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Extraction method and structural and composition characteristics of extracellular polymeric substances in granular sludge from an enhanced biological phosphorus removal system

Yajing Li[a,b,*], Shaopo Wang[a,b], Zhu Li[a], Liping Sun[a,b]

[a]*School of Environmental and Municipal Engineering, TCU, Tianjin 300384, China, Tel. +86 022 23085306; emails: yajingli79@163.com (Y. Li), wspfr@sina.com (S. Wang), Tel. +86 022 23085117; emails: Sitaralizhu@163.com (Z. Li), slpyqs@vip.sina.com (L. Sun)*
[b]*Tianjin Key Laboratory of Aquatic Science and Technology, Tianjin 300384, China*

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ABSTRACT

In this study, based on the granular sludge formation mechanism of "microsphere-collision-bonding," biological phosphorus removal granules were divided into the following three structural levels: submicron particles, microsphere particles, and mature granules. Therefore, extracellular polymeric substances (EPS) were classified into microsphere level extracellular polymers (MEPS) and granular level extracellular polymers (GEPS), which provided a novel approach to identifying the role of EPS in microbial aggregations of granular sludge. With regard to this sludge, compared with another three common and efficient methods for EPS extraction, the ultrasonic-cation exchange resin method effectively separated these two extracellular polymers based on the new classification paradigm of EPS. The results also showed that the contents of protein, polysaccharide and the total polysaccharides/total proteins (PN/PS) ratios in the MEPS were much higher than those in the GEPS. In addition, the MEPS contained higher molecular weight proteins than the GEPS. The ultrasonic-cation exchange resin method was used to extract EPS from granular sludge with different carbon sources to evaluate its versatility. The results showed that the variety and size of proteins were clearly different in the different carbon source feeds and this was demonstrated to be an efficient and reliable extraction method.

Keywords: Biological phosphorus removal granules; Extracellular polymeric substances (EPS); Classification; Extraction strategy; Characterization

1. Introduction

Extracellular polymeric substances (EPS) are important in biological wastewater treatment systems as they are ubiquitously present and usually influence the treatment performance of systems [1–3]. To date, it is arguably which component of EPS has a major role in functional studies of microbial aggregates [4–6]. The composition and content of EPS in microbial aggregates are highly variable, due to factors such as substrate type, growth phase, sludge type, operating conditions, extraction technique, and analytical method [7–10]. Hence, in-depth research of these extracellular polymers in microbial aggregates is of high importance to understand and control microbial aggregations, effluent qualities and process dynamics of biological wastewater treatment systems.

EPS are usually classified by the method used for extraction, which is a crude method of classification. EPS in biological phosphorus removal granules should be classified

* Corresponding author.

according to the formation mechanism of granular sludge, and then appropriate extraction methods should be selected to extract EPS. To date, EPS in biological phosphorus removal granules are rarely classified by theoretical consideration of their function and composition. Wu et al. [11,12] proposed the theory of "microsphere-collision-bonding" for the formation of granular sludge in the enhanced biological phosphorus removal (EBPR) system. The formation mechanism and possible steps of granular sludge are as follows: (1) With the release of phosphorus in the EBPR anaerobic phase, a series of precipitation reactions caused by the increase in phosphate concentration promoted the formation of a large number of positively charged particles in the system. The negatively charged microbial cells in the anaerobic segment are adsorbed on the surface of the positively charged particles, or some of these positively charged particles have an uneven surface structure, which will have an electron-hole attraction, making the microbial cells adsorb on its surface. With these two steps, multilayer wrapped microspheres are formed in the system. (2) The denser microspheres move under the action of flow shear force and collide with each other. As the surface of the microspheres is covered with viscous EPS, they easily adhere to each other after a collision, making the spheres large enough to form granular sludge. (3) Due to the shear force of water flow, the granular sludge further enhances the stability of the structure wrapped by the EPS. The positively charged particles in the microspheres are gradually exhausted due to biochemical reactions during granulation. Microorganisms located inside the granules gradually die due to the lack of substrate or oxygen, while microorganisms located on the surface or superficial layer of the granules proliferate. Mature granular sludge with a complex community and functional structure is formed. Based on this, biological phosphorus removal granules were divided into the submicron level (0.5–5 μm), microsphere level (10–100 μm), and granule level (>200 μm) in this study. The EPS were then classified into microsphere level extracellular polymers (MEPS) and granular level extracellular polymers (GEPS).

Due to the complexity of this system, it is almost impossible to extract all the EPS components using one method. To date, there is no "one size fits all" method for EPS extraction. In our study, two types of EPS were extracted with sequential extraction methods based on the evolution of particle size distribution. Four combination methods were employed and compared to identify the most efficient method with less chemical pollution, bacterial lysis and easier to process. Evidence suggests that different substrates in wastewater have significant effects on microbial communities and their metabolism, and thereby influence the production of EPS. In order to investigate whether the same extraction method for sludge with different carbon sources could achieve the same extraction efficiency, EBPR granular sludge systems were operated in parallel with two different carbon sources in this study. This was performed in the hope of determining a new extraction methodology for separating two types of EPS.

