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Novel insights into enzymatic-enhanced anaerobic digestion of waste activated sludge by three-dimensional excitation and emission matrix fluorescence spectroscopy

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HIGHLIGHTS

- Hydrolytic enzymes were applied to enhance the anaerobic digestion of WAS.
- EPS and DOM in WAS were characterized using EEM fluorescence spectroscopy.
- The destruction mechanisms of WAS enhanced by hydrolytic enzymes was explored.

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ABSTRACT

In our previous study, it has been proposed that the hydrolysis of waste activated sludge (WAS) can be enhanced by hydrolytic enzymes. In this study, fluorescence spectral characteristics of extracellular polymeric substances (EPSs) and dissolved organic matter (DOM) during anaerobic digestion were investigated using three-dimensional excitation–emission matrix (EEM) fluorescence spectroscopy to explore the destruction mechanisms of WAS enhanced by additional enzymes (protease, α-amylase and the mixture). Two individual fluorescence peaks associated with protein-like fluorophores (aromatic and tryptophan protein-like substances) were identified in the EEM fluorescence spectra of the EPS after 1 and 6 d, and only aromatic protein-like substances were observed after 12 d of anaerobic digestion for all treatments. As for the DOM, three individual fluorescence peaks were identified, but the peaks associated with visible humic acid-like fluorophores disappeared after 12 d. The EEM fluorescence intensity of EPS decreased during the entire anaerobic process, whereas that of the DOM increased at 1 d and then decreased till the end. In the EPS, the residual protein-like substances were found to be the lowest during the entire anaerobic process when treated with protease. Correspondingly, the protein-like substances in the DOM increased rapidly from 1 to 6 d, and decreased to the lowest level after 12 d for the protease treatment.

1. Introduction

Anaerobic digestion, in which three steps (hydrolysis, acidification and methanogenesis) are generally involved, is widely applied to stabilize organic matters of the sludge (Bouskova et al., 2005). The additional commercially available enzymes can promote sludge disintegration and cell destruction or lysis, thus strongly improve the efficiency of anaerobic digestion (Watson et al., 2004; Yang et al., 2010). Compared with other chemical and physical methods, such as alkaline, ozone, microwave and ultrasonic methods, anaerobic digestion enhanced by enzymes has advantages in minimal byproduct formation, low energy requirement, and mild operation conditions (Ruggaber and Talley, 2006).

Extracellular polymeric substances (EPSs), the major constituents of organic matter in sludge (Liu and Fang, 2002), are excreted by bacteria for adherence to surfaces as well as for protection from predation, phagocytosis, and adverse conditions such as desiccation and sudden pH changes (Flemming and Wingender, 2001), and they have been implicated in determining the sludge structure, sludge charge, flocculation process, settling and dewatering properties. Considering the crucial roles of EPS in the structure and the function of sludge, the biochemical composition and physicochemical properties of EPS is a critical factor to explore the destruction mechanisms of waste activated sludge (WAS) enhanced by enzymes. Dissolved organic matter (DOM), of which the majority is soluble microbial products originated from bound EPS, is ubiquitous in wastewater and sludge (Imai et al., 2002; Tang

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et al., 2010). The DOM comprising the biopolymers of polysaccharides and proteins might be used to represent the soluble biopolymers. Thus, characterization of DOM during enzymatic anaerobic digestion would also be useful for understanding the WAS destruction mechanisms during anaerobic digestion.

The EPS and DOM contain large quantities of aromatic structures and unsaturated fatty chains (Wingender et al., 1999), and their intrinsic spectrometric characteristics can provide information concerning the structure, functional groups, and heterogeneity of the components during WAS anaerobic digestion. Consequently, spectrometry can be an ideal method for bioprocess monitoring. With the application of Fourier transform infrared spectroscopy (Sheng et al., 2005) and fluorescence spectroscopy (Esparza-Soto and Westerhoff, 2001), the structural and functional properties of the WAS can be obtained.

