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Comparison of different treatment methods for protein solubilisation from waste activated sludge

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Abstract

Biomaterials recovery from wasted sludge has become an increasing interesting research topic. The purpose of this study was to systemically evaluate different sludge disintegration methods (ultrasonic, alkaline, and thermal treatments) for protein solubilisation from waste activated sludge (WAS). Compared to control without treatment, the soluble protein

concentration increased by 11, 23 and 12 times under the optimal treatment conditions (ultrasonic treatment of 1 W mL^{-1}, alkaline treatment of pH 12 and thermal treatment at 80°C). The increased soluble protein were significantly correlated with the release of total organic carbon (TOC), total dissolved nitrogen (TDN) and total organic nitrogen (TON) in soluble EPS, and the degradation of above parameters in tightly bound EPS. For all sludge samples treated by various methods, tyrosine-like protein with molecular weight less than 20 kDa predominated, and alkaline treatment at pH 12 showed the highest protein dominance. Further surface analysis of sludge by X-ray photoelectron spectroscopy indicated this might be related with the significant protein-N conversion occurred at pH 12. The economic analysis indicated alkaline treatment at pH 12 was economically feasible with a net saving of 25.57 USD per ton wet sludge compared to conventional sludge treatment and disposal method.

Keywords

Treatments; Protein solubilisation; Extracellular polymeric substances; Molecular weight distribution; N-containing component

Abbreviations

C	Specific heat of sludge
DOM	Dissolved organic matter
DOC	Dissolved organic carbon
EE	Electrical energy

43	Em	Emission
44	EPS	Extracellular polymeric substances
45	Ex	Excitation
46	HCl	Hydrochloride acid
47	HMW	High molecular weight
48	L1	Region I in loosely bound extracellular polymeric substances
49	L2	Region II in loosely bound extracellular polymeric substances
50	L3	Region III in loosely bound extracellular polymeric substances
51	L4	Region IV in loosely bound extracellular polymeric substances
52	L5	Region V in loosely bound extracellular polymeric substances
53	LB EPS	Loosely bound extracellular polymeric substances
54	LC-OCD-OND	Liquid chromatography organic carbon and nitrogen detection
55	LMW	Low molecular weight
56	NaOH	Sodium hydroxide
57	N1	Inorganic-N
58	N2	Protein-N
59	N3	Pyridine-N

60	N4	Pyrrole-N
61	N5	Quaternary-N
62	N6	Nitrile-N
63	ρ	Density of sludge
64	P	Ultrasonic power
65	pKa	Acid dissociation constant at logarithmic scale
66	Q	Heat energy
67	S1	Region I in soluble extracellular polymeric substances
68	S2	Region II in soluble extracellular polymeric substances
69	S3	Region III in soluble extracellular polymeric substances
70	S4	Region IV in soluble extracellular polymeric substances
71	S5	Region V in soluble extracellular polymeric substances
72	SB EPS	Soluble Extracellular polymeric substances
73	T1	Region I in tightly bound extracellular polymeric substances
74	T2	Region II in tightly bound extracellular polymeric substances
75	T3	Region III in tightly bound extracellular polymeric substances
76	T4	Region IV in tightly bound extracellular polymeric substances

77	T5	Region V in tightly bound extracellular polymeric substances
78	t	Sonication time
79	T_i	Initial temperature
80	T_f	Final temperature
81	TB EPS	Tightly bound extracellular polymeric substances
82	TDN	Total dissolved nitrogen
83	TKN	Total Kjeldahl nitrogen
84	TON	Total organic nitrogen
85	TS	Total solids
86	TSS	Total suspended solids
87	USD	The United States dollar
88	V	Volume of sludge treated
89	VS	Volatile solids
90	VSS	Volatile suspended solids
91	WAS	Waste activated sludge
92	XPS	X-ray photoelectron spectroscopy

93

94

1. Introduction

A large amount of waste activated sludge (WAS) produced during municipal and industrial wastewater treatment process has been considered as an inevitable drawback for bioprocesses, and its disposal and treatment poses a big challenge to the operation of wastewater treatment plants (Yan *et al.*, 2013). WAS has a complex floc structure that consists of different microorganisms, organic and inorganic matter agglomerated together in a polymeric network formed by cations and microbial extracellular polymeric substances (EPS) (Frølund *et al.*, 1996). In view of fact that WAS contains a number of economically useful organic substances, such as proteins, enzymes, nucleic acids, polysaccharides, recovering useful biomaterials (i.e. methane, hydrogen, and volatile fatty acids) from excess sludge through physiochemical or biological processes has received great attention recently (Li *et al.*, 2016, Wang *et al.*, 2015a, Wang *et al.*, 2015b).

Protein accounts for about 50% of the dry weight of bacterial cells in waste activated sludge (Shier and Purwono, 1994). The steps for protein recovery from sludge typically included screening, treatments, filtration, protein precipitation from protein solution, drying of protein precipitate and the recovery of final protein product (crude protein) (Chishti *et al.*, 1992). The recovered protein (i.e. crude protein) can be used as feed stuff (Hwang et al. 2008) and wood adhesive (Pervaiz and Sain, 2011), which would help to mitigate global energy shortage. Prior to protein recovery, solubilisation of WAS is necessary. Methods for sludge solubilisation have been investigated for years, including physical (Tian *et al.*, 2016), chemical (Xiang *et al.*, 2017) and biological methods (Luo *et al.*, 2012, Yang *et al.*, 2010), with thermal, ultrasonic, and chemical treatments typically used to solubilize both intracellular (within the microbial cells) and extracellular (within the polymeric network) materials before sludge digestion step.

