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# Enzyme extraction by ultrasound from sludge flocs

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**Abstract**

Enzymes play essential roles in the biological processes of sludge treatment. In this article, the ultrasound method to extract enzymes from sludge flocs was presented. Results showed that using ultrasound method at 20 kHz could extract more types of enzymes than that at 40 kHz and ethylenediamine tetraacetic acid (EDTA) methods. The optimum parameters of ultrasound extraction at 20 kHz were duration of 10 min and intensity of 552 W/g TSS. Under the optimum condition, ultrasound could break the cells and extract both the extracellular and a small part of intercellular enzymes. Ultrasound intensity was apparently more susceptive to enzyme extraction than duration, suggesting that the control of intensity during ultrasound extraction was more important than that of duration. The Pearson correlation analysis between enzyme activities and cation contents revealed that the different types of enzymes had distinct cation binding characteristics.

**Key words**: enzymes; extracellular polymeric substances; extraction method; sludge flocs; statistical analysis; ultrasound

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## Introduction

Proteins, polysaccharides, and lipids were the major organic matters in sewage sludge, which must be hydrolyzed to smaller units by enzymes before subsequent degradation (Frølund *et al*., 1995; Cadoret *et al*., 2002; Sheng and Yu, 2006). Therefore, enzymes played essential roles in the biological processes of sludge treatment (Tcuber and Brodisch, 1977). The concentration of enzymes, location of the enzymes, and product transport mechanisms all influenced the reaction rate in the biological processes (Morgenroth *et al*., 2002). Thus, measurement of enzyme activities was the most direct way to study mechanisms and biological reaction. Additionally, measurement of enzyme activities was an alternative method to assess microbial biomass and activity of sludge, and acted on indicators of specific processes such as COD and phosphorus removal (Richards *et al*., 1984; Nybroe *et al*., 1992).

In recent years, there had been a growing interest on the study of enzymes from sludge flocs (Teuber, and Brodisch, 1977; Richards *et al*., 1984; Frølund *et al*., 1995; Goel *et al*., 1998; Cadoret *et al*., 2002; Nielsen *et al*., 2002; Whiteley *et al*., 2002). It was found that a large proportion of enzymes were immobilized in sludge flocs by adsorption in the extracellular polymeric substances (EPS) matrix (Frølund *et al*., 1995). EPS and cells formed bioaggregates, such as biofilms and sludge flocs (Nielsen and Jahn, 1999). The researchers (Goel *et al.*, 1998; Confer and Logan, 1998; Whiteley *et al*., 2002) collected sludge samples from one sewage treatment plant, and they reported that most extracellular enzymes were bound with cell, pellet, or the organic particulate matters in sludge flocs. To date, however, no standardized methods for enzymes extraction from sludge flocs exist.

Enzymes were considered to be an integrated part of EPS matrix (Frølund *et al*., 1995). Therefore, enzymes in the sludge flocs could also be extracted by EPS extraction methods. Different methods had been developed for EPS extraction (Frølund *et al*., 1996; Liu and Fang, 2003; Comte *et al*., 2006a, 2006b), but not all of them could be used for the enzymes extraction as some of them could lead to enzymes inactivation or just partial extraction (Gessesse *et al*., 2003). For example, cation exchange resin (CER) was regarded as an efficient standard method to extract EPS (Henze, 2007). However, sole CER could not efficiently extract all the enzymes in the sludge flocs (Frølund *et al*., 1995; Gessesse *et al*., 2003). The reason was that CER was highly selective for $Ca^{2+}$ and $Mg^{2+}$ bound EPS (Park and Novak, 2007). Therefore, CER could only extract the enzymes bound with $Ca^{2+}$ and $Mg^{2+}$.