After the selection of the EPS extraction method in the EBPR system, the differences in these two types of EPS were thoroughly investigated. The exact composition of the EPS and how the composition of EPS is modified depending on environmental changes were investigated. The detection and analysis of all involved compounds and their interactions can provide a reference for establishing the response relationship between EPS components and sludge properties, and further provide theoretical support for a selection of the EPS extraction method in other biological wastewater systems.

2. Materials and methods

2.1. Origin of biomass

The biological phosphorus removal granules used were obtained from two laboratory-scale sequencing batch reactors (SBRs) with a working volume of 12 L operated under anaerobic/aerobic conditions, with four 6 h cycles per day. Each cycle consisted of an anaerobic period (2 h) and aerobic period (3 h), with the remainder of the cycle time for settling, decanting, and idle periods. The sludge retention time was controlled at approximately 15 d by drawing the sludge from the reactor at the end of the aerobic period. The mixed liquor suspended solids (MLSS) was kept at 3,000 mg L^{-1}. During the aerobic stage, the air was sparged to maintain the dissolved oxygen (DO) level around 2.0 ± 0.2 mg L^{-1}. The SBRs were supplied with synthetic wastewater containing volatile fatty acids (400 mg L^{-1}) as the chemical oxygen demand basis; one used acetic acid as the carbon source (R1) and the other used propionic acid as the carbon source (R2), NH_4Cl (20 mg L^{-1} as NH_4^+–N basis), KH_2PO_4 (25 mg L^{-1} as PO_4^{3-}–P basis), and a trace element solution. The trace element solution consisted of the following compounds per liter: 0.1 mg $ZnCl_2$, 0.5 mg $MgSO_4$, 0.5 mg $FeCl_3{\cdot}6H_2O$, 0.1 mg $MnSO_4{\cdot}H_2O$, 0.1 mg KI, 0.5 mg $CaCl_2$, 0.1 mg H_2BO_4, and 0.1 mg $NiCl_2$. It took about 6 months before stable phosphorus removal was achieved in the two SBRs. The average PO_4^{3-}–P concentration in the effluent of R1 and R2 in the stable period was 0.62 and 0.54 mg L^{-1}, and the average PO_4^{3-}–P removal rate was 97.52% and 97.84%, respectively. Thereafter, the experiments reported below were conducted.

2.2. Chemical analysis

Proteins were quantitated by the modified Lowry method [13]. Carbohydrates were measured using the anthrone method [14]. DNA was measured by the diphenylamine colorimetric method [1]. The yield of EPS was measured as total organic carbon (TOC) using a TOC analyzer (Shimadzu, Japan). The MLSS and volatile suspended solid (VSS) contents in sludge were analyzed according to standard methods [15].

2.3. Microscopy

The morphology of sludge before and after EPS extraction was examined with a high-resolution scanning electron microscopy (SEM) (model JEM 7800F, Japan). Sludge samples were fixed with 2.5% glutaraldehyde in 0.1 M PBS. The samples were subsequently washed and dehydrated in a series of ethanol solutions (50%, 70%, 80%, 90%, and 100%). Dewatered samples were dried by the critical point method.

The dried sludge samples were further sputter-coated with gold for SEM observation.

2.4. Particle size and molecular weight (MW) distribution

The particle size distribution of activated sludge was determined with a laser particle size meter (MS2000, Malvern, UK) over a 0.01–2,000 μm size range. The measurement mode was the volume percentage mode. The particle size distribution curve used logarithmic coordinates, and the area corresponding to the curve was the particle volume in the size range of the total particle volume percentage. The data were obtained based on the average of three replicates.

The MW distribution of extracellular polymers was determined by Shimadzu high-performance liquid chromatography and gel permeation chromatography. The liquid chromatograph consisted of a high-pressure pump (LC-20AT Shimadzu, Japan), column oven (CTO-20A Shimadzu, Japan), differential refraction detector (RID-20A Shimadzu, Japan) and a gel chromatographic column (OH pak GF-510 HQ Shodex Asahipak, Japan). The SEC column was calibrated using standard proteins (Protein Standard Mix 15-600 kDa, 69385, Sigma-Aldrich, USA). The column oven was set to a constant temperature of 40°C with ultrapure water at a flow rate of 1 mL min^{-1} as the mobile phase.

2.5. Infrared spectrometry

The Fourier-transform infrared spectroscopy (FTIR) spectrum was obtained using an FTIR spectrometer (Nicolet iS10, Thermo Scientific Co., USA) with a resolution of 4 cm^{-1} and a scan range of 400–4,000 cm^{-1}. Freeze-drying of sludge samples was conducted using a lyophilizer (FD-1A-50, Beijing Boyikang Laboratory Instruments Co., Ltd., China).

2.6. EPS extraction protocol

Ultrasound is a form of physical energy and uses shear force and cavitation pressure to peel off the EPS from particle surfaces [16]. It can be selected as the first step to disperse the granular sludge from the granular level to the microsphere level for one type of EPS harvesting. The NaOH method promotes the dissolution of EPS from granular