Since the WAS anaerobic digestion bioreactor is a complex system, it is difficult to acquire the entire fluorescence characteristics of the EPS and DOM in WAS using conventional fluorescence spectral measurements, which are limited to special fluorescence region, and other substances may also fluoresce in this region (Li et al., 2008). Interacting and overlapping with the spectra of other fluorophores can cause analytical problems, thus interpretation of data becomes difficult (Marose et al., 1998). Excitation–emission matrix (EEM) fluorescence spectroscopy is a rapid, selective and sensitive technique, and can be an ideal method to characterize complex environmental samples. The outstanding advantages of EEM fluorescence spectroscopy are that the fluorescence characteristics can be entirely acquired by changing excitation wavelength and emission wavelength simultaneously, and the samples will not be destructed (Baker, 2001; Sheng and Yu, 2006).

As reported in our previous study (Yang et al., 2010), the additional enzymes could strongly enhance WAS hydrolysis. However, the destruction mechanisms of WAS during anaerobic digestion has not yet been investigated. Meanwhile, the report of using EEM spectrometric characteristics of EPS and DOM to explore the destruction mechanisms of WAS disintegration enhanced by enzymes was hardly available. Therefore, the objectives of this research were to obtain the EEM fluorescence characteristic of the EPS and DOM during WAS anaerobic digestion enhanced by enzymes and attempt to well understand this process from a novel insight.

2. Materials and methods

2.1. WAS and enzymes

WAS were obtained from the secondary sedimentation tank of the second municipal wastewater treatment plant in Changsha, China. Fresh sludge was concentrated by settling for 4 h, further filtered through a 0.71-mm metal sieve and then stored at 4 °C for later use. The characteristics of sludge were as follows: pH 6.7 ± 0.2, total chemical oxygen demand (COD) 8430 ± 250 mg L^{-1}, soluble COD 120 ± 10 mg L^{-1}, total suspended solids (TSSs) 8820 ± 200 mg L^{-1}, volatile suspended solids (VSSs) 6050 ± 80 mg L^{-1}, total carbohydrate 590 ± 60 mg L^{-1} and total protein 5560 ± 180 mg L^{-1}.

The enzymes were preparations of commercial neuter protease and α-amylase (both purchased from Solarbio Biotechnology). The activities of neuter protease and α-amylase were 6000 and 3700 U g^{-1}, respectively.

2.2. Batch experiments

In accordance with the previous study (Yang et al., 2010), anaerobic digestion were carried out in 500 mL reactors with 400 mL of WAS. Additional protease, α-amylase and the mixed enzymes with the ratio of 1:3 (w/w, protease/α-amylase) was respectively added into three reactors at the dosages of 0.06 g g^{-1} dry sludge. Oxygen was removed from the headspace by nitrogen gas sparging for 4 min to maintain strict anaerobic condition. The reactors capped with rubber stoppers were agitated in water-bath shaker (100 rpm) and the temperature was kept constantly at 50 °C. The test without enzyme addition was defined as the blank test, in which other operation parameters were similar to the enzymatic anaerobic digestion tests. The anaerobic digestion process lasted for 12 d. After start-up, sludge was sampled every day and analyzed for soluble protein, soluble carbohydrate and volatile fatty acids (VFAs), and the EEM fluorescence spectroscopy of the EPS and DOM was analyzed after 1, 6 and 12 d.

2.3. EPS extraction

The EPS of WAS were extracted using the thermal treatment method described by Chang and Lee (1998). The WAS samples were firstly centrifuged at 3200 rpm for 30 min in order to remove the bulk solution. After discarding the supernatant, the remaining sludge samples were washed and re-suspended with 0.9% NaCl solution, and then followed by subjecting to heat treatment for 1 h at 100 °C and centrifuging again under the same operating conditions. Finally, the supernatants were then filtrated through 0.45-μm membrane filter (Whatmann, USA) and were used as the EPS fraction for EEM fluorescence spectral analyses.

