# Effect of different hydrolytic enzymes pretreatment for improving the hydrolysis and biodegradability of waste activated sludge

Jiahao Chen, Shihu Liu, Yingmu Wang, Wei Huang and Jian Zhou

## ABSTRACT

In this study, the effects of lysozyme, protease and $\alpha$-amylase pretreatments for improving the hydrolysis and biodegradability of waste activated sludge (WAS) were investigated. The results showed that lysozyme was more effective in increasing the soluble chemical oxygen demand (SCOD) concentration in the liquid phase of sludge and improving the release of protein and carbohydrate from sludge flocculation to enhance sludge hydrolysis. After 8 h hydrolysis, the net SCOD increase in a reactor with lysozyme was 2.23 times and 2.15 times that of the reactors with protease and $\alpha$-amylase, respectively. Meanwhile, lysozyme and protease could improve the lysis of microorganism cells and the dissolution of extracellular polymeric substances (EPS) to a certain extent, and lysozyme was more effective. Furthermore, the compositional characteristics of dissolved organic matter (DOM) and EPS were analyzed by EEM fluorescence spectroscopy and fluorescence regional integration (FRI) analysis. Tryptophan-like protein was the main component of sludge, which accounted for 31% and 38% of DOM and EPS, respectively. Lysozyme could decrease the percentage of non-biodegradable materials in sludge, such as humic acid-like substances and fulvic acid-like substances, so it could improve the biodegradability of sludge. This study can provide valuable information for future studies about hydrolytic enzyme pretreatments for WAS disposal.

**Key words** | anaerobic digestion, EEM, extracellular enzymes, extracellular polymeric substances, WAS

**Jiahao Chen**
**Shihu Liu**
**Yingmu Wang**
**Wei Huang**
**Jian Zhou** (corresponding author)
Key Laboratory of the Three Gorges Reservoir's Eco-Environments, Ministry of Education,
Chongqing University,
Chongqing 400045,
China
E-mail: *zhoujiantt@126.com*

**Shihu Liu**
Chongqing Water Group Co. Ltd,
Chongqing 400015,
China

**Jian Zhou**
Faculty of Urban Construction and Environmental Engineering,
Chongqing University,
174 Shazheng Street, 400045 Chongqing,
China

## INTRODUCTION

With the rapid economic development of China, a large amount of waste activated sludge (WAS) produced from municipal wastewater treatment plants (WWTPs) has been generated (Yang *et al.* 2015). Recently, because of its large production, the high cost for WAS disposal and potentially environmental pollution, WAS has become a serious public issue (Zhao & Kugel 1996). Anaerobic digestion (AD), which was regarded as the most energy efficient process for WAS disposal, has been widely used for organic reduction and stabilization with biogas production recovery (Souza *et al.* 2009; Martinez *et al.* 2012; Wang *et al.* 2013). However, the application of AD is limited by its long solids retention time and low degradation ratio of organic matter. Previous studies reported that the hydrolysis process was the rate-limiting step in the AD process (Tiehm *et al.* 2001). To accelerate the hydrolysis process in AD, various pretreatment technologies have been used to improve the biodegradability of WAS, including mechanical, thermal, chemical and biological methods (Carrère *et al.* 2010; Lizama *et al.* 2017).

Biological pretreatments (especially hydrolytic enzyme pretreatments) have showed an absolute advantage in accelerating the hydrolysis process of sludge, compared with mechanical, thermal and chemical methods (Teo & Wong 2014). Hydrolytic enzymes, a kind of effective catalyst in the AD process, can accelerate the hydrolysis of extracellular polymeric substances (EPS), disrupt the microorganism cell structure, and dissolve the insoluble materials in sludge. Meanwhile, as biological products, hydrolytic enzymes can be degraded easily and they are harmless to the environment. Therefore, hydrolytic enzyme pretreatment, which can

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shorten the hydrolysis time and reduce the WAS disposal cost, is an environment-friendly technology (Kim *et al.* 2002; Ahuja *et al.* 2004; Parawira 2012; Kavitha *et al.* 2013). Lysozyme, protease and $\alpha$-amylase are the most common hydrolytic enzymes for accelerating the hydrolysis process and promoting the biodegradability of WAS (Yu *et al.* 2013; He *et al.* 2014). Lysozyme can efficiently disrupt the microorganism cell structure, which is composed of peptidoglycan, and accelerate the release of cytoplasm (Guo & Xu 2011). Proteases and $\alpha$-amylase can hydrolyze macromolecules, like protein and carbohydrate, to simpler ones (i.e., peptides, two peptides, amino acids and simpler carbohydrate molecules), when breaking the sludge floc and dissolving the organic solids of WAS (Yu *et al.* 2003). Furthermore, they can be degraded into some other low-molecular weight organic acids, $H_2$, ammonia, and $CO_2$ in further processes (Guo & Xu 2011). However, the studies on hydrolytic enzyme pretreatment for WAS are still limited. The differences in performance and mechanisms of lysozyme, proteases and $\alpha$-amylase for improving the hydrolysis of WAS need a comprehensive comparison from different aspects. The comparison needs to be analyzed not only by conventional parameters, including EPS which played a significant role in sludge flocculation (Monique *et al.* 2008), dissolved organic matter (DOM), volatile fatty acids (VFAs) and soluble chemical oxygen demand, but also by EEM fluorescence spectroscopy and fluorescence regional integration (FRI) analysis. On the other hand, the addition of hydrolytic enzymes, a kind of protein, would increase the quantities of organic species in the pretreatment processes. But there are a few studies which have analyzed the influence of the organic matter brought by hydrolytic enzymes themselves to pretreatment processes (Guo *et al.* 2014; Xin *et al.* 2016).