Ultrasound could effectively disintegrate sludge flocs and released enzymes embedded in the sludge flocs (Tiehm *et al*., 2001; Whiteley *et al*., 2002). On the other hand, ultrasound could enhance the enzyme activities in the sludge flocs (Yu *et al*., 2007, 2008), suggesting that ultrasound is a good method to extract enzymes from sludge flocs. To our knowledge, no previous work has been conducted

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on the enzymes extraction by ultrasound method. The objective of this article was to optimize the process parameters of ultrasonic extraction. A better knowledge on this issue could not only provide a suitable enzyme extraction method but further deepen our understanding on the roles of enzymes in wastewater treatment processes. Protease, α-amylase, and α-glucosidase had been reported to hydrolyze proteins and polysaccharides (Goel *et al*., 1998). Alkaline phosphatase hydrolyzed phosphomonoesters to provide an alternative source of phosphorus for the cells, while acid phosphatase was reported to be involved in internal cell metabolism (Kloeke and Geesey, 1999). Thus, the five enzymes were selected for this study due to their essential roles in the sludge treatment.

## 1 Materials and methods

### 1.1 Sludge samples

Activated sludge samples were collected from the aerated basin and returned pump house of a municipal wastewater treatment plant (WWTP) in Shanghai, China. The plant treated 75000 $m^3$/d of wastewater (93% domestic and 7% industrial sewage) using anaerobic-anoxic-oxic process. The collected samples were transported to laboratory within 30 min after sampling.

### 1.2 Ultrasound equipments

Two types (20 and 40 kHz) of ultrasound equipments were applied to extract enzymes from sludge flocs in this study. Table 1 lists the parameters of the ultrasound equipments.

**Table 1** Parameters of the two ultrasound equipments

| Parameter | Equipment I | Equipment II |
|---|---|---|
| Producer | Shanghai Sonxi Co., Ltd., China | Kunshan Ultrasonic Equipment Co., Ltd., China |
| Model | FS-600 | KQ-300 DE |
| Type | Probe | Water-bath |
| Frequency (kHz) | 20 | 40 |
| Electricity supply (V, kHz) | 220, 50 | 220, 50 |
| Maximum energy output (W) | 600 | 300 |
| Treated volume (mL) | 10000 | 0.5–500 |

### 1.3 Enzymes extraction protocol

Ultrasound protocol was selected to extract enzymes from sludge flocs. Since the ethylenediamine tetraacetic acid (EDTA) method had high EPS extraction efficiency, it was selected as a counterpart (Yu *et al*., 2007). Figure 1 illustrates the extraction protocol applied in this work. In brief, the sludge flocs were settled for 1.5 h at 4°C with supernatant decanted carefully by a siphon. The decanted fraction of sludge flocs was taken as slime that contained few enzymes (Frølund *et al*., 1995; Nielsen and Jahn, 1999). The sludge sediment was collection, whose characteristics are listed in Table 2. The sediment was then centrifuged at 2000 ×*g* for 15 min. The collected bottom sediment was re-suspended to its original volume using a pH 7 buffer solution consisting of $Na_3PO_4$, $NaH_2PO_4$, NaCl, and KCl. The molar ratio of these components was 2:4:9:1. The conductivities of the buffers were adjusted with distilled water to match those of the sludge sediment samples listed in Table 2. The suspension was centrifuged