Ultrasonication can form cavitation bubbles in the liquid phase and it may also induce chemical reactions by forming OH·, HO_2·, and H· radicals at high frequencies, thus solubilize proteins from sludge (Salsabil *et al.*, 2009). Feng *et al.* (2009) reported soluble protein increased from 538.33 to 1000 mg L^{-1} with specific energy input increased from 0 to 26000 KJ kg^{-1} dry solids. Alkaline treatment is one of the most widely used chemical methods for protein solubilisation from sludge, with advantages of simple manufacturing of device, easy operation and high efficiency (Weemaes and Verstraete, 1998). Alkali can induce solubilisation of membrane proteins, saponification of the membrane lipids and damage the microbial cell (Mendonca *et al.*, 1994). Yuan *et al.* (2006b) found during alkaline treatment of waste activated sludge, soluble protein increased by 200 mg L^{-1} at pH 8, 600 mg L^{-1} at pH 9, 1000 mg L^{-1} at pH 10 and 1010 mg L^{-1} at pH 11. While for thermal treatment, mild temperature treatment has drawn more attention recently compared to high temperature treatment (> 100 ℃) and high pressure (> 10 M), as the latter required high energy input and dedicated equipment (Xue *et al.*, 2015). Zhang *et al.* (2015a) reported soluble protein increased from 50 mg L^{-1} at 35˚C to 300 mg L^{-1} at 80˚C, 600 mg L^{-1} at 100˚C and 1600 mg L^{-1} at 120˚C during thermal treatment of dewatered activated sludge. However, most of these studies just considered how to increase the soluble protein content, and the energy consumption and cost accompanied are largely ignored. Therefore, cost and energy input need to be carefully analyzed when investigating the feasibility of each treatment method. Moreover, contradictory results about the best treatment method for protein solubilisaiton existed, i.e. Yu *et al.* (2014) reported protein solubilisation efficiency was in sequence of electrochemical> thermal-alkaline>thermal>alkaline, while Chishti *et al.* (1992) reported alkaline treatment was the best method for protein recovery from sludge. A more

systematic and detailed investigation of these treatments on protein solubilisation is necessary, with purpose of obtaining a more appropriate and economically feasible treatment method.

The organic material solubilized by ultrasonic, thermal, and alkaline treatments can be both intracellular material (cytoplasm) and extracellular organic compounds contained in the bacterial flocs (Gonze *et al.*, 2003). Extracellular polymeric substances (EPS) was a more significant contributor to the sludge mass compared to the intracellular material, as it accounts up to 80% of sludge biomass (Liu and Fang, 2003) and is a significant component in microbial aggregates for keeping them together in a three-dimensional matrix (Sheng *et al.*, 2006). EPS can be categorized as soluble EPS (SB EPS), loosely bound EPS (LB EPS), and tightly bound EPS (TB EPS), and are composed of biopolymers, i.e. proteins, carbohydrates, humic acid-like substances, uronic acids, nucleic acids (Xiao *et al.*, 2016). To our best knowledge, most previous studies on protein solubilisation have not specifically and precisely addressed the contribution of EPS. For instance, the correlation between solublilized protein and EPS content, composition, and stratification are limited. Consequently, the essential role played by EPS still needs further identification during protein solubilization with different treatment methods. Moreover, some studies have characterized protein in EPS in terms of protein content (Yuan *et al.*, 2006a), protein composition (Jorand *et al.*, 1998), and molecular weight (Görner *et al.*, 2003). Different treatment methods may alter the related protein molecular weight and type (Dewit and Klarenbeek, 1984, Wang *et al.*, 2016, Xiang and Wang, 2015). For example, the protein molecular weight would largely affect the properties of final protein product, i.e. wood adhesive through affecting the contact surface and interacting groups (Liu *et al.*, 2017), while the protein type can affect the final amino acid composition of crude protein, thus affecting its application as feed stuff (Chishti *et al.*, 1992). Nevertheless, little is known about the effects of the above

163 mentioned treatment methods on the changes of protein characteristics i.e. protein molecular
164 weight and type (Xin *et al.*, 2009, Zhang *et al.*, 2015a).

165 Protein is made up of organic nitrogen (Aquino and Stuckey, 2004). Ultrasonic treatment can
166 decrease the organic nitrogen in particles and increase it in soluble phase (Bougrier *et al.*, 2005).
167 The nitrogen content in solid/liquid correlated with protein solubilisation or decomposition
168 extent (Réveillé *et al.*, 2003, Tian *et al.*, 2014). Understanding the nitrogen species distribution in
169 liquid and solid fractions would offer mechanistic insights into nitrogen transformation during
170 protein solubilisation. There are, however, very few reports on the detailed transformation
171 pathways of nitrogenous species in solid and liquid phases during protein solubilisation by
172 different treatment methods. It is not clear if the treatment methods would affect the nitrogenous
173 species transformation pathways. Moreover, the correlation between N-containing compounds
174 and protein solubilisation has not been investigated in details.

175 The objectives of this study were to evaluate and compare different treatment methods for
176 protein solubilisation. The qualitative characteristics of protein, and their relationship with EPS
177 fractions were investigated. The changes of protein molecular weight and type were studied. The
178 transformation pathways of nitrogenous species in solid and liquid phases during protein
179 solubilisation were investigated. Economic assessment of different treatment methods on protein
180 solubilisation was conducted to investigate the feasibility of each treatment method.

181 **2. Materials and methods**

182 **2.1. Source and characteristics of waste activated sludge**

183 Waste activated sludge was obtained from a local wastewater treatment plant in Singapore.
184 The sludge was concentrated by settling for approximately 24 h at 4 °C prior to use. The total

185 solids (TS), volatile solids (VS), total suspended solids (TSS), and volatile suspended solids
186 (VSS) of the sludge were 11.49 ± 1.11, 8.77 ± 0.59, 9.70 ± 0.41 and 7.92 ± 0.40 g L^{-1}. The pH of
187 WAS was 6.3.

188 **2.2. Sludge treatment methods**

189 For thermal treatment, temperature below 100℃ can be considered as mild thermal treatment
190 (Gavala *et al.*, 2003). A series of experiments were conducted in a 150 mL thermal reactor
191 equipped with a thermometer and magnetic stirrer. For each test, the sludge sample was first
192 preheated to designed temperatures (25, 40, 60 and 80 °C) and maintained at desired temperature
193 for 30 mins, with stirring speed controlled at 200 rpm to avoid temperature gradients.