The objective of this study was to illustrate the differences in performance and mechanisms of lysozyme, proteases and $\alpha$-amylase to dissolve the sludge flocculation, disrupt the microorganism cell structure, and disintegrate the insoluble macromolecular compounds in sludge for improving WAS hydrolysis and biodegradability. Also, the influence of the organic matter brought by hydrolytic enzymes themselves to pretreatment processes was analyzed. Besides, the component changes of EPS and DOM were analyzed by the EEM fluorescence spectra with FRI analysis to evaluate the improvement in biodegradability of WAS. This study can provide valuable information for future studies on hydrolytic enzyme pretreatments.

## METHODS

### Sludge and enzymes origin

WAS used in this experiment was collected from the secondary sedimentation tank of the Tang Jiatuo WWTP, Chongqing, China. Fresh sludge was sieved by a 1.0 mm grid sieve first, and concentrated by settling for 2 h, then stored at 4 °C for the following experiments. The characteristics of raw sludge (WAS after sieving and settling) were as follows: total suspended solids (TSS) 27,550 ± 138 mg/L, volatile suspended solids (VSS) 18,442 ± 153 mg/L, total COD (TCOD) 23,308 ± 271 mg/L, soluble COD (SCOD) 101.30 ± 9.3 mg/L, soluble carbohydrate 6.74 ± 0.7 mg COD/L, soluble protein 59.35 ± 2.3 mg COD/L, pH 7.2 ± 0.2.

The hydrolytic enzymes used in this study were commercial lysozyme (purchased from Amresco, USA), protease and $\alpha$-amylase (purchased from Gentihold, China). The activities of lysozyme, protease and $\alpha$-amylase were 20,000 (U)/g, 60,000 (U)/g and 8,000 (U)/g, respectively.

### Experiment setup and operation

Four 500 ml Erlenmeyer flasks were prepared to determine the effects of different hydrolytic enzyme pretreatments for AD of WAS. Each of them were loaded separately with 200 ml raw sludge and placed in a water-bath at 35 ± 1 °C. After that, lysozyme, protease and $\alpha$-amylase with a dosage of 4% (w/w, hydrolytic enzymes/TSS), which were dissolved in 10 ml distilled water prior, were added to the flasks (labeled as reactors L, P and A), respectively. Meanwhile, 10 ml distilled water without hydrolytic enzymes was added to the control reactor (labeled as reactor C). During the experiment, all flasks were shaken at 120 rpm and 35 ± 1 °C in water-bath shaker with a strict anaerobic condition. Meanwhile, the experiment time of 8 h was chosen, because the activity life time of hydrolytic enzymes is usually limited (Odnell *et al.* 2016). The anaerobic condition was maintained by rubber stoppers, after injecting $N_2$ for 5 min into the flasks. All tests in this study were conducted in triplicate, and the mean value and standard deviation were adopted.

### Analytical methods

#### Samples collection and EPS extraction

The sludge samples were collected from above Erlenmeyer flasks when hydrolytic enzyme pretreatment time was

0 min, 15 min, 30 min, 60 min, 120 min, 240 min, 360 min and 480 min, respectively. The sludge samples were centrifuged at 6,000 g for 10 min at 4 °C first. The supernatant, which was filtrated with 0.45 μm cellulose acetate membrane first, was used for testing the DOM in the sludge. Meanwhile, precipitation after centrifugation was used for EPS extraction and testing the TSS and VSS. The steps of EPS extraction were as follows: the sludge samples, which were washed with distilled water prior to extraction, were re-suspended with distilled water to keep the same volume of samples. The re-suspended sludge samples were heated at 65 °C for 30 min, and then centrifuged at 8,000 g for 15 min at 4 °C. After that, the supernatant, which was filtrated with 0.45 μm cellulose acetate membrane first, was used for testing the EPS.

### Chemical analysis

The SCOD, TCOD, TSS and VSS were determined according to the standard methods (APHA 2005). Soluble protein and soluble carbohydrate in DOM and EPS were measured according to Lowry's method (using bovine serum albumin as the standard substance) (Frølund *et al.* 1995) and phenol–sulfuric acid method (using glucose as the standard solution) (Chaplin & Martin 2006), respectively. The VFA components in DOM, including acetic, propionic, n-butyric, iso-butyric, n-valeric and iso-valeric acids, were quantified by a gas chromatograph (Agilent, 7890A, USA) equipped with a flame ionization detector and HP-INNOWAX column.

### EEM fluorescence spectroscopy and FRI analysis

The component changes in DOM and EPS were measured by three-dimensional EEM fluorescence spectroscopy (F-7000 FL Spectrophotometer, Hitachi, Japan). The scan was made with an excitation (Ex) wavelength from 200 nm to 400 nm, and corresponding to emission (Em) wavelengths from 200 nm to 500 nm, with a 5 nm sampling interval. The scan speed was set at 12,000 nm/min, and the photomultiplier detector voltage was 700 V. The FRI technique was adopted for EEM spectral data analysis (Chen *et al.* 2003). According to the differences of excitation-emission wavelengths of organic matter, EEM peaks can be divided into five regions, including simple aromatic proteins (tyrosine-like Region I and tryptophan-like Region II), fulvic acid-like substances (Region III), soluble microbial by-product-like substances (Region IV), and humic acid-like substances (Region V). Therefore, the component changes of organic matters can be quantitated by the fluorescent intensities of characteristic peaks on an EEM plot (Chen *et al.* 2003).

## RESULTS AND DISCUSSION

### DOM variation affected by hydrolytic enzyme pretreatments

#### SCOD variation

Previous studies reported that the hydrolysis process was the rate-limiting step in the AD process of WAS. The SCOD variation could reflect the hydrolysis degree of WAS to some extent (Hatziconstantinou *et al.* 1996). Therefore, the performance of hydrolytic enzyme pretreatments for improving the hydrolysis process of WAS could be estimated preliminarily by SCOD variation in DOM.