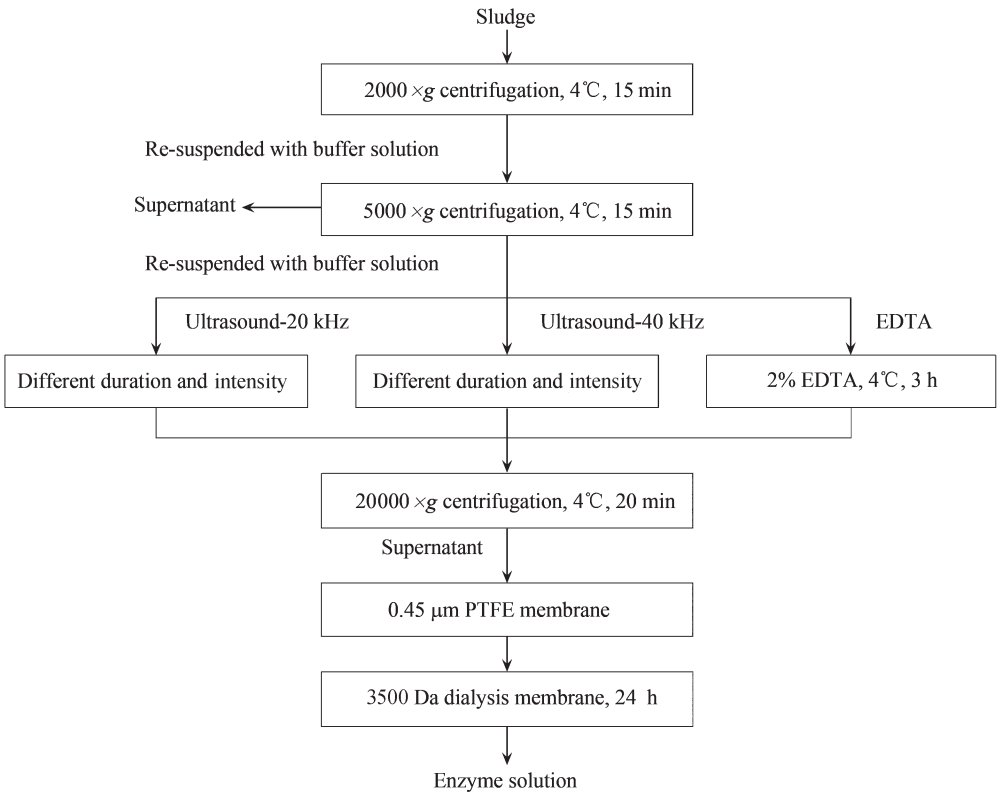


**Fig. 1** Enzymes extraction protocol from sludge flocs.

**Table 2** Characteristics of the sludge sediment samples

| Sludge type | TSS (g/L) | VSS (g/L) | COD (mg/L) | SCOD (mg/L) | Conductivity (μS/cm) |
|---|---|---|---|---|---|
| Aerated tank | 17.4 ± 0.6 | 15.6 ± 0.1 | 19200 ± 100 | 138 ± 3 | 574 ± 5 |
| Returned sludge | 6.30 ± 0.3 | 6.00 ± 0.0 | 11500 ± 100 | 129 ± 2 | 439 ± 7 |

TSS: total suspended solids; VSS: volatile suspended solids; COD: chemical oxygen demand; SCOD: soluble COD.

again at 5000 ×*g* for 15 min with solid phase collection. Collected sediment was re-suspended again with buffer solution to the original volumes for further extraction.

Sludge flocs (50 mL) was put into a polyethylene bottle (5 cm × 5 cm × 4 cm) and treated using the two ultrasound equipments under different conditions to keep the same ultrasonic density, and placed in an ice water bath to prevent possible thermal effects. As for frequency selection of ultrasound, the sludge flocs were treated under the conditions of 138 W/g TSS for 2 min at the frequency of 20 and 40 kHz, respectively. For the duration selection of ultrasound, the sludge flocs were treated under the ultrasound duration range 2–20 min at the intensity of 138 W/g TSS. For the intensity selection of ultrasound, the sludge flocs were treated under the ultrasonic intensity range 138–698 W/g TSS W at the duration of 10 min. For EDTA process, the sludge flocs were extracted using EDTA (2%, *W/V*) at 4°C for 3 h.

The extracted solutions of the three methods were centrifuged at 20000 ×*g* for 20 min. The supernatant was used as the enzyme sources. The particulates and low molecular-weight metabolites in the enzyme solutions were removed with a 0.45-μm polytetrafluoroethylene (PTFE) membrane (Mosu Scientific Equipment Co., Shanghai, China) and an dialysis membrane of MWCO of 3500 Da (Shanghai Sangon Biotechnology Co., Ltd., China), respectively.

### 1.4 Enzyme and sludge characterization assay

All enzymes were measured in duplicates. Protease was analyzed using the Lowry method with casein as the standard (Lowry *et al*., 1951). The α-amylase was determined by the Bernfeld method with glucose as the standard (Bernfeld, 1955). The α-glucosidase was measured according to Goel *et al*. (1998) with *p*-ntrophenyl-α-D glucoyranoside (Sigma N 1377, USA) as the standard. The alkaline phosphatase and acid phosphatase were measured according to Goel *et al*. (1998) with *p*-nitrophenylphosphate disodium salt (Sigma N 3254, USA) as the standard.