194 For alkaline treatment, pH of sludge samples (150 mL) was adjusted to 8, 10, and 12 with
195 hydrochloride acid (HCl) (5 mol L^{-1}) and sodium hydroxide (NaOH) (5 mol L^{-1}). After pH
196 adjustment, sludge samples were shaken at 150 revolutions per minute (rpm) for 30 min in an
197 incubator at room temperature (25°C) (Sartorius Stedim Biotech, Germany).

198 For ultrasonic treatment, the energy intensity was set at 0 W mL^{-1} (no sonication), 0.25 W
199 mL^{-1} (specific energy input of 3.04 kJ g^{-1} TS), 0.5 W mL^{-1} (specific energy input of 6.10 kJ g^{-1}
200 TS) and 1 W mL^{-1} (specific energy input of 10.71 kJ g^{-1} TS) by adjusting the input power with a
201 fixed sludge volume (150 mL) for 2 mins, which was the optimum treatment duration based on
202 previous study (Xiao *et al.*, 2016). Ultrasonic treatment was performed with an ultrasonicator
203 (Q700, Misonix Qsonica, Newton, CT, USA). The tip of the sonication probe was centrally
204 placed 1 cm into sludge samples held in a beaker. Temperature of sludge samples in the beaker
205 was maintained at 25 ± 1°C with ice bath where necessary.

206 **2.3. Analytical methods**

2.3.1. Characteristics of waste activated sludge

TS, VS, TSS and VSS of sludge were measured according to the standard methods (APHA, 2005).

2.3.2. EPS extraction

EPS of sludge samples was extracted with the method described by Li and Yang (2007). The definitions of SB EPS, LB EPS and TB EPS were based on the extraction steps described in Li and Yang (2007). Briefly, sludge sample (15 mL) was centrifuged at 4000 × *g* at 4℃ for 15 mins, and the supernatant was collected for SB EPS content analysis. The sludge pellet left was then re-suspended with 15 mL of 0.05% sodium chloride (NaCl) solution. The sludge sample was re-suspended with a vortex mixer and then incubated at 70℃ water bath for 1 min, followed by centrifugation at 4000 *g* at 4℃ for 10 mins. The organic matter in the supernatant was readily extractable EPS, and was regarded as the LB EPS of the biomass. The residual sludge pellet was re-suspended to its original volume by adding 0.05% NaCl solution, put at 60℃ water bath for 30 mins and the sludge mixture was centrifuged at 4000 *g* at 4℃ for 15 mins. The supernatant collected was regarded as the TB EPS extraction of the sludge. The SB EPS, LB EPS and TB EPS substances were then filtered through a 0.45 μm cellulose nitrate membrane and then subjected to EPS analysis.

2.3.3. Analysis of extracted EPS

2.3.3.1. Dissolved organic carbon, total dissolved nitrogen, protein, ammonium, nitrite, nitrate measurements

The EPS content was characterized by measuring different parameters. Dissolved organic carbon (DOC), and total dissolved nitrogen (TDN) were measured using a TOC/TN analyser (Shimadzu, Japan). Protein was measured with the modified Lowry method (Frølund *et al.*, 1995). Ammonium, nitrite, and nitrate concentrations were measured with flow injection analysis (Lachat Instruments, Singapore). Total dissolved organic nitrogen (TON) concentrations were calculated by the difference of TDN and total inorganic nitrogen species (nitrate + nitrite + ammonium) concentrations (He *et al.*, 2015).

2.3.3.2. Three dimensional excitation emission (3D EEM)

The protein type in SB EPS, LB EPS and TB EPS were determined with a luminescence spectroscope (Model LS-S5, PerkinElmer®, Waltham, MA, USA), with 230-520 nm excitation wavelength at intervals of 10 nm and 230-545 nm emission wavelength at intervals of 0.5 nm. The excitation and emission slit bandwidths were 10 nm for spectra, and were recorded at 10,000 nm min^{-1}scan rate. The unit of fluorescence intensity was Raman Unit (RU) (Singh *et al.*, 2010).

The EEM spectrum was delineated into five regions based on methods described in Chen et al. (2003), which were associated with tyrosine-like proteins (Region I; excitation (Ex) wavelengths less than 250 nm), tryptophan-like protein (Region II, emission (Em) wavelength less than 380 nm), fulvic acid-like materials (Region III, Ex/Em wavelengths: 230-250 /380-545 nm), microbial by-product-like materials (Region IV, Ex/Em wavelengths: 250-280 /230-380 nm), and humic acid-like organic compounds (Region V, Ex/Em wavelengths: 280-520/ 380 - 545 nm).

2.3.3.3. Molecular weight distribution of protein in different fractions of EPS

The high molecular weight (> 20 KDa) distribution of protein (HMW protein) in SB EPS, LB EPS and TB EPS were quantified with a size-exclusion organic carbon and nitrogen detection (LC-OCD-OND) (DOC-LABOR, Karlsruhe, Germany). Details of the measurement procedure can be found in Xiao *et al.* (2017). Injection volume of samples was 1000 µL. Concentrations of low molecular weight protein (LMW protein) was calculated by subtracting HMW protein from the total protein concentration determined by the modified Lowry method (Frølund *et al.*, 1995).

2.3.4. Sludge solids characterization

For solids characterization, sludge samples were first dried for 24 h, and then cooled down to room temperature. The dry sludge samples were then ground and filtered through a sieve (60 mesh) with details described in Tian *et al.* (2014). The filtered sludge samples were stored in a desiccator at room temperature (25℃) until further use.

Elemental composition (i.e. nitrogen) of the solids was determined with an elemental analyser (vario EL cube CHNOS, Germany). The Total Kjeldahl Nitrogen (TKN) of solid power was measured with an automatic TKN analyser (KjelFlex K-360, Metrohm, Switzerland). This measurement has been estimated to represent total organic nitrogen in solid sludge samples (Bougrier *et al.*, 2005).