Figure 1 shows the SCOD variation in DOM affected by hydrolytic enzyme pretreatments. During the pretreatment, the SCOD concentration in all reactors was on the upward trend. Meanwhile, there was more significant SCOD production released from sludge in reactor L, compared with reactors P, A and C (Figure 1(a)). When hydrolytic enzymes were inoculated into WAS (0 min), the SCOD in DOM of sludge was obviously changed. The SCOD concentration in reactors P, L and A was 878.27 mg/L, 233.16 mg/L and 420.22 mg/L, respectively. And the SCOD concentration in the control reactor was only 95.54 mg/L. Hydrolytic enzymes are organic species with a certain SCOD concentration. So, adding the hydrolytic enzymes into WAS would cause the increase of SCOD concentration (at time 0), and this part of SCOD production was not released from the sludge. Meanwhile, the SCOD in reactors P and A kept a decreasing tendency during the hydrolysis process from 0 min to 30 min. Previous studies reported that the enzymes would be entrapped by, adsorbed to or bound to the sludge (Wawrzynczyk *et al.* 2008). Also, enzymes' action on sludge kept a decreasing tendency after entrapment (Wawrzynczyk *et al.* 2008). Therefore, the hydrolytic enzymes, which dissolved in the liquid phase of sludge, entrapped by sludge flocculation could be the main factor in the decrease of SCOD in DOM of sludge. When hydrolysis time arrived at 480 min, the net SCOD concentration increase in DOM of sludge in reactor C was 923.72 mg/L (Figure 1(b)), which accounted for 3.96% of TCOD. In contrast, the net SCOD increase in concentration in DOM of sludge in reactor L reached 1,696.71 mg/L, which accounts for 7.28% of TCOD, and was 83.68% higher than the control reactor. The net

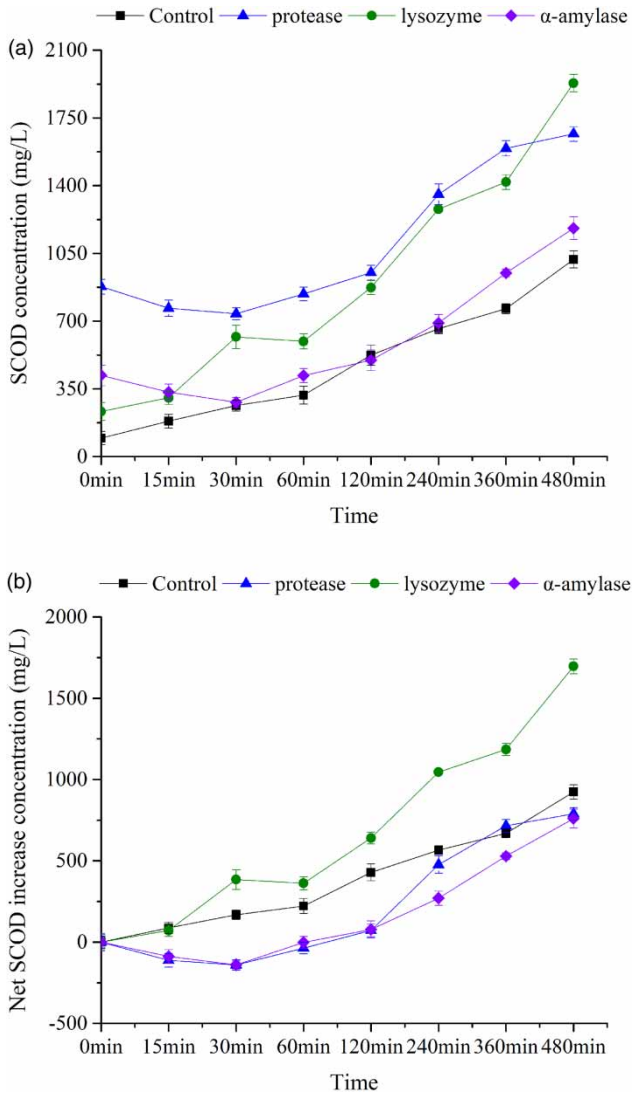


**Figure 1** | SCOD (a) and net SCOD increase (b) variation in DOM affected by hydrolytic enzyme pretreatments.

SCOD increase in concentration in reactors P and A only reached 44.80% and 46.50% of reactor L, respectively. The above results showed that lysozyme could increase the SCOD in DOM of sludge and improve the sludge hydrolysis efficiently. It was hard to get such a high net SCOD increase by adding the protease and $\alpha$-amylase. Meanwhile, it was important to take the organic matter, which was brought by the hydrolytic enzymes themselves, into consideration when analyzing the performance of the hydrolytic enzyme pretreatments.

## Protein and carbohydrate variation

The main components of WAS were carbohydrate and protein, which comprised a large proportion of SCOD in sludge (Yang *et al.* 2010). Therefore, the SCOD variation in DOM of sludge could be further analyzed by protein and carbohydrate variation in DOM. Figure 2 shows the protein and carbohydrate variation in DOM affected by hydrolytic enzyme pretreatments. When hydrolytic enzymes were inoculated in WAS (0 min), the protein concentrations in reactors P, L and A were 412.32 mg COD/L, 162.49 mg COD/L and 97.50 mg COD/L, respectively. The protein concentration in reactor C was only 53.58 mg COD/L. Meanwhile, the carbohydrate concentrations in reactors P, L and A were 131.08 mg COD/L, 10.42 mg COD/L and 128.17 mg COD/L, respectively. The carbohydrate concentration in reactor C was only 7.58 mg COD/L. Combined with the protein and carbohydrate concentration of hydrolytic enzymes (Table 1), the DOM increase in sludge at 0 min was caused by the organic matter from hydrolytic enzymes themselves. Meanwhile, at this moment, almost all protease and $\alpha$-amylase were dissolved in the liquid phase of sludge. In contrast, most of the lysozyme was entrapped by sludge flocculation, with a very small amount of the lysozyme dissolved in the liquid phase of sludge.