All of the chemical analysis was carried out using reagents of analytical grade. The COD of the filtrate was referred to as SCOD. The COD and SCOD were measured using a HACH DR/2000 Spectrometer (Hach Co., USA). Cation contents were measured using ICP-OES (2100 DV, Perkin-Elmer, USA). Conductivity was determined by a conductivity meter (DDSJ-308A, Leici Co., Ltd., Shanghai, China). Other sludge parameters, including total suspended solids (TSS) and volatile suspended solids (VSS) were analyzed according to the standard methods (APHA *et al*., 1998).

### 1.5 Statistical analysis

Statistical analysis was carried out using the software SPSS version 11.0 for Windows (SPSS, Chicago, USA). The Pearson correlation coefficient (*R*) was evaluated in linear correlations between any two parameters. Pearson coefficient equals –1 or +1 denote perfect negative or positive correlation. Correlations were considered statistically significant at a 95% confidence interval ($p < 0.05$).

## 2 Results and discussion

### 2.1 Effect of ultrasonic frequency on enzyme extraction

Figure 2 depicts the enzyme activities extracted by ultrasound and EDTA methods. As for the two sources of sludge flocs, the α-amylase showed the highest activity, followed by alkaline phosphatase, acid phosphatase, protease, and α-glucosidase. All the methods tested could extract high level of α-amylase, where the EDTA method had the highest extraction efficiency. In contrast, the ultrasound method at the frequency of 40 kHz and EDTA method had low extraction efficiency for the other four enzymes. Therefore, the frequency of 20 kHz was selected to further optimize the extraction parameters of enzymes. In addition, it was found that the enzyme activities in the

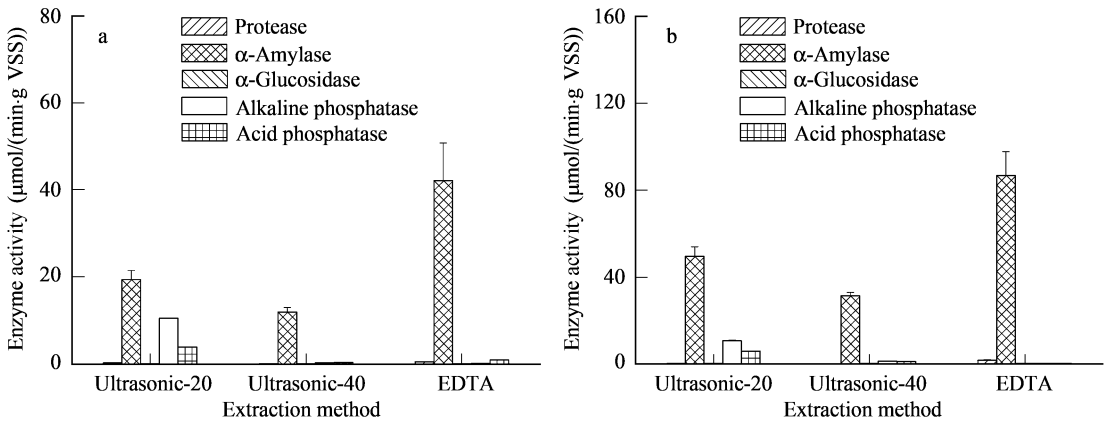


**Fig. 2** Comparisons of extraction methods for sludge flocs from (a) aerated basin and (b) returned sludge (error bars represent standard deviation).

sludge flocs from returned sludge were higher than those from aerated basin, suggesting that the biological activities in returned sludge were higher than those in aerated basin sludge flocs.

Figure 3 presents the DNA contents extracted by different methods in the sludge flocs. The DNA contents extracted using the three methods were all less than 5.2 mg/g VSS. Hence, the three extraction methods did not break the cells embedded in the sludge flocs (Liu and Fang, 2003; Zhang *et al*., 1999), suggesting that the enzymes extracted by the three methods were all extracellular enzymes.