N 1s X-ray photoelectron spectroscopy was used to determine the evolution of N-containing compounds in sludge solid residues. Samples were selected based on the highest protein solubilisation efficiency with each treatment method, i.e. ultrasonic treatment at 1 W mL^{-1}, thermal treatment at 80 °C and alkaline treatment at pH 12. The studies were conducted using a Kratos Axis Supra spectrophotometer with a dual anode monochromatic Kα excitation source. N

1s XPS spectra of all solid powder samples were corrected against an adventitious carbon C 1s core level at 284.8 eV. All XPS peaks were fitted using Shirley background together with Gaussian-Lorentzian function using CASA XPS software. According to methods described in Tian *et al.* (2013) and Kelemen *et al.* (2002), the N peaks can be assigned to inorganic–N, protein-N, pyridine–N, pyrrole–N, quaternary–N and nitrile–N at respective binding energy values of 402.5, 400, 398.8, 400.3, 401.4, 399.7 eV.

2.3.5. Energy consideration and economic analysis

The theoretical computation of energy balance in this study was calculated according to the experimental data. The costs for thermal and ultrasonic treatments were mainly electricity associated with energy input, while for chemical treatment, the cost was mainly on NaOH dosage. For thermal treatment, the heat input was calculated based on Eq. 1 with details described in Passos and Ferrer (2014).

Energy (input, heat) $\mathbf{Q} = \boldsymbol{\rho} * \boldsymbol{V} * \boldsymbol{C} * (T_f - T_i)$ (1)

Where Q is the heat energy required to heat the sludge (kJ), ρ is the density of sludge (kg m^{-3}), V is the volume of sludge treated (m^3), C is the specific heat of sludge (kJ kg^{-1} °C) (4.2 kJ kg^{-1} °C), T_i and T_f are the initial and final temperatures (°C) of the sludge, respectively.

For ultrasonic treatment, electrical energy (EE) required for ultrasonic pretreatment was calculated based on ultrasonic input power and sonication time using the following equation (Kavitha *et al.*, 2016):

$\text{Energy}_{(\text{input, electricity for sonication})}$ EE= P*t (2)

Where EE is electrical energy (KW·s), P is ultrasonic power (KW m^{-3}), and t is sonication time (s).

3. Results and discussion

3.1. The components of different fractions of EPS

The changes of DOC, TDN, TON, protein and ammonium concentrations in different fractions of EPS (i.e. SB EPS, LB EPS, and TB EPS) were measured at various treatment conditions as shown in Fig.1. For each treatment, the trends of DOC, TDN, TON and protein concentrations in SB EPS were similar, that is, increased as treatment intensity increased. In LB EPS, the concentrations of DOC, TDN, TON and protein increased as pH and ultrasonic intensity increased, but decreased at high temperatures, i.e. 60°C and 80°C. For TB EPS, with pH and temperature increased, the concentrations of DOC, TDN, TON and protein decreased. The relationship between solubilized protein in SB EPS (defined as soluble protein) and concentrations of other dissolved organic matter was investigated based on Pearson's correlation and shown in Table 1. The results indicated soluble protein was positively related to TOC, TDN and TON in SB EPS, while negatively correlated to those compounds in TB EPS. The results suggested organic compounds in TB EPS (i.e. TOC, TDN and TON) were solubilized and converted into bulk liquid with treatments (Xiao *et al.*, 2016, Zhang *et al.*, 2015b). This result suggests attacking TB EPS layer and degradation of related organic compounds therein, i.e. TOC, TDN and TON, were the key steps for protein solubilization. Compared to control without treatment, the soluble protein increased by 11, 23 and 12 times under the optimal conditions (ultrasonication of 1W mL^{-1}, alkaline treatment of pH 12 and thermal treatment at 80°C).

In order to know whether soluble proteins were degraded by treatments, ammonium concentrations were measured. The changes of ammonium in bulk solution were different with

various treatment methods. It increased with increased temperature and ultrasonic intensity. This could probably be related with protein degradation in WAS (Negral *et al.*, 2013), as the peptide bonds of proteins in WAS started to be cleaved, and eventually led to the release of ammonium (Tian *et al.*, 2013). Similarly, for thermal treatment, ammonium concentration increased as temperature was higher than 25°C, which implies that part of soluble protein being degraded by heat (Xue *et al.*, 2015). In alkaline treatment, it increased as pH increased from 6.3 to 8, but decreased at pH 10 and 12. This was likely due to the conversion of ammonium to volatile ammonia at strong alkaline conditions (pKa value for NH_3 is 9.25 at 25 °C) (Liu *et al.*, 2015). Therefore, it seemed proteins were solubilized and partially degraded due to the treatments conducted. Moreover, the soluble protein in the supernatant was from both EPS and cell lysis, which can be seen from the variation of proteins in supernatant and EPS. The increment of proteins in the supernatant at each treatment all exceeded the decrement of proteins in TB EPS. Theoretically, the difference between the two values should be proteins from cell lysis.

Moreover, TOC, TDN, TON and protein in respective fraction of EPS was correlated with each other (Table 1), and this indicated chemical compositions of organic compounds (i.e. protein, TOC, TON, TDN) in each fraction of EPS (i.e. SB EPS, LB EPS or TB EPS) were similar. The results of Zhang *et al.* (2014) supported this finding that the chemical compositions of each fraction of EPS (e.g. TB EPS) was similar and relatively stable with time regardless of processes types of wastewater treatment plants.