2.4. Analytical methods

2.4.1. EEM fluorescence spectroscopy

Fluorescence measurements were conducted using a luminescence spectrometry (F-7000 FL spectrophotometer, Hitachi, Japan). In this study, emission scans were performed from 250 to 550 nm at 5 nm increments, with excitation wavelengths from 220 to 400 nm at 5 nm intervals. The detector was set to high sensitivity by adjusting the voltage of the photomultiplier tube to 700 V, and the scanning speed was maintained at 2400 nm min^{-1}. The slit widths for excitation and emission were 2.5 nm. A 290 nm emission cutoff filter was used in scanning to eliminate second order Raleigh light scattering. The spectrum of distilled water was recorded as the blank. To solve the overlap problem of the fluorescence spectra, the spectral data were analyzed using the parallel factor analysis (PARAFAC) algorithm to quantitatively determine the components of fluorophores.

2.4.2. Other analytical methods

Sludge samples were filtered through a Whatmann GF/C glass microfiber filter with 1.2 μm pore size, the filtrate was analyzed for soluble protein and soluble carbohydrate. Sludge samples were firstly centrifuged at 10,000 rpm for 5 min, the supernatants were then filtrated through 0.45-μm membrane filter (Whatmann, USA), and were used to analyze for VFAs. The determination was the same as described in our previous publications (Yang et al., 2010; Luo et al., 2011).

3. Results and discussion

3.1. Hydrolysis and acidification products during anaerobic digestion

The profiles of soluble protein and carbohydrate concentrations during anaerobic digestion after enzymatic-enhanced treatments are illustrated in Fig. 1a and b. Apparently, soluble protein and car-

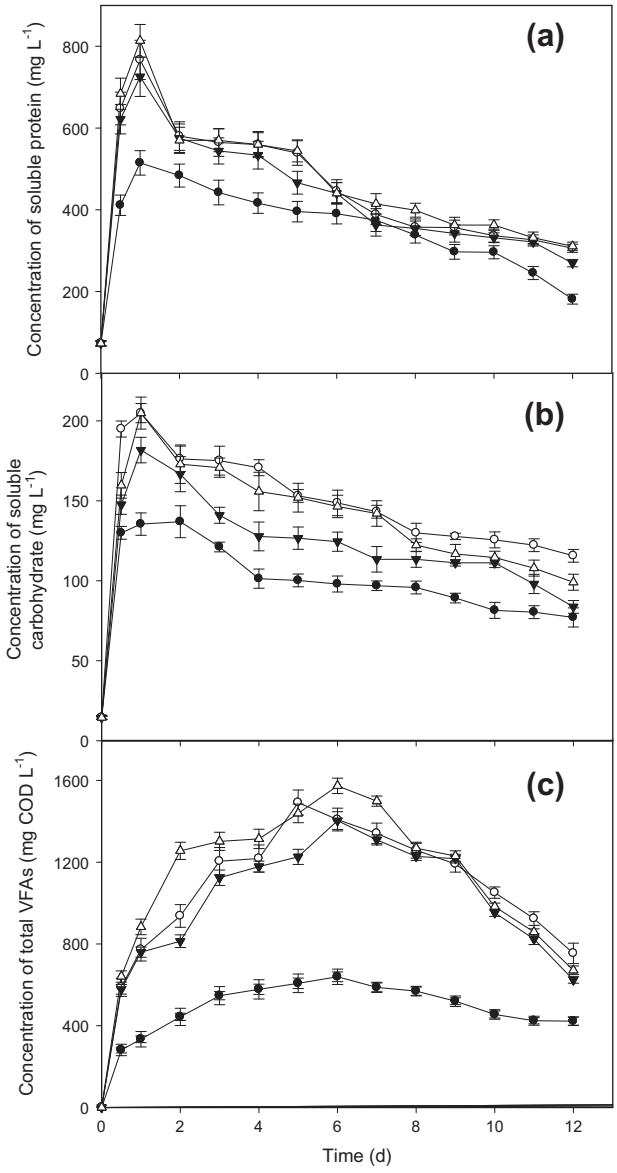

Fig. 1. Soluble protein (a), soluble carbohydrate (b), and total VFAs (c) concentrations after enzymatic-enhanced treatments. (●) Blank, (○) protease, (▼) amylase and (△) mixed enzyme.