The theoretical approach gave evidence that low ultrasound frequency had better extraction efficiency. The cavitation bubble was produced by ultrasound, and its radius was inversely proportional to the ultrasonic frequency (Tiehm *et al*., 2001). The ultrasonic cavitation bubble radius can be approximated as:

$$R_r \approx 3.28 f_r^{-1} \quad (1)$$

where, $R_r$ (mm) is the resonant bubble radius, $f_r$ (kHz) is the resonance frequency.

Tiehm *et al*. (2001) reported that the degree of sludge disintegration increased proportionally to the logarithm of the bubble radius calculated by the aforementioned equation. Upon bubble collapse, hard mechanical jet streams are produced and the energy released by a jet stream is a function of the bubble size at the moment of collapse. Therefore, the low ultrasound frequency created large cavitation bubbles and strong shear forces. In conclusion, the theoretical and experimental approaches all supported that the disintegration of sludge flocs at 20 kHz was more effective than that at 40 kHz. Since a large proportion of enzymes was immobilized in the sludge flocs by adsorption in EPS matrix (Frølund *et al*., 1995), the enzyme extraction efficiency at 20 kHz was higher than that at 40 kHz.

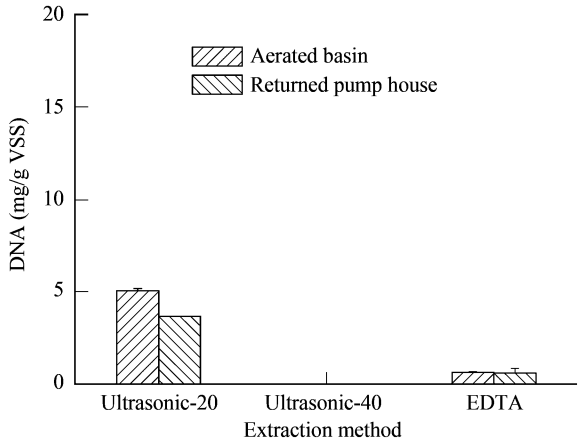


**Fig. 3** Comparisons of extraction method on DNA extraction efficiency (error bars represent standard deviation).

### 2.2 Effect of ultrasound duration and intensity on enzyme extraction

The optimum ultrasound duration and intensity at 20 kHz were determined with aerated basin sludge flocs. Figure 4 displays that under the duration of 10 min and intensity of 552 W/g TSS, the extracted enzyme types were much more and extracted enzyme activities were high, and virtually no improvement was observed at 20 min and 690 W/g TSS.

Figure 5 shows DNA extraction efficiency of the sludge flocs with duration at the level of 10 min and with intensity at the level of 138 W/g TSS, respectively. The DNA contents at the level of 138 W/g TSS were all less than 7 mg/g VSS, while at the level of 10 min they were generally more than 7 mg/g VSS. It should be noted that DNA contents at the level of 414 and 552 W/g TSS were very high (> 10 mg/g VSS). According to the previous results (Zhang *et al*., 1999; Liu and Fang, 2003), both of the two schemes broke the cells and released the extracellular and intercellular enzymes during the extraction. Therefore, ultrasound intensity was more susceptive to enzymes extraction than duration, suggesting that as for efficient enzyme extraction by ultrasound method, the control of intensity was apparently more important than that of duration. This result was consistent with the release of enzymes (Fig. 4).

Considering the higher enzyme extracted types and activities, the optimum parameters of ultrasound extraction were duration of 10 min and intensity of 552 W/g TSS,