3.2. Molecular weight distribution of protein in different fractions of EPS

The molecular weight distribution of protein, namely high molecular weight (HMW; > 20 kDa) and low molecular weight (LMW; 0-20 kDa) is shown in Table 2. For all the sludge samples treated by different methods, low molecular weight protein was more dominant (as indicated by green colour) than high molecular weight protein (as indicated by red colour) in all EPS fractions. Most of the soluble protein in bulk liquid was proved to be LMW protein in SB EPS rather than HMW protein in SB EPS (Table S1). The results were closely related to the treatment methods applied. For example, ultrasonication can cause cleavage of chains of larger molecular weight (Jambrak *et al.*, 2014) and split molecules into LMW substances (Grönroos *et al.*, 2004). Tan *et al.* (2012) reported most of the proteins were hydrolysed into small fractions by the alkaline treatment, and these low molecular weight fractions were transferred into the supernatant. Silva *et al.* (2017) also found thermal treatment can increase the solubilisation of low molecular weight proteins, but reduce the solubilisation of high molecular weight proteins, as heat can intensify interactions between amino acids radicals of the proteins through electrostatic interactions, hydrogen bonding, and hydrophobic interactions, thus hindering the accessibility to HMW protein (Silva *et al.*, 2017). Compared to the raw sludge, LMW protein in SB EPS increased for all treated sludge samples, which is consistent with the results shown in Fig. 1d. These smaller molecular weights of protein may result in a larger contact surface, thus interacting more groups, which would improve wet strength of protein adhesives on wood (Liu *et al.*, 2017). It was noted compared to WAS without treatment, the LMW protein concentration increased by 9, 19 and 12 times under the optimal condition (ultrasonication of 1W mL^{-1}, alkaline treatment of pH 12 and thermal treatment at 80°C).

3.3. 3D EEM analysis

3D-EEM fluorescence spectroscopy is a rapid and sensitive technique to measure the fluorescence compounds in EPS, and the two peaks with fluorescence intensity can be associated with the protein-like substances (Liu *et al.*, 2016). To investigate the effects of different treatments on the changes of protein types in different EPS fractions, 3D-EEM fluorescence spectroscopy was employed. The fluorescence region integration (FRI) results are summarized in Table 3. For all the EPS fractions, the fluorescence intensities of tryptophan-like protein and humic acid-like organic compounds (as indicated by green colour) were relatively higher than the other organic compounds (i.e. tyrosine-like proteins, fulvic acid-like materials and microbial by-product-like materials) (as indicated by red colour). In fact, EPS is consisted of large quantities of unsaturated fatty chains with various types of functional groups and aromatic structures (Wingender *et al.*, 1999). The dominance of tryptophan-like protein and humic-acid like substances may be related to the changes of the aromatic structures and acidic functional groups of macromolecular structure of EPS induced by various treatments (Sheng and Yu, 2006). For instance, Yang *et al.* (2013) concluded that similar amount of tryptophan-like protein and humic-acid like substances was present during ultrasonic treatment.

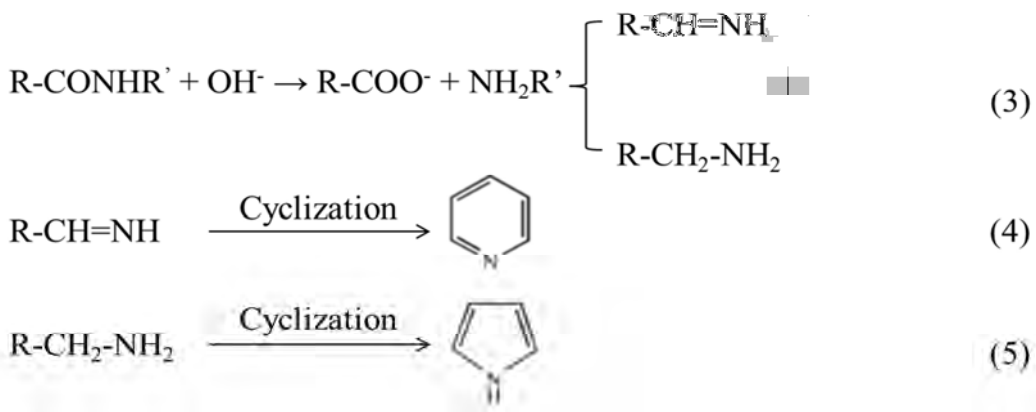
The fluorescence intensities of all the five organic compounds increased in SB EPS as treatment intensity increased. Pearson's correlation analysis showed the soluble protein in bulk liquid showed relatively higher correlations with tyrosine-like protein than the other organic compounds (Table S1). Moreover, the alkaline treatment at pH 12 can result in the highest production of tyrosine-like protein compared to other treatment methods. Tyrosine was an important amino acid composition of poultry requirement, and the typical suggested level of tyrosine requirement for chicken was 1.43% (Chishti *et al.*, 1992). The recovery of this type of

protein (with tyrosine as its main component) from WAS may be used as feed stuff and thus help to mitigate the energy crisis.

3.4. Evolution of N-containing compounds in solid residues by different treatment methods

Total organic nitrogen in solid residues was reduced after all the treatments (Table S2), which suggested ultrasonic, thermal and alkaline treatment could disintegrate organic nitrogen compounds in sludge solid matrix into ammonium and/or soluble protein in the liquid phase as shown in Fig.1. In this study, XPS technique had been deployed to analyse the distribution of N-containing compounds and composition in raw sludge and treated sludge solid samples. This would help to elucidate the nitrogen transformation during different treatment processes. As shown in Fig. 2 and Table 4, organic protein-N and pyridine–N were predominant N-containing compounds in all sludge solid residues. For ultrasonic treatment, up to 96.4% of N was left in solid residue with only slight decreases of protein-N and pyridine-N in solid residue compared to the raw sludge. Hence, ultrasonication applied in this study may not sufficient to release N compounds. For alkaline treatment, significant protein-N conversion occurred under basic conditions (Liu *et al.*, 2012a), and pyridine–N was the major N-containing components in the solid faction of sludge after treatment. Strong basic condition favoured the solubilisation of sludge proteins and their subsequent degradation (Liu *et al.*, 2012b, Liu *et al.*, 2012c). As the protonated amine in protein can be easily converted to volatile ammonia in gas phase at pH 12 (pKa value for NH_3 is 9.25 at 25 °C) (Liu *et al.*, 2015), total N in alkaline treated sludge solid fraction was removed significantly (Table S3). Moreover, the abundant OH^- could drive the reactions in Eq. 3. The cyclization of amine-N intermediates obtained from protein decomposition may generate heterocyclic-N compounds, i.e. pyridine-N (Eqs. 4 & 5) (He *et al.*, 2015). This high depletion of protein-N in solid phase for alkaline treatment at pH 12 might

contribute to its highest protein solubilisation efficiency as mentioned above. For thermal treatment, the pyridine–N content decreased, while the protein – N content increased. Similar results were also reported by Liao *et al.* (2011) applying XPS to characterize the surface composition of the thermophilic and mesophilic sludge. Their results showed as temperature increased, the element component of C=O (chemical composition of peptide bond in protein–N) increased while the element component of N-C (chemical bond in pyridine-N) decreased. However, the detailed formation mechanism remains less clear and will need to be investigated in the future.