bohydrate increased rapidly in the initial 1 d, and then gradually declined till the end of anaerobic process. As to the mixed enzyme system, the soluble protein concentration increased from 73 to 813 mg L^{-1}, and the soluble carbohydrate increased from 14 to 205 mg L^{-1} after 1 d. Correspondingly, the soluble protein increased from 73 to 514 mg L^{-1}, and the carbohydrate increased from 14 to 136 mg L^{-1} for the blank system. Afterwards, the concentration of soluble protein and soluble carbohydrate gradually decreased till the end of anaerobic process. The transformation of large molecule organics (such as protein and carbohydrate) to low molecule organics could be enhanced by hydrolytic enzymes, thus the concentration of total VFAs after enzyme treatments was always higher than that of the blank during the entire process. For instance, the total VFAs was 1574 and 640 mg COD L^{-1} respectively for the mixed enzyme and the blank system after 6 d. This result was consistent with the report proposed by Yu et al. (2003) that more soluble organics were correspondence with higher short-chain fatty acid accumulation.

3.2. EEM fluorescence spectra of EPS during anaerobic digestion

In this study, three-dimensional EEM spectroscopy was applied for characterizing the EPS extracted from WAS. The EEM fluorescence spectra of EPS samples extracted from raw sludge are illustrated in Fig. 2a. The correct number of components was determined by using second-order calibration method coupled with PARAFAC algorithms, in which the observed peaks were identified by comparing their fluorescence properties (excitation/emission (*Ex*/*Em*)) with those of pure compounds, such as aromatic protein, tryptophan protein and humic acid. With the PARAFAC, three main peaks could be identified from the fluorescence spectra of EPS samples of the raw sludge (Fig. 2b). The first main peak was located at the *Ex*/*Em* of 225/340 nm (Peak A), and the second was observed at the *Ex*/*Em* of 275/340 nm (Peak B). The two peaks located at the same *Em* had been reported as protein-like peaks, in which the fluorescence was associated with aromatic protein-like substances (Peak A) and tryptophan protein-like substances (Peak B) (Yamashita and Tanoue, 2003; Sheng and Yu, 2006). Peak C with relatively low fluorescence intensity (FI) was located at the *Ex*/*Em* of 320/405 nm, and the peak present in the region of *Ex* > 280 nm and *Em* > 380 nm represented humic acid-like substances (Chen et al., 2003; Wang et al., 2009).

EEM spectra of the EPS from WAS after enzymatic-enhanced treatments are illustrated in Fig. 3. They were similar in the peak locations at different times, but had different FI after enzymatic treatments. Compared with the EEM fluorescence spectra of the EPS in the raw sludge, Peak C at the *Ex*/*Em* of 320/405 nm was absent after 1 and 6 d, and two main peaks could be readily identified from Fig. 3a and b, located at the *Ex*/*Em* of 225/335–340 and 270–280/335–350 nm, respectively. However, only one peak located at the *Ex*/*Em* of 225–235/350–360 nm remained after 12 d of anaerobic digestion (Fig. 3c). Peak locations of the EPS samples after enzymatic treatments showed a slight difference, of which the Peak A after amylase treatment demonstrated a blue shift of 5–15 nm, while Peak B was red-shifted (5–15 nm). The location shift of fluorescence peak provided spectral information on the chemical