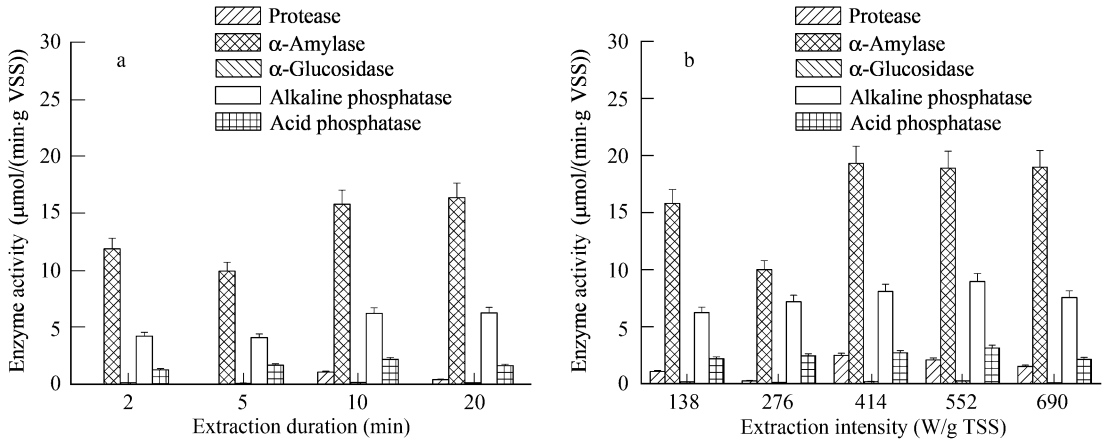


**Fig. 4** Enzyme extraction efficiency of sludge flocs from aerated basin (error bars represent standard deviation). (a) at the level of 138 W/g TSS; (b) at the level of 10 min.

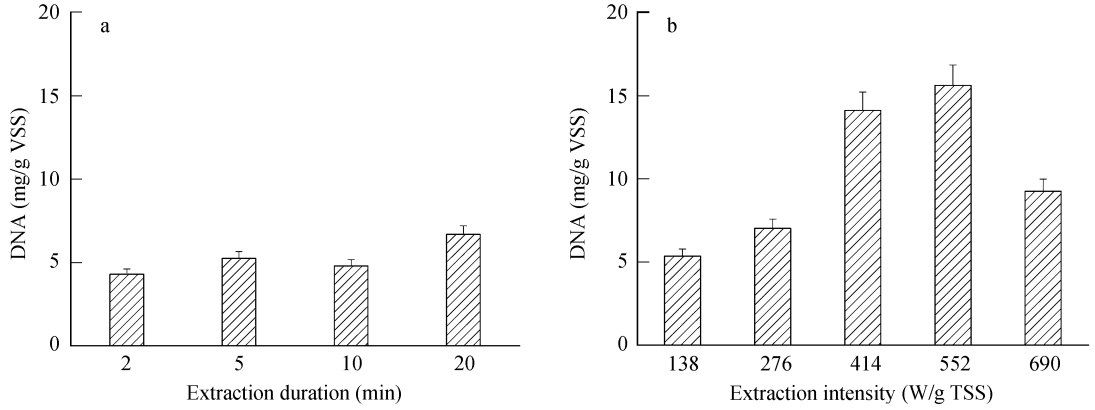


**Fig. 5** DNA extraction efficiency (error bars represent standard deviation). (a) at the level of 138 W/g TSS; (b) at the level of 10 min.

which released both extracellular as well as a small part of intercellular enzymes, and thus had high extraction efficiency. After ultrasound extraction, α-amylase, alkaline phosphatase, acid phosphatase, and protease were all released from the sludge flocs. Strangely, α-glucosidase was not extracted at all by either ultrasound or EDTA methods. This phenomenon may be partially owing to the low activity of α-glucosidase in the sludge flocs. Gessesse *et al*. (2003) investigated the extraction efficiency of protease by CER, Triton X-100, and EDTA from sludge flocs, respectively. They found that the 5% Triton X-100 had the highest extraction efficiency for protease, corresponding to approximate 4000 μmol/(min·g VSS). Since EDTA could break cells and release an amount of intracellular materials (Comte *et al*., 2006a; Yu *et al*., 2007), it was surmised that 5% Triton X-100 might also release an amount of intracellular enzymes. Compared with Triton X-100, the ultrasound method used in this study had lower extraction efficiency. The difference may be due to an amount of release of intracellular enzymes by Triton X-100. The extracellular enzymes played more important roles in wastewater treatment process than intracellular enzymes (Confer and Logan, 1998; Goel *et al*., 1998). Therefore, as a method to extract principally extracellular enzymes, ultrasound has a more important meaning than the extraction methods of intracellular enzymes.