During the reaction (from 0 min to 30 min), the carbohydrate concentrations in reactors P and A decreased to 82.08 mg COD/L and 24.47 mg COD/L from 131.08 mg COD/L and 128.17 mg COD/L, respectively (Figure 2(c)). After that, the carbohydrate concentrations in all reactors kept an increasing tendency over time. In this period (from 0 min to 30 min), the carbohydrate in DOM was brought by hydrolytic enzymes themselves. And the decrease of carbohydrate in DOM could reflect the decrease of protease and $\alpha$-amylase in liquid phase of sludge. Because most of the lysozyme was entrapped by sludge flocculation, there was no carbohydrate decrease in reactor L in this period. In contrast, the protein concentrations in reactor P and A increased to 503.92 mg COD/L and 223.28 mg COD/L from 412.32 mg COD/L and 97.50 mg COD/L, respectively (Figure 2(a)), with a net protein increase of 91.6 mg COD/L and 125.78 mg COD/L, respectively, in this period. And the net protein increase in reactor C was only 57.68 mg COD/L (Figure 2(b)). When hydrolysis time arrived at 30 min, the ratio of protein/SCOD of reactor P and A was 68.30% and 79.67%, respectively, and far above the 42.19% of control one. It meant that a large amount of protein was dissolved in the liquid phase from sludge flocculation, in this period. Combined with the protein and carbohydrate variation during the whole reaction (480 min), protease and $\alpha$-amylase had positive effects on protein and carbohydrate released from sludge in initial

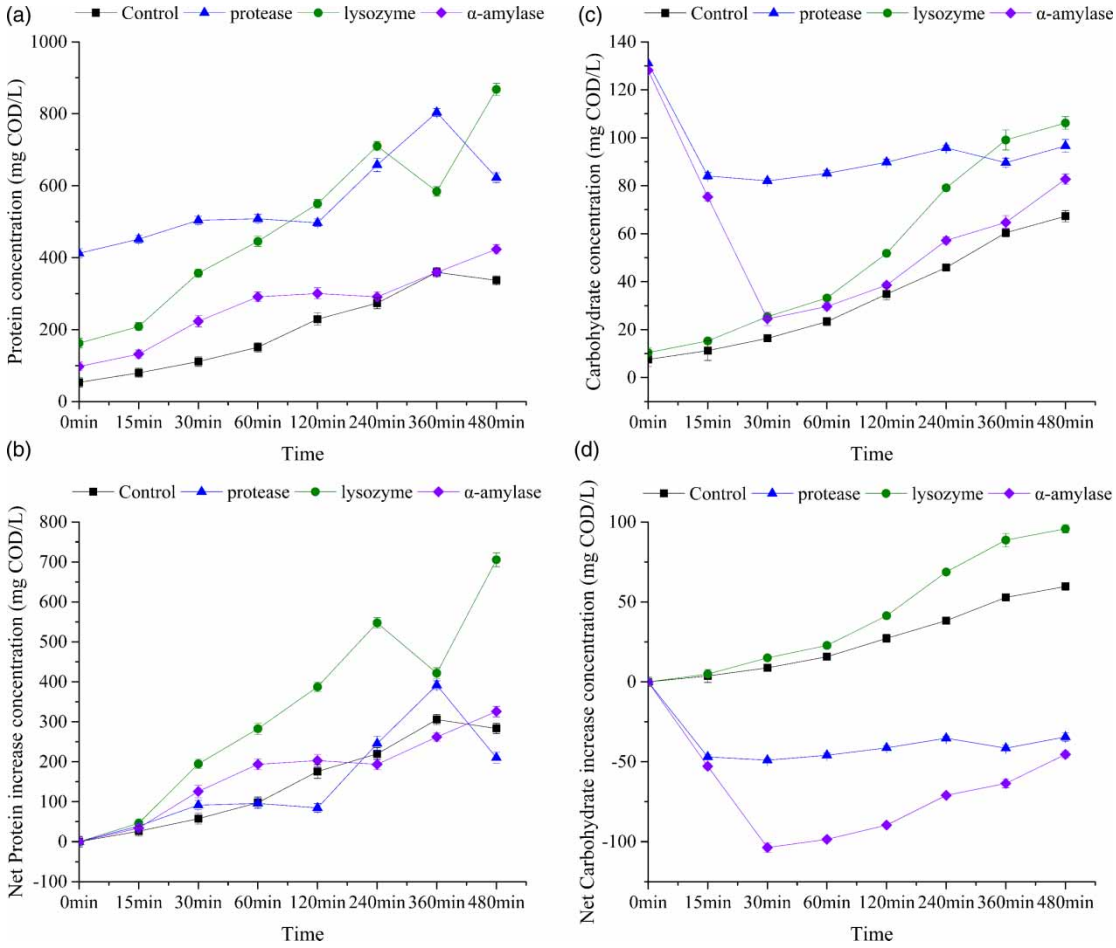


**Figure 2** | Protein (a), net protein increase (b), carbohydrate (c) and net carbohydrate increase (d) variation in DOM affected by hydrolytic enzyme pretreatments.