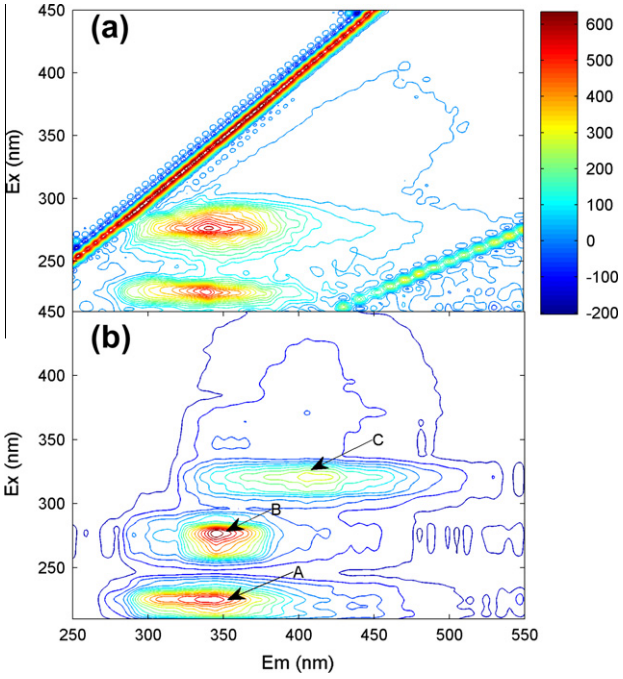

Fig. 2. EEM fluorescence spectra of the EPS of the raw sludge (a) and EEM spectra of three components decomposed by PARAFAC (b).

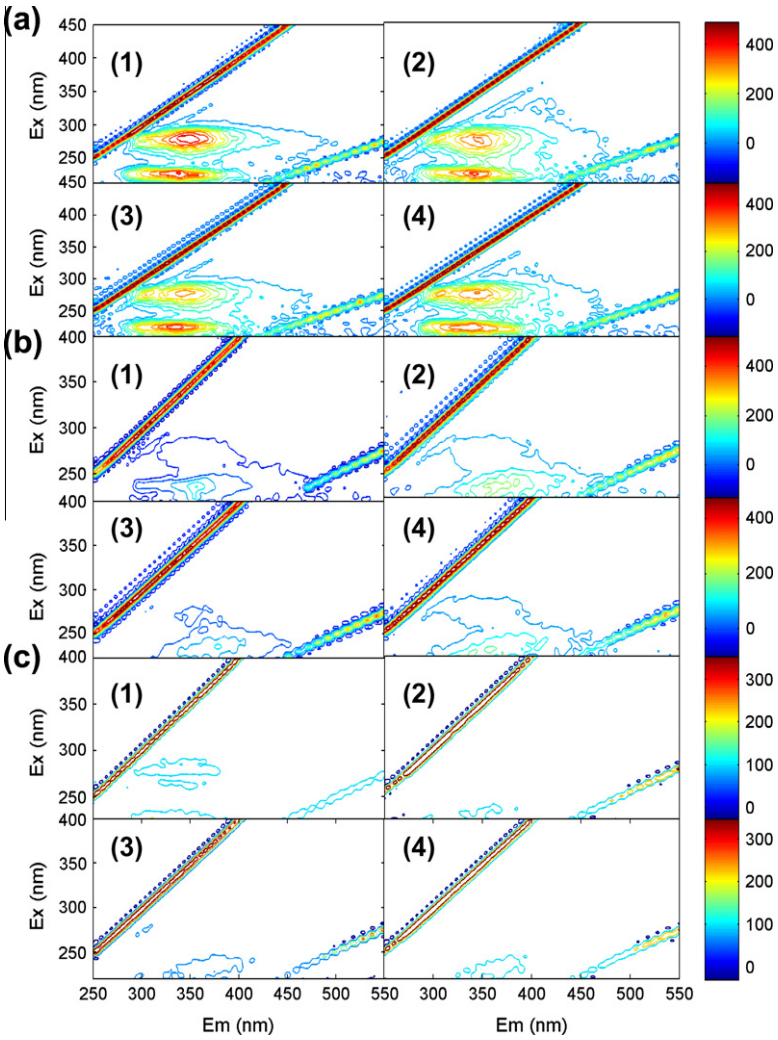

Fig. 3. EEM fluorescence spectra of the EPS after enzymatic-enhanced treatments: (a) 1 d, (b) 6 d and (c) 12 d (1 blank, 2 protease, 3 amylase and 4 mixed enzyme).

structure changes of the EPS samples. A red shift is related to the increase of carbonyl, hydroxyl, alkoxyl, amino, and carboxyl groups in the structure of fluorophores (Chen et al., 2002), while a blue shift is ascribed to the elimination of particular functional groups such as carbonyl, hydroxyl and amine, the reduction in the degree of π-electron systems, and the decrease in the number of aromatic rings and conjugated bonds in a chain structure (Coble, 1996; Swietlik et al., 2004).