3.5. Economic analysis

Table 5 shows the summarized economic assessment for different treatments for protein solubilisation and the subsequent potential protein recovery when compared to the conventional sludge treatment and disposal process. The installation of treatment systems would also increase the overall capital investment, which was not included in this economic assessment. All the treatment methods can significantly increase protein release from sludge matrix, and showed

418 economic feasibility compared to conventional sludge treatment and disposal method, i.e.
419 alkaline treatment at pH 12 showed highest net saving of 25.57 USD per ton wet sludge.

420 The cost for protein recovery and isolation from released protein are still quite high and the
421 market price for crude protein is relatively low. With the shortage of grain proteins continues,
422 various alternative sources of protein need to be considered and the relative market price may
423 also increase in the near future. Moreover, more efforts are required in seeking cost-effective
424 methods that can separate, concentrate and purify proteins continuously, and can be easily
425 scaled-up are of great commercial interest (Melo *et al.*, 2001).

4. Conclusions

This study systemically evaluated different treatment methods for protein solubilisation. The following conclusions can be drawn:

(1) The concentration of soluble protein increased by 11, 23 and 12 times under the optimal treatment conditions (ultrasonic treatment of 1 W mL^{-1}, alkaline treatment of pH 12 and thermal treatment at 80°C);

(2) EPS analysis indicated the increased soluble protein was correlated with the release of TOC, TDN and TON in SB EPS, and the degradation of above parameters in TB EPS, thus suggesting attacking TB EPS layer was the key step for protein solubilisation;

(3) For all the treatment investigated, tyrosine-like protein at molecular weight less than 20 kDa predominated, thus suggesting the feasibility of its application for wood adhesives or feed stuff.

(4) The alkaline treatment at pH 12 gave the highest dominant protein, which might be related with the significant removal of protein-N in solid phase as indicated by X-ray photoelectron spectroscopy;

(5) Alkaline treatment at pH 12 showed highest net saving of 25.57 USD per ton wet sludge.

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449

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627

Table 1. Correlation between solubilized protein and dissolved organic matters in different fractions of EPS.

	PN SB	**PN LB**	**PN TB**	**TOC SB**	**TOC LB**	**TOC TB**	**TDN SB**	**TDN LB**	**TDN TB**	**TON SB**	**TON LB**	**TON TB**
PN SB	1	0.444	-0.588	0.982[a]	0.447	-0.655[b]	0.977[a]	0.220	-0.702[b]	0.938[a]	0.438	-0.693[b]
PN LB	0.444	1	0.403	0.403	0.977[a]	0.341	0.386	0.941[a]	0.268	0.293	0.977[a]	0.272
PN TB	-0.588	0.403	1	-0.625	0.411	0.993[a]	-0.644[b]	0.596	0.983[a]	-0.681[b]	0.417	0.984[a]
TOC SB	0.982[a]	0.403	-0.625	1	0.401	-0.686[b]	0.993[a]	0.145	-0.739[b]	0.984[a]	0.391	-0.732[b]
TOC LB	0.447	0.977[a]	0.411	0.401	1	0.347	0.364	0.955[a]	0.279	0.283	0.998[a]	0.288
TOC TB	-0.655[b]	0.341	0.993[a]	-0.686[b]	0.347	1	-0.705[b]	0.544	0.995[a]	-0.734[b]	0.354	0.995[a]
TDN SB	0.977[a]	0.386	-0.644[b]	0.993[a]	0.364	-0.705[b]	1	0.119	-0.758[b]	0.978[a]	0.355	-0.732[b]
TDN LB	0.220	0.941[a]	0.596	0.145	0.955[a]	0.544	0.119	1	0.491	0.008	0.958[a]	0.497
TDN TB	-0.702[b]	0.268	0.983[a]	-0.739[b]	0.279	0.995[a]	-0.758[b]	0.491	1	-0.786[a]	0.289	0.999[a]
TON SB	0.938[a]	0.293	-0.681[b]	0.984[a]	0.283	-0.734[b]	0.978[a]	0.008	-0.786[a]	1	0.273	-0.780[a]
TON LB	0.438	0.977[a]	0.417	0.391	0.998[a]	0.354	0.355	0.958[a]	0.289	0.273	1	0.297

TON TB	-0.693[b]	0.272	0.984[a]	-0.732[b]	0.288	0.995[a]	-0.732[b]	0.497	0.999[a]	-0.780[a]	0.297	1

[a] Correlation is significant at the 0.01 level (2-tailed). [b] Correlation is significant at the 0.05 level.

Table 2. The concentration of protein characterized with different molecular weight in EPS fractions of WAS by different treatment methods (Unit: ppm-C).

		SB EPS		LB EPS		TB EPS	
		HMW protein MW>20 kDa	LMW protein 0 kDa < MW < 20 kDa	HMW protein MW>20 kDa	LMW protein 0 kDa < MW < 20 kDa	HMW protein MW>20 kDa	LMW protein 0 kDa < MW < 20 kDa
Ultrasonic ($W\ mL^{-1}$)	**0**	5.42	22.77	12.17	71.36	5.20	107.65
	0.25	33.17	82.55	16.05	92.49	4.32	108.34
	0.5	59.57	178.45	19.90	111.20	4.21	106.25
	1	78.25	227.58	8.07	134.89	5.32	106.66
pH	**6.3**	5.42	22.77	12.17	71.36	5.20	107.65
	8	18.57	138.46	10.21	116.77	4.51	98.44
	10	18.54	152.36	8.53	122.33	2.67	99.53
	12	104.40	446.77	34.58	155.03	9.98	54.46
Temperature (°C)	**25**	5.42	22.77	12.17	71.36	5.20	107.65
	40	9.82	51.50	7.98	97.58	5.36	96.27
	60	22.43	270.61	6.25	57.49	5.39	31.15

80	41.72	296.97	6.87	54.78	5.91	14.29

Table 3. EEM FRI of soluble organics in different EPS fractions (unit: 10^6 RU).

		S1	S2	S3	S4	S5
Ultrasonic (W mL^{-1})	0	0.72	1.00	0.76	0.32	1.11
	0.25	1.32	2.33	1.22	0.84	2.19
	0.5	2.31	4.22	1.78	1.51	3.35
	1	3.15	6.63	2.09	2.54	4.85
Alkaline	pH 6.3	0.72	1.00	0.76	0.32	1.11
	pH8	1.52	2.38	1.38	0.87	2.43
	pH10	2.01	3.60	1.52	1.36	3.07
	pH12	6.31	12.37	5.73	5.08	14.93
Temperature(°C)	25	0.72	1.00	0.76	0.32	1.11
	40	1.11	1.90	1.01	0.63	1.82
	60	3.09	5.67	2.13	2.13	5.00
	80	4.33	8.40	3.11	3.10	6.97
		L1	L2	L3	L4	L5
Ultrasonic (W mL^{-1})	0	3.11	3.40	2.96	1.31	4.07
	0.25	2.89	3.14	2.80	1.22	3.22
	0.5	4.01	5.22	3.59	2.06	5.25
	1	3.66	5.50	3.12	2.28	4.58
Alkaline	pH 6.3	3.11	3.40	2.96	1.31	4.07
	pH8	4.23	4.80	3.95	1.88	5.12
	pH10	1.71	3.35	1.16	1.72	2.00

	pH12	2.22	4.53	1.52	2.22	3.22
	25	3.11	3.40	2.96	1.31	4.07
	40	4.44	4.72	4.19	1.77	5.93
	60	1.57	2.89	1.31	1.31	2.25
Temperature(°C)	80	1.15	2.23	0.90	1.11	1.49
		T1	T2	T3	T4	T5
	0	1.46	2.86	1.08	1.02	2.23
	0.25	1.48	2.87	1.09	1.05	2.11
	0.5	1.31	2.61	1.04	0.94	2.21
Ultrasonic (W mL^{-1})	1	1.31	2.53	1.14	0.94	2.71
	pH 6.3	1.46	2.86	1.08	1.02	2.23
	pH8	1.29	2.48	0.99	0.88	2.06
	pH10	1.82	5.15	1.49	1.75	5.08
Alkaline	pH12	1.20	3.18	1.26	0.80	3.82
	25	1.46	2.86	1.08	1.02	2.23
	40	1.89	4.84	1.38	1.83	4.22
	60	0.85	2.62	0.91	0.56	3.33
Temperature(°C)	80	0.77	2.36	0.89	0.44	3.22

Minimum Maximum

S1, S2, S3, S4, and S5 denote Region I, Region II, Region III, Region IV and Region V in SB EPS, respectively; L1, L2, L3, L4, and L5 denote Region I, Region II, Region III, Region IV and Region V in LB EPS, respectively; T1, T2, T3, T4, and T5 denote Region I, Region II, Region III, Region IV and Region V in TB EPS, respectively.

Table 4. Normalized relative intensities (%) of N-containing compounds in sludge solid residues after different pretreatment conditions according to N 1s XPS spectra.

	Binding energy (eV)	Raw	Ultrasonic (1 W mL^{-1})	Thermal (80 °C)	Alkaline (pH 12)
Total N (%)		100	96.4	98.2	86.0
Inorganic-N (%) N1	402.5	-	-	-	-
Protein-N (%) N2	400	38.3	35.8	45.1	0
Pyridine-N (%) N3	398.8	61.7	60.6	53.1	86
Pyrrole-N (%) N4	400.3	-	-	-	-
Quaternary-N (%) N5	401.4	-	-	-	-
Nitrile-N (%) N6	399.7	-	-	-	-

Table 5. Energy balance and cost evaluation.

		Ultrasonic (W mL^{-1})			**Alkaline**			**Thermal (°C)**		
	Raw	0.25	0.5	1	pH 8	pH 10	pH 12	40	60	80
Energy consumption (kWh/ton wet sludge)[a]	0	9.12	17.89	30.93	0	0	0	17.50	40.83	64.17
Chemical dosage (g g^{-1}TS)	0		_		0.022	0.046	0.1		_	
Chemical cost (USD/ ton wet sludge)[b]	0		_		0.093	0.18	0.47		_	
Energy cost (USD/ton wet sludge)[c]	0	2.10	4.11	7.11	-	-	-	4.03	9.39	14.76

Protein concentration (mg L^{1})	56.72	232.84	478.92	615.35	315.96	343.87	1109.00	123.37	589.63	681.47
Credit from protein recovery[c] (USD/ ton wet sludge)[d]	8.10	36.96	76.02	97.68	50.15	54.58	176.04	19.58	93.59	108.17
Cost of sludge transport and disposal (USD/ton wet sludge)[e]	150									
		0	0	0	0	0	0	0	0	0
Net saving compared to	-141.90	-115.14	-78.09	-59.43	-99.94	-95.60	25.57	-134.45	-65.80	-56.59

conventional

treatment

(USD/ton wet

sludge)[f]

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[a] Energy consumption was based on 1 ton wet sludge

[b] Refer to Ruiz-Hernando *et al.* (2014) that the average price of NaOH was 333 USD/ton

[c] Refer to Kavitha *et al.* (2016) that 1 kWh energy can cost 0.23 USD.

[d] Assumed 90% protein can be recovered from the released protein, refer to Chishti *et al.* (1992), and the cost for protein recovery and isolation was about USD 28/lb and USD 40/lb was assumed for an isolated protein price, refer to Cater *et al.* (1974)

Credit from protein recovery = Market credit from isolated protein – cost for protein isolation process.

[e] Refer to Foladori *et al.* (2010)

[f] Net saving cost = increase in protein recovery (USD) – cost of sludge transport and disposal (USD) – energy cost (USD)

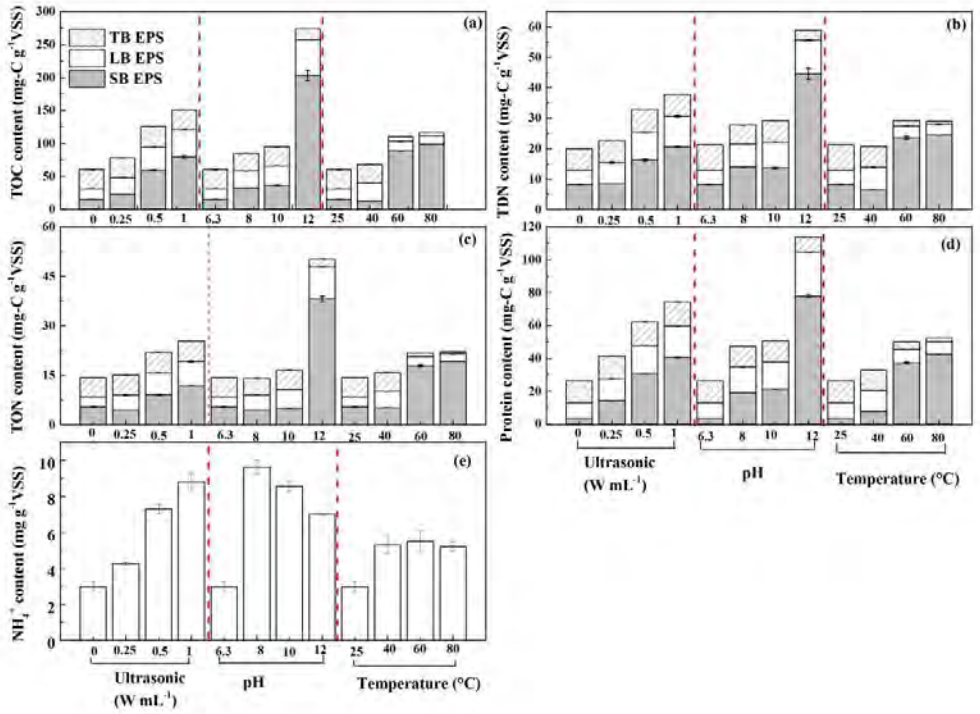

Fig.1. Variances of (a) TOC, (b) TDN, (c) TON, (d) protein and (e) NH_4^+ concentrations by ultrasonic, alkaline and thermal treatments of waste activated sludge.

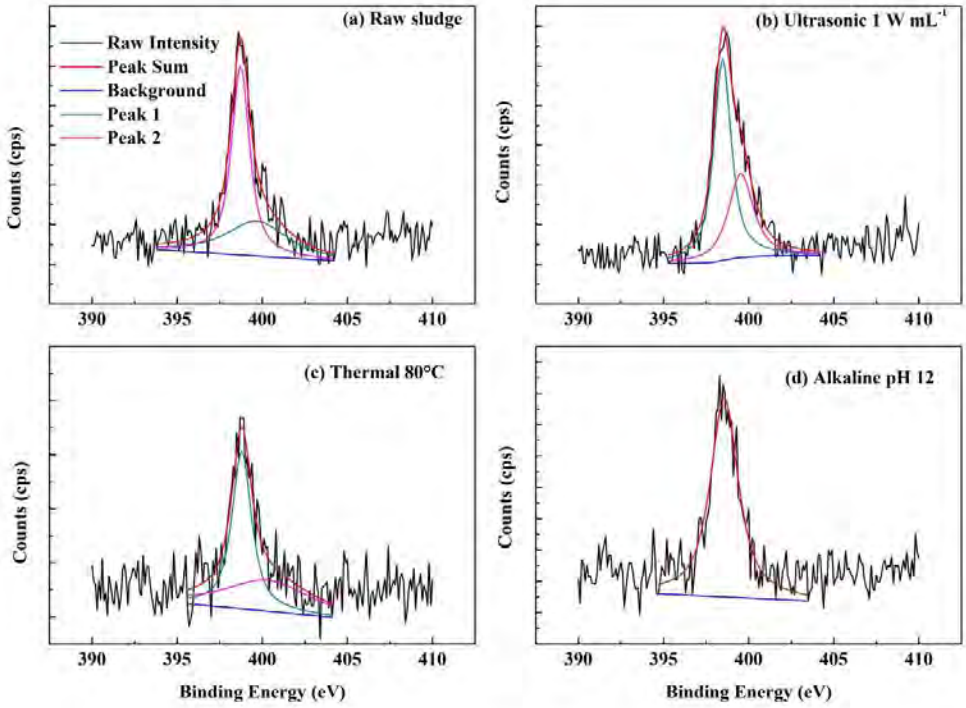

Fig.2. Evolution of N 1s XPS spectra for solid residues derived from various pretreatments: (a) raw sludge; (b) ultrasonic pretreatment at 1 WmL^{-1}; (c) thermal pretreatment at 80 °C; (d) alkaline pretreatment at pH 12. Peak 1 represents integration for pyridine-N; peak 2 represents integration for protein-N.

Highlights

- Protein solubilisation was negatively correlated to TOC, TDN and TON in TB EPS;
- Molecular weight of soluble protein was mainly less than 20 kDa for all samples;
- X-ray photoelectron spectroscopy was used to investigate N transformation in solid;
- The feasibility of method for protein recovery was assessed with economic analysis.