategy is combined with other methods such as heat treatment and co-fermentation substrate optimization, which remain to be studied in future.

### 3.6. *Implications for WWTPs*

This study reveals for the first time that FNA can be used as an ideal pretreatment tool for alkaline fermentation. As mentioned above, although numerous investigations were conducted to promote SCFA generation from WAS anaerobic fermentation, the suitable technology that can achieve a high SCFA yield with a low treatment time and reasonable construction and operation costs has seldom proposed. The findings obtained in this work may provide a scientific basis for developing such a technology. More importantly, the finding achieved in this study might have momentous implications for the operation of WWTPs. It is well-known that the performance of BNR depends on the presence of available biodegradable carbon sources such as SCFA. However, the amount of influent SCFA is often deficient in many WWTPs. To keep high levels of BNR, additional supply of SCFA is inevitably required in these cases. On the other hand, society is increasingly aware that WWTPs can be a valuable energy source rather than "waste" (Li et al., 2014; Xu et al., 2012). It is a big challenge that maintains good performance of BNR and recovers maximal energy concurrently in WWTPs. Therefore, the development of suitable technology to address this challenge is urgently needed.

Fig. 4 demonstrates an enhanced "nutrient removal-energy recovery" concept with the proposed FNA pretreatment + alkaline fermentation method applied in a WWTP from an integrated environmental and economic perspective. After concentrating in a thickener, WAS generated in both primary and secondary settling tanks is first pretreated with FNA for 2 d. Then, a part of pretreated WAS is used for SCFA production while the remainder is employed to generate methane. It should be noted that nitrogen and phosphorus were also released into the fermentation liquid (Fig. S5, Supporting Information). The released nitrogen and phosphorus will increase the nitrogen and phosphorus loading ratios of WWTPs if they are not removed from the fermentation liquid. Thus, the produced SCFA-rich fermentation liquid should be introduced to a nitrogen and phosphorus recovery and separation system before it can be introduced to the influent of WWTP for the improvement of BNR. Previous studies have demonstrated that by the formation of struvite precipitation both nitrogen and phosphorus released could be efficiently recovered (Tong and Chen, 2007; Zhang and Chen, 2009). The generated energy source, methane, can be collected and further utilized. Anaerobic digestion liquor generated in the methane production reactor,

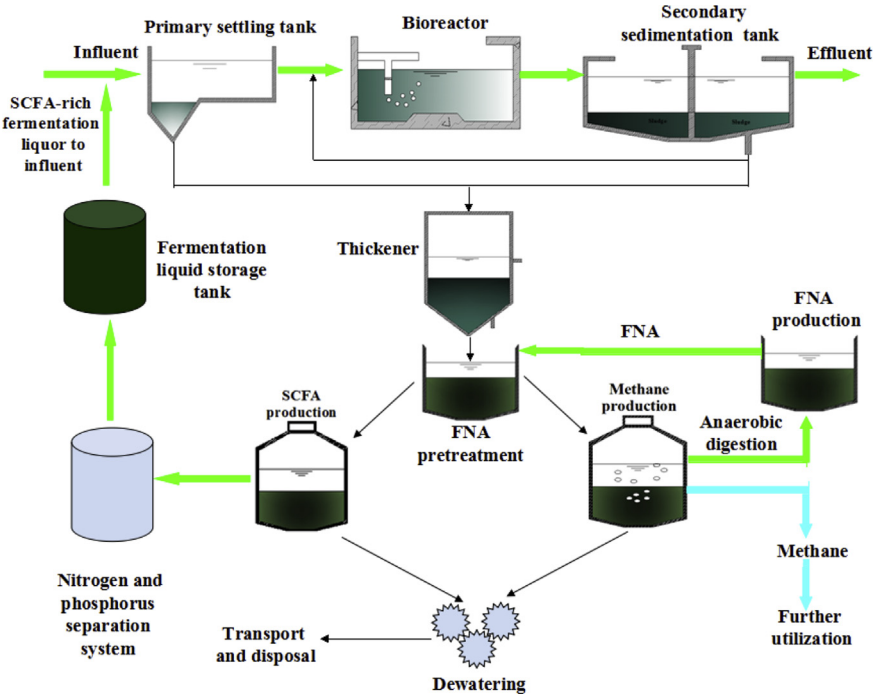


**Fig. 4 – The proposed FNA pretreatment combined with alkaline fermentation technology for enhancing nutrient removal as well as energy recovery in a WWTP.**

which typically contains 0.8–1.5 g ammonia-nitrogen/L (Wang et al., 2013b), is then employed to produce FNA. It was reported that more than 90% of ammonia in the anaerobic digestion liquor could be bio-converted to nitrite in an enriched ammonia oxidizing bacteria nitritation reactor (Bartroli et al., 2010). In addition, Wang et al. proved that FNA pretreatment also enhanced WAS methane production in the anaerobic digestor (Wang et al., 2013b). Therefore, good performance of wastewater BNR is guaranteed, considerable energy is recovered, and massive WAS is reduced.

Here, a desktop scaling-up study of WWTP was performed to further interpret this concept (Table S7, Supporting Information). According to our calculation based on the data obtained in this study and typical values documented in the literature, about 43% of produced WAS should be used to generate SCFA, while 57% of WAS is left to produce methane. In addition, the optimal FNA concentration (i.e., 1.54 mg/L) can be also achieved readily by controlling temperature (20 °C) and pH (6.0) in the FNA pretreatment reactor. It seems that the proposed FNA pretreatment + alkaline fermentation technology is practically feasible. However, the values presented here should be considered indicative only and required to be modified when applying in the real WWTPs.

## 4. Conclusion

This paper reports a new pretreatment (i.e., FNA pretreatment) for pH 10 fermentation. The results showed that the optimal FNA pretreatment concentration and time were 1.54 mg/L and 2 d, respectively, while the optimal time of pH 10 fermentation was 2 d. Under this condition, 370.1 mg COD/g VSS was achieved, which was 4.7- and 1.5-fold of that in the blank (uncontrolled) and sole pH 10 systems, respectively. Compared with pH 10, FNA accelerated disruption of both EPS and cell envelope. Moreover, FNA pretreatment combined with pH 10 fermentation caused positive synergy on sludge solubilization, hydrolysis, and acidification processes. Thus more SCFA was generated but less fermentation time was required.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2015.04.012.

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