The high enzyme activities of ultrasound extraction from the sludge flocs may be attributed to the fact that ultrasound can significantly break up the sludge matrix and thus release a large amount of enzymes embedded in the sludge flocs matrix (Yu *et al*., 2007, 2008). Some other hypotheses have been proposed to explain how the enzyme activity is enhanced by the ultrasound treatment (Schlafer *et al*., 2000; Liu *et al*., 2003). For example, Liu *et al*. (2003) indicated that the enhancement of enzyme transport and hydrolysis activities seemed to be an ultrasound-induced metabolic response of cells by modifying the cellular metabolisms or facilitating the uptake of nutrient. Gessesse *et al*. (2003) found that negligible enzymes were extracted in the presence of buffer alone, suggesting that the enzymes were not extracted by shear force alone. Therefore, the mechanism deserves more research since it is far from being fully understood.

### 2.3 Pearson correlation between enzymes and cations

The sludge flocs were comprised of different types of EPS with distinct cations binding characteristics (Novak *et al*., 2003; Park and Novak, 2007). Most of enzymes were immobilized in the EPS matrix (Frølund *et al*., 1995). If enzymes were released during the ultrasound extraction, cations binding with corresponding enzymes should be also released. Therefore, it is essential to measure cations in the sludge flocs.

The changes of cation content in the extracted enzyme solution with different ultrasound duration and intensity are showed in Fig. 6. It was noted that $Ca^{2+}$ and $Mg^{2+}$ were

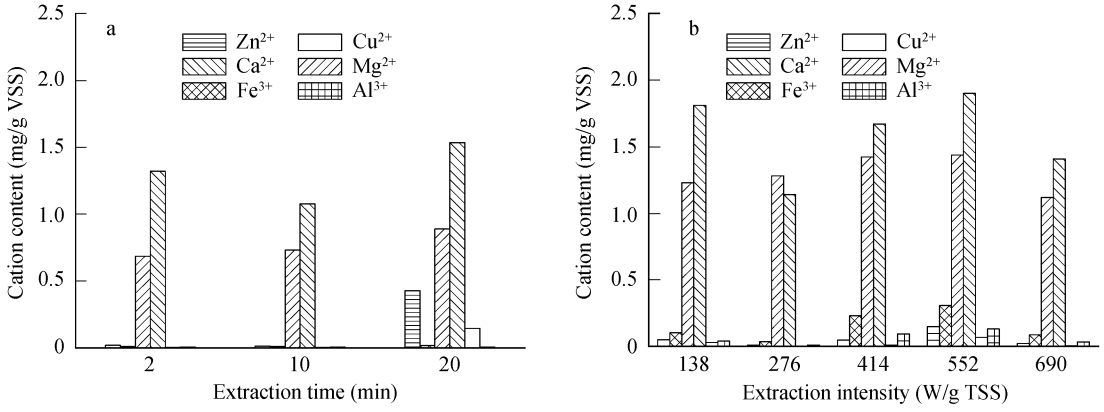


**Fig. 6** Extraction efficiency of cation content with (a) at the level of 138 W/g TSS; (b) at the level of 10 min.

predominant in the extracted enzyme solution compared with the other four cations. Basically, the cation contents in the extracted enzyme solution increased with the ultrasound duration and intensity, and reached the highest at 20 min and 552 W/g TSS. This trend was in agreement with the enzymes extraction, suggesting that the disintegration degree of sludge flocs increased with duration time and power during the extraction and released cations as well as enzymes. Interestingly, when the ultrasound duration was less than 20 min at the level of 138 W/g TSS, only the cation contents increased with duration. However, the contents and types (including $Zn^{2+}$ and $Cu^{2+}$) of cations all increased at the level of 20 min. The contents and types ($Zn^{2+}$, $Cu^{2+}$, $Fe^{3+}$, and $Al^{3+}$) of cations all increased with ultrasound intensity. These results suggested that higher ultrasound power released more types of enzymes while lower ultrasound duration just released higher activities of enzymes. Therefore, it could be inferred that ultrasound power had a more significant effect on the disintegration degree of sludge flocs than ultrasound duration. Gronroos *et al*. (2005) also observed that the energy efficiency with high ultrasound intensity together with short duration was higher than that with low ultrasound intensity and long duration. Our results indicated that the higher ultrasound intensity released more quantities and types of cations than higher ultrasound duration.