The fluorescence parameters of the spectra including peak locations, FI and different peak intensity ratios are summarized in Table 1. The locations of Peak A after 6 and 12 d were red-shifted (5–20 nm) to longer wavelengths compared with that after 1 d. Correspondingly, the locations of Peak B after 6 d were red-shifted (5–15 nm), and it was disappeared after 12 d. The FI of Peaks A and B of the EPS was gradually decreased during the entire anaerobic digestion, while Peak C disappeared after 1 d. It has been reported that sludge flocs could be divided into four layers, slime, loosely bound extracellular polymeric substances (LB-EPSs), tightly bound EPS (TB-EPS) and pellet (Yu et al., 2008). During anaerobic digestion, the EPS was gradually degraded by hydrolytic enzyme, thus the slime, LB-EPS, TB-EPS and pellet, which were mainly composed of protein- and humic acid-like substances, were gradually released to supernatant. The slime and LB-EPS were firstly degraded, and TB-EPS and pellet fractions were the last to release.

The peak intensity ratios could provide additional information about the chemical nature of the macromolecules and further imply the structural differences (Coble, 1996). As seen from Table 1, the ratio of A/B of different enzymatic treatments increased markedly from 1 to 6 d, but Peak B disappeared after 12 d, indicating that the release and transformation of tryptophan protein-like sub-

Table 1
Fluorescence spectra parameters of the EPS during anaerobic digestion.

Samples	Peak A		Peak B		Peak C		A/B
	Ex/*Em*	FI	*Ex*/*Em*	FI	*Ex*/*Em*	FI	
Raw sludge	225/340	534	275/335	570	330/415	67	0.93
t = 1 d							
Blank	225/340	490	280/350	433			1.13
Protease	225/340	378	275/335	330			1.14
Amylase	225/335	411	280/345	327			1.26
Mixed enzyme	225/340	371	280/345	339			1.09
t = 6 d							
Blank	235/355	309	275/345	160			1.94
Protease	235/360	178	275/345	74			2.40
Amylase	235/360	242	275/355	90			2.68
Mixed enzyme	225/350	225	275/350	90			2.49
t = 12 d							
Blank	230/350	242					
Protease	225/350	100					
Amylase	235/360	142					
Mixed enzyme	230/360	123					

stances (Peak B) to supernatant was more rapidly than that of aromatic protein-like substances (Peak A). The FI of characteristic peaks on EEM plot have a good relationship with the concentration of fluorophores (Ni et al., 2009; Li et al., 2011). Thus according to Table 1, the residual protein-like substances in the EPS, including aromatic protein-like (Peak A) and tryptophan protein-like substances (Peak B) after different treatments was as follows: protease < mixed enzyme < amylase < blank during the entire anaerobic process. The EPS was gradually degraded by hydrolytic enzyme, contributing to the release of protein- and humic acid-like substances to supernatant, suggesting the biodegradable fluorophores were used as a substrate gradually metabolized by microorganisms. Each enzyme specifically attacks certain targeted bonds, proteins can be released by protease, amylase can also cause small quantity of proteins release along with the carbohydrates (Sesay et al., 2006), contributing to the lowest residual protein-like substances in the EPS when treated with protease during the entire anaerobic process.