**Table 1** | SCOD, protein and carbohydrate concentration[a] of hydrolytic enzymes (1.05 g/L)

| | SCOD (mg/L) | Protein (mg COD/L) | Carbohydrate (mg COD/L) |
|---|---|---|---|
| Protease | 786.91 ± 22.90 | 402.53 ± 14.19 | 111.70 ± 5.65 |
| Lysozyme | 1,721.16 ± 28.53 | 1,300.83 ± 20.60 | 8.91 ± 0.82 |
| $\alpha$-amylase | 308.78 ± 14.14 | 59.15 ± 2.27 | 108.97 ± 4.15 |

[a]The concentration of standard substance of hydrolytic enzymes used in this study.

period (from 0 min to 30 min). But there were no obvious effects on protein and carbohydrate release after 30 min. When hydrolysis time arrived at 480 min, the net soluble protein and carbohydrate increase in reactors P and A accounted for 0.8% and 1.3% of TCOD, respectively. In contrast, the protein and carbohydrate concentrations in reactor L were significantly higher than the control one, during the whole reaction. When hydrolysis time arrived at 480 min, the net soluble protein and carbohydrate increase in reactor L accounted for 3.65% of TCOD. Earlier studies have shown that protease and $\alpha$-amylase in sludge would be rapid inactivation in 1 h to 2 h, because of the endogenous proteases in the liquid phase of sludge. And lysozyme could keep above 50% of activity life time after 5 h dissolved in sludge (Odnell *et al.* 2016). Therefore, the rapid inactivation of protease and $\alpha$-amylase in sludge was the main reason to lead to the unsustainable release of protein and carbohydrate in sludge in reactors P and A. Meanwhile, after the lysis of the cell by lysozyme, the whole intracellular organic matter of the lysed microbial cell (i.e., endogenous hydrolytic enzymes) was released to the sludge. It means that the hydrolysis by endogenous hydrolytic enzymes would appear in the sludge (Odnell *et al.* 2016). Although the activity life time of lysozyme itself was short in sludge (just several hours), the effect lasts much longer. Meanwhile, due to the longer life time of lysozyme in sludge, the net protein and carbohydrate increase in reactor L were far above that in reactors P and A. This indicated that lysozyme

was more efficient in enhancing the release of protein and carbohydrate from WAS.

## VFA variation

Hydrolytic enzymes could promote the dissolution of organic solids in WAS. At the same time, protein and carbohydrate could be further degraded into small molecule organic matter by it (Yu *et al.* 2003). Therefore, the protein and carbohydrate in DOM of sludge could fluctuate in a definitive range. VFAs, which are closely related to protein and carbohydrate, were the important intermediate products in sludge AD. Also, it could reflect the hydrolysis process of WAS (Luo *et al.* 2014). Therefore, the performance of hydrolytic enzymes for improving the WAS hydrolysis could be further analyzed by VFA variation in DOM. The concentrations of VFAs in this study were converted into mg COD/L by conversion factors in Table 2.

Figure 3(a) shows the VFA variation in DOM affected by hydrolytic enzyme pretreatments.

There were no obvious VFAs after 60 min reaction. And when hydrolysis time arrived at 360 min, the VFA concentration in all reactors rose quickly. After 480 min reaction, the total VFA concentrations in reactors P, L and A were 459.71 mg COD/L, 465.68 mg COD/L and 396.96 mg COD/L, respectively, which accounted for 1.97%, 1.99% and 1.70% of TCOD, respectively. And the total VFA concentration in reactor C was only 294.50 mg COD/L, which account for 1.26% of TCOD. Meanwhile, from the net VFA concentration of reactors P, L and A, with the control data deducted in Figure 3(b), it was more easy to get a high VFA production at an even earlier time (360 min). Combined with protein and carbohydrate variation (Figure 2), there was a large amount of DOM released from sludge for VFA production in reactor L. So therefore the production of VFAs could be promoted effectively in reactor L. In contrast, it was hard to get such a net protein and carbohydrate increase in reactors P and A, compared with

**Table 2** | Conversion factors for protein, carbohydrate and VFAs

| Compound | Formula | g COD/g | g C/g COD |
|---|---|---|---|
| Protein | $(C_4H_{6.1}O_{1.2}N)_x$ | 1.56 | 0.35 |
| Carbohydrate | $C_6H_{12}O_6$ | 1.07 | 0.38 |
| Acetic acid | $C_2H_4O_2$ | 1.07 | 0.38 |
| Propionic acid | $C_3H_6O_2$ | 1.51 | 0.32 |
| Butyric acid | $C_4H_8O_2$ | 1.82 | 0.3 |
| Valeric acid | $C_5H_{10}O_2$ | 2.04 | 0.29 |

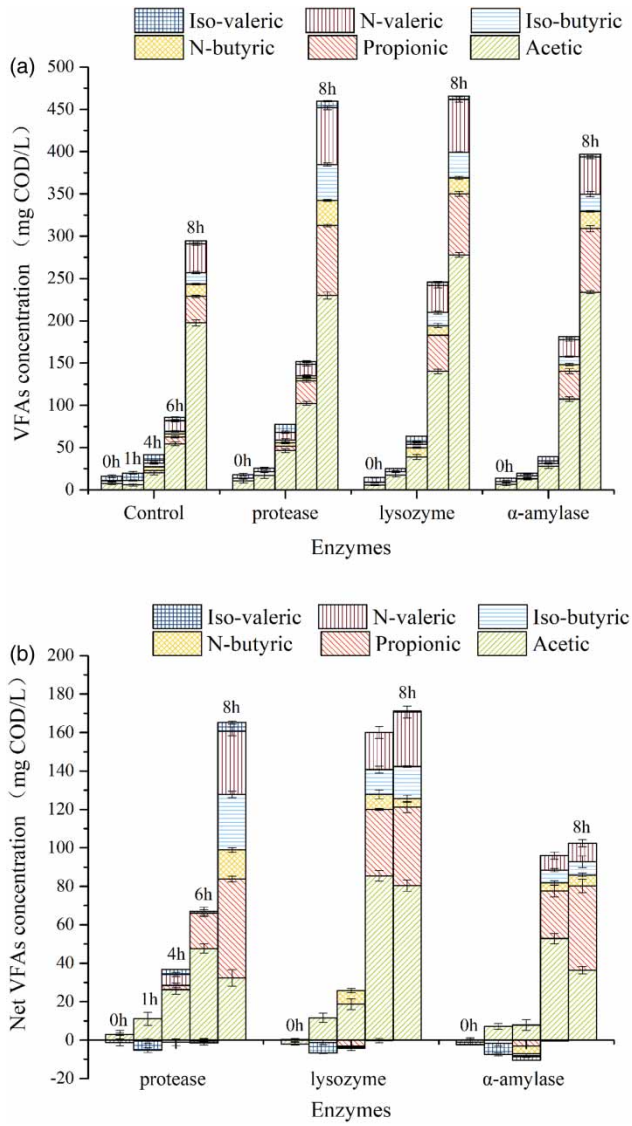


**Figure 3** | Components and variation of VFAs (a) and net VFAs with control data deducted (b) in DOM affected by hydrolytic enzyme pretreatments.

reactor L (Figure 2). But as protease and $\alpha$-amylase could promote the further degradation of protein and carbohydrate to small molecule organic matter (Yu *et al.* 2003), it could accelerate the production of VFAs. The degradability of protein and carbohydrate was 39.70% and 52.24%, respectively. Carbohydrate was easier to degrade by microorganisms (Pinnekamp 1989), but protein was the main component of EPS in sludge. Therefore, protease was more effective in promoting the production of VFAs than $\alpha$-amylase.

The main degradation products of sludge, which were mainly composed of protein and carbohydrate, were straight chain and/or branching chain fatty acids from 2–5 carbon

atoms, including acetic, propionic, n-butyric, iso-butyric, n-valeric and iso-valeric acids (Yuan *et al.* 2006). Figure 3(a) shows the variation of the components of VFAs in DOM affected by hydrolytic enzyme pretreatments. Acetic acid was the main component of the degradation products of sludge, with a percentage range of 50–65%, followed by propionic acid (10–18%) and iso-valeric acid (10–14%). When hydrolysis time arrived at 480 min, the sum of iso-valeric and n-valeric acids in reactors L, P, A and C were 74.84 mg COD/L, 66.30 mg COD/L, 47.17 mg COD/L and 37.49 mg COD/L, respectively. The sum of iso-valeric and n-valeric acids in reactors L and P were significantly higher than the sum of iso-valeric and n-valeric acids in reactors A and C. The VFAs with lower molecular weight (i.e., acetic and propionic) may be fermented directly by carbohydrate and protein, but the VFAs with higher molecular weight, like iso-valeric and n-valeric acids, were closely related to the fermentation of protein in sludge (Zehnder 1988). There were more iso-valeric and n-valeric acids produced in reactor L and P, which meant more proteins were degraded in reactor L and P. After the lysis of the cell by lysozyme, the whole intracellular organic matter of the lysed microbial cell (i.e., endogenous hydrolytic enzymes) released to the sludge. It means that the hydrolysis of protein by endogenous hydrolytic enzymes would appear in the sludge (Odnell *et al.* 2016). Therefore, lysozyme and protease were more effective in promoting the hydrolysis of protein, and improving the production of VFAs.

## EPS variation affected by hydrolytic enzyme pretreatments

EPS, the main components of WAS, was one of the key factors of sludge hydrolysis. So, it was important to analyze the compositional characteristics of EPS during the hydrolytic enzyme pretreatments (Liu *et al.* 2015). Figure 4(a) shows the EPS variation affected by hydrolytic enzyme pretreatments. The main components of EPS in WAS were protein (2,436.49 mg COD/L) and carbohydrate (240.44 mg COD/L), with a percentage of 57.43% and 6.38%, respectively. When lysozyme was inoculated into WAS (0 min), the protein in EPS could be changed obviously. The SCOD and protein concentrations in EPS were 5,201.58 mg/L and 3,495.00 mg COD/L, respectively, which were far above the concentrations in raw sludge (the WAS without pretreatment). In contrast, when protease and $\alpha$-amylase were inoculated into the WAS, respectively, the components in EPS could be changed slightly. After hydrolytic enzymes hydrolyzed for 4 h, there was no significant change of the

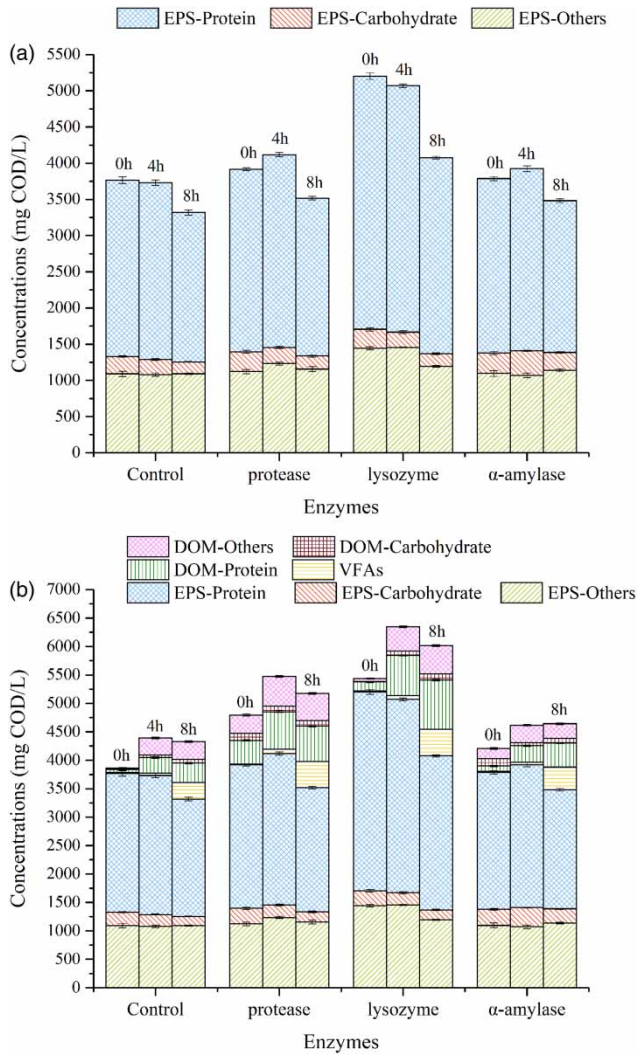


**Figure 4** | DOM and EPS variation affected by hydrolytic enzyme pretreatments. (a) Carbohydrate, protein and others distribution in EPS. (b) Carbohydrate, protein, VFAs and others distribution in DOM and EPS.

components in EPS of all reactors. Meanwhile, after hydrolytic enzymes hydrolyzed for 8 h, there was an obvious decrease of EPS in all reactors. The net SCOD decrease (compare with 0 h) in reactors L and P was 992.15 mg/L and 601.67 mg/L, respectively. But in reactors A and C, it was only 442.30 mg/L and 414.40 mg/L, respectively. Hydrolytic enzymes, a kind of organic matter, have a certain SCOD concentration. The lysozyme, which was absorbed immediately by sludge flocculation after being inoculated into sludge, could cause the increase in EPS. In contrast, when protease and $\alpha$-amylase were inoculated into WAS, almost all protease and $\alpha$-amylase were dissolved in the liquid phase of sludge, so there was no significant increase in EPS. Furthermore, all reactors would take a relatively

long time to achieve the dissolution of EPS. Also, lysozyme and protease were more effective in promoting the dissolution of EPS.

Figure 4(b) shows the distribution of carbohydrate, protein, VFAs and others in DOM and EPS affected by hydrolytic enzyme pretreatments. After hydrolytic enzymes were hydrolyzed for 4 h, there was no significant change of the components in EPS of all reactors. In contrast, there was a significant increase in DOM. The net SCOD increase in reactors L and P was 913.43 mg/L and 679.55 mg/L, respectively. But in reactors A and C, it was only 530.37 mg/L and 406.41 mg/L, respectively. Meanwhile, after hydrolytic enzymes were hydrolyzed for 8 h, there was an obvious decrease of EPS with an increase of DOM in all reactors, including the significant increase of VFAs. In the initial period (0 h–4 h), the increase of DOM was caused by the lysis of microorganism cells. Compared with protease and $\alpha$-amylase, lysozyme could disrupt the microorganism cell structure, which was composed of peptidoglycan, efficiently and accelerate the release of cytoplasm (Guo & Xu 2011). It meant a more effective promotion of the lysis of the microorganism cell. After that, the increase of DOM was caused by the dissolution of EPS, during the hydrolytic enzyme hydrolysis from 4 h to 8 h. Differing from the mechanism of lysozyme, the main roles of protease and $\alpha$-amylase in WAS were promoting the further degradation of protein and carbohydrate into small molecule organic matter (Yu *et al.* 2003), which could improve the hydrolysis process of sludge. But compared with protein, carbohydrate was not the main component of EPS, with a percentage of 6.38%. This meant that $\alpha$-amylase could not promote the dissolution of EPS as effective as lysozyme and protease, because it only attacks alpha-linked carbohydrates. The results above indicated that lysozyme and protease could improve the lysis of microorganism cells and dissolution of EPS to a certain extent, and lysozyme was more effective at it.

## Carbon mass balance analysis of DOM and EPS affected by hydrolytic enzyme pretreatments

The carbon content in pretreatments was not measured directly, but was calculated from the COD of protein, carbohydrate and VFAs in DOM and EPS of sludge (Table 2) (Feng *et al.* 2009). Carbon mass balance analysis was processed on the basis of the results above. The C-balance was applied to evaluate the carbon content of DOM and EPS in sludge after different hydrolytic enzyme pretreatments. Figure 5 shows the carbon mass balance analysis of

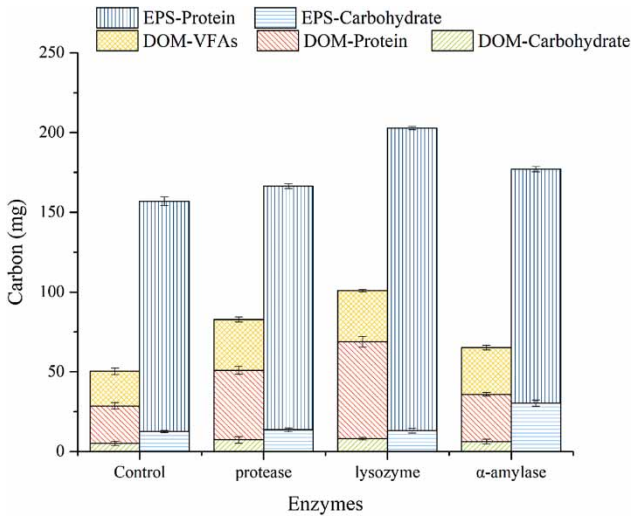


**Figure 5** | Carbon mass balance analysis of EPS and DOM with hydrolysis time of 8 h.

EPS and DOM with a hydrolysis time of 8 h. The carbon content of DOM and EPS in sludge reached the highest in reactor L (with 303.75 mg carbon), after hydrolytic enzymes hydrolyzed for 8 h. Meanwhile, the carbon mass of protein in DOM reached 60.76 mg, which accounted for 60.21% of the total carbon in DOM of reactor C, and it was 157.57% higher than the control reactor (23.59 mg). Furthermore, the carbon content of EPS in reactor C reached 303.75 mg, which was 46.58% higher than the control reactor (207.22 mg). The results showed that the carbon content increase in DOM of reactor C was mainly caused by the lysis of microorganism cells, but not the dissolution of EPS. It meant that lysozyme could promote the lysis of microorganism cells effectively, and improve the hydrolysis of sludge.

## FRI analysis of DOM and EPS affected by hydrolytic enzyme pretreatments

EEM fluorescence spectroscopy was adopted to study the variation of organic matter with fluorescence characteristics, including protein, humic acid, fulvic acid and so on (Marhuenda-Egea *et al.* 2007). The EEM fluorescence spectroscopy could display the distribution of biodegradable matter and non-biodegradable matter in DOM and EPS. Previous studies reported that VFAs were the easiest accessible organic matters in sludge. Regions I and II in DOM of sludge were accessible and easily biodegradable, followed by Regions I and II in EPS of sludge, which were biodegradable compounds of slowly accessible matter. Regions III and V in DOM and EPS of sludge were related to the least biodegradable and accessible matter. Finally, Region IV was

soluble microbial by-product-like substances, which were protein-like compounds, and had a positive effect on biodegradability of sludge (Jimenez *et al.* 2014).

Figure 6(b) shows the distribution of FRI in EPS affected by hydrolytic enzyme pretreatments. In EPS of WAS, the main parts were Region II (tryptophan-like protein) and Region IV (soluble microbial by-product-like substances), with a percent fluorescence response ($P_{i,n}$) of 38% and 27%, respectively. Meanwhile, the $P_{i,n}$ value of Region I (tyrosine-like protein), Region III (fulvic acid-like substances) and Region V (humic acid-like substances) were 21%, 11% and 3%, respectively. During the whole reaction, there was no significant change in the FRI distribution in EPS of all reactors. The results indicated that tryptophan-like protein and soluble microbial by-product-like substances were the main compounds in EPS. After hydrolytic enzyme hydrolysis, the dissolution of EPS by hydrolytic enzymes, which led to the significant increase of DOM in the liquid phase of sludge, could not change the components and distribution of organic matter in EPS of sludge.

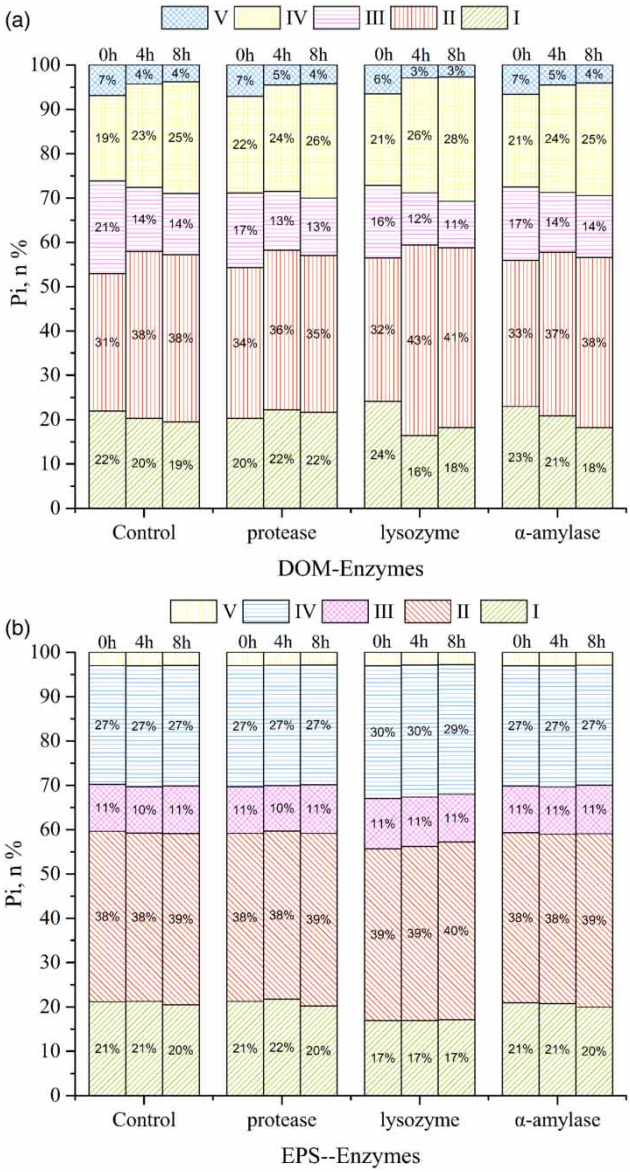


**Figure 6** | Distribution of FRI in DOM (a) and EPS (b) affected by hydrolytic enzyme pretreatments.

Differing with EPS, there was a significant change in DOM after hydrolytic enzyme hydrolysis (Figure 6(a)). In DOM of sludge, the main part was Region II (tryptophan-like protein), with a $P_{i,n}$ value of 31%, followed by Region I (tyrosine-like protein), Region III (fulvic acid-like substances) and Region IV (soluble microbial by-product-like substances), with a $P_{i,n}$ value of 22%, 21% and 19%, respectively. Meanwhile, the Region V (humic acid-like substances) was the least part, with a $P_{i,n}$ value of 7%. The biodegradable materials (Regions I, II and IV) were increased to some extent, and the non-biodegradable materials (Regions III and V) were decreased, with the hydrolytic enzymes' incubation time ongoing. The $P_{i,n}$ value of non-biodegradable materials in reactors P, A and C were 17%, 18% and 18%, respectively, after hydrolytic enzyme hydrolysis. In contrast, the $P_{i,n}$ value of non-biodegradable materials in reactor L was only 14%. This meant that humic acid-like substances and fulvic acid-like substances were obviously decreased in DOM of sludge, after lysozyme hydrolysis. The results above indicated that comparing with protease and $\alpha$-amylase, lysozyme was more effective in improving the hydrolysis and biodegradability of WAS.

## CONCLUSION

Comparison of different hydrolytic enzymes (lysozyme, protease and $\alpha$-amylase) pretreatments, lysozyme was more effective in improving the release of protein and carbohydrate from the sludge floc to enhance sludge hydrolysis. Lysozyme and protease could promote the hydrolysis of protein and improve the production of VFAs. Meanwhile, they could improve the lysis of microorganism cells and dissolution of EPS to a certain extent, but lysozyme was more effective in this. Furthermore, lysozyme could decrease the percentage of non-biodegradable materials in WAS, such as humic acid-like substances and fulvic acid-like substances, and improve the biodegradability of WAS. The results showed that lysozyme was more suitable than

protease and $\alpha$-amylase as an advanced technology for sludge pretreatment.

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