Table 3 lists the Pearson correlation between enzyme activities and cation contents in the extracted enzyme solution. Among six types of cations measured in the sludge flocs, $Fe^{3+}$ and $Al^{3+}$ correlated significantly with protease ($R^2 > 0.830$, $p < 0.01$). Additionally, the significant correlations ($R^2 > 0.557$, $p < 0.01$) were found between $Fe^{3+}$, $Ca^{2+}$, $Al^{3+}$ and α-glucosidase, and between $Fe^{3+}$, $Mg^{2+}$, $Ca^{2+}$, $Al^{3+}$ and acid phosphatase. However, the α-amylase and alkaline phosphatase correlated significantly ($R^2 > 0.542$, $p < 0.01$) only with $Ca^{2+}$ and $Mg^{2+}$, respectively. In contrast, no significant correlation could be found between $Zn^{2+}$, $Cu^{2+}$ and the five types of enzymes. Based on Pearson correlation analyses, we presumed that in the sludge flocs, the protease was bound with $Fe^{3+}$ and $Al^{3+}$, the α-glucosidase was bound with $Fe^{3+}$, $Ca^{2+}$ and $Al^{3+}$, the α-amylase and alkaline phosphatase were bound with $Ca^{2+}$ and $Mg^{2+}$ respectively, and the acid phosphatase was bound with $Fe^{3+}$, $Mg^{2+}$, $Ca^{2+}$ and $Al^{3+}$.

Correlation between enzymes and cations might be applied to extrapolate whether EPS extraction methods could extract enzymes effectively. For example, the sulfide method could only extract $Fe^{3+}$-bound EPS (Park and Novak, 2007), thus it should have a good efficiency for protease, α-glucosidase and acid phosphatase. The CER method could extract $Ca^{2+}$- and $Mg^{2+}$-bound EPS (Park and Novak, 2007), and should extract effectively enzymes (except protease) including α-amylase, α-glucosidase, alkaline phosphatase, and acid phosphatase. The results of Gessesse *et al*. (2003) had also verified that CER method could extract a little protease.

**Table 3** Pearson correlation between enzyme activities and cation contents ($n = 10$)

| Cation | Protease | α-Amylase | α-Glucosidase | Alkline phosphatase | Acid phosphatase |
|---|---|---|---|---|---|
| $Zn^{2+}$ | 0.002 | 0.031 | 0.045 | 0.029 | 0.007 |
| $Fe^{3+}$ | 0.859** | 0.410* | 0.691** | 0.464* | 0.593** |
| $Mg^{2+}$ | 0.482* | 0.518* | 0.482* | 0.806** | 0.885** |
| $Ca^{2+}$ | 0.408* | 0.542** | 0.640** | 0.518* | 0.557** |
| $Cu^{2+}$ | 0.000 | 0.037 | 0.076 | 0.037 | 0.015 |
| $Al^{3+}$ | 0.830** | 0.399* | 0.711** | 0.437* | 0.564** |

* Correlation is significant at the 0.05 level (2-tailed); ** correlation is significant at the 0.01 level (2-tailed).

## 3 Conclusions

This study demonstrated that ultrasound method could effectively extract enzymes from sludge flocs. It was found that ultrasound at 20 kHz could extract more types of enzymes than at 40 kHz and with EDTA method. Meanwhile, ultrasound intensity was apparently more susceptive than ultrasound duration for enzymes extraction from sludge flocs. The optimum parameters of ultrasound extraction at 20 kHz were duration of 10 min and intensity of 552 W/g TSS. Under the optimum extraction conditions, ultrasound could break the cells and extract both the extracellular and a small part of intercellular enzymes from sludge flocs. In addition, the Pearson correlation analysis between enzyme activities and cation contents revealed that the different types of enzymes had distinct cations binding characteristics. Therefore, the correlation between enzymes and cations might be applied to extrapolate whether EPS extraction method could also extract enzymes effectively.

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