EPS significantly affects the degradation of sludge by enzymatic-enhanced treatment. The added and original enzymes are usually entrapped by, adsorbed by, or bound to the sludge (Wawrzynczyk et al., 2008). Cadoret et al. (2002) proposed that sludge hydrolysis rates depended on the diffusion of enzyme surface active site into sludge matrix particles. Thus the EPS resistance can reduce the contact between the enzyme and the substrate as well as the diffusion efficiency of the substrate in the EPS. As a consequence, the hydrolytic enzyme adsorbed by, or bound to EPS released into solution through the disruption of the EPS matrix, which inevitably led to the enhanced solubilization of sludge.

3.3. EEM fluorescence spectra of DOM during anaerobic digestion

The EEM fluorescence spectra of DOM might be used to evaluate the sludge destruction during enzymatic-enhanced anaerobic digestion. EEM fluorescence spectra of the DOM after enzymatic treatments are shown in Fig. 4, and the EEM fluorescence spectra shapes of DOM after different digestion times were similar among the four treatments. There were three main peaks observed in all DOM samples after 1 and 6 d, while one peak disappeared after 12 d of anaerobic digestion. The two peaks with higher FI were located at *Ex*/*Em* of 220–225/335–345 and 270–285/340–345 nm, and the peak with relatively low FI was at 320–325/405–410 nm.

The supernatant of the raw sludge was low-fluorescent (data not shown), thus increased fluorescence signal could be attributed solely to the generation and accumulation of fluorophores. The EEM FI of DOM sharply increased with enzymatic treatment after 1 d, indicating the protein- and humic acid-like substances were released to the supernatant accompanying the sludge destruction

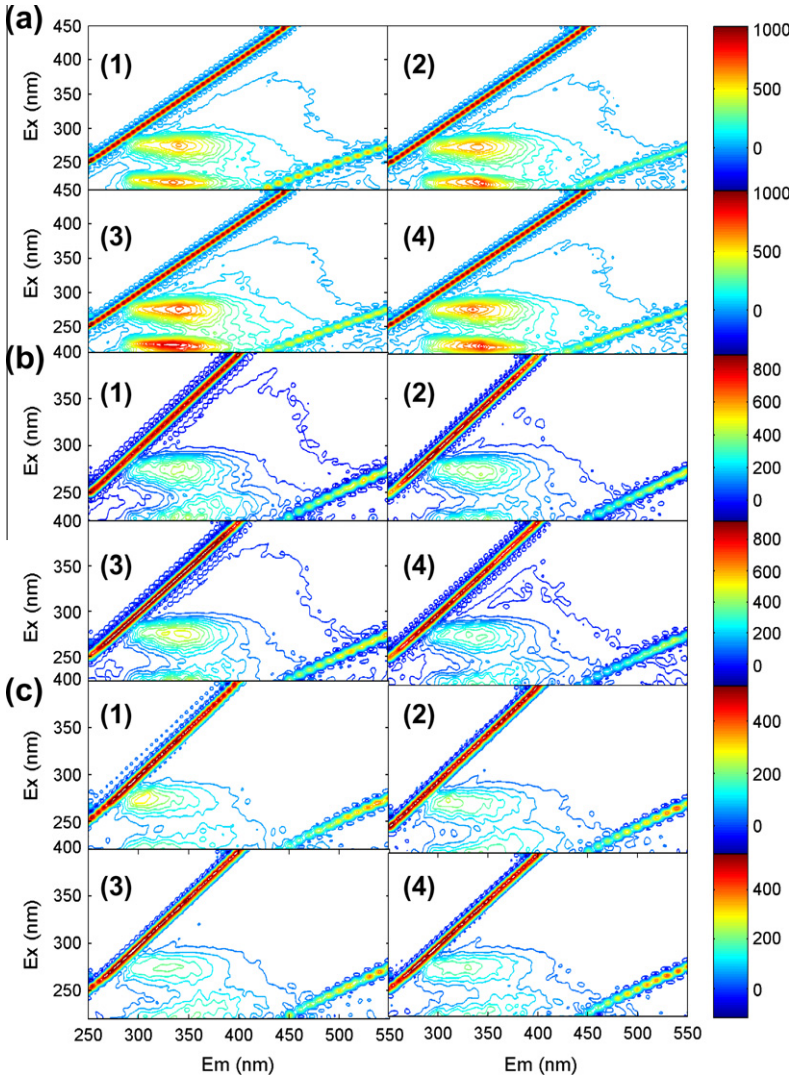

Fig. 4. EEM fluorescence spectra of the DOM after enzymatic-enhanced treatments: (a) 1 d, (b) 6 d and (c) 12 d (1 blank, 2 protease, 3 amylase and 4 mixed enzyme).

during the anaerobic digestion. It could be obtained from Yu et al. (2010) that the humic acid-like substances mainly existed in the slime and LB-EPS. Thus with the degradation of WAS, the outer layer of the EPS gradually released to the supernatant, leading to the emergence of Peak C in DOM after 1 d (Fig. 4a).

The EEM fluorescence spectra of the DOM after 6 and 12 d of anaerobic digestion are shown in Fig. 4b and c. In addition, Peak C located at the *Ex*/*Em* of 315–325/405–410 nm disappeared after 12 d, and Peak A at the *Ex*/*Em* of 220–225/335–345 nm was not obvious. The extracellular proteins were mainly substrate-utilization-associated, while the humic-acid-like substances were non-growth-associated (Li et al., 2008). Only monomeric and oligomeric substrates were able to cross bacterial membranes through cell-specific active transport processes, thus the sludge should undergo mass transfer into the bacterial cells in order to take part in metabolic reactions (Cadoret et al., 2002). The complex organic matter (such as protein and carbohydrate) in activated sludge was broken down into low molecular-weight intermediates (such as amino acid and glucose) by the action of hydrolytic enzymes. These low molecular-weight compounds were in turn assimilated by the cells and used as an autochthonous substrate that was subsequently used and degraded in microbial metabolism. A portion of organics was released as metabolic products in the ways of VFAs, CO_2, et al., resulting in the decrease of fluorophores (Figs. 1a and 4) and the increase of VFAs (Fig. 1c) in the supernatant.

According to Fig. 4, the protein-like substances in the DOM after different enzymatic treatments, including aromatic protein-like (Peak A) and tryptophan protein-like substances (Peak B), was ordered by blank < mixed enzyme < protease < amylase after 1 and 6 d. However, the order changed as protease < mix-enzyme < amylase < blank after 12 d of anaerobic digestion. The protein-like substances were the result of a net balance between competing rates of release and degradation. The particulate organics, protein and carbohydrate could be released to the solution and then degraded into low molecule products by the action of specific hydrolytic enzymes during anaerobic digestion. Nevertheless, the degradation rate of protein could be enhanced by protease (Pei et al., 2010; Yang et al., 2010), which caused more protein consumption and transformation to low molecule organics, thus resulted in the highest protein-like substances in the supernatant when treated with amylase, the second with protease and the lowest for the blank after 1 and 6 d. However, the blank system ranked the first and the protease system ranked the last after 12 d of anaerobic digestion.

4. Conclusions

As one of the rapid, sensitive and selective analytical methods, EEM fluorescence spectroscopy was appropriate for characterizing the EPS and DOM during WAS anaerobic digestion enhanced by hydrolytic enzymes. The protein-like (aromatic and tryptophan protein-like substances) and humic acid-like substances were the mainly fluorophores in the sludge, of which the release and transformation of tryptophan protein-like substances was more rapidly than that of aromatic protein-like substances. The EEM FI of EPS declined during the entire process, whereas that of DOM increased at 1 d and then decreased till the end of anaerobic digestion which was attributed to the substrate utilization in microbial metabolism. The destruction mechanisms of WAS enhanced by hydrolytic enzymes was proposed. The EPS was partially disrupted by the action of hydrolytic enzymes, and the complex DOM was also broken down into low molecular-weight intermediates, thus the hydrolytic enzyme could enhance WAS solubilization and destruction.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2012.12.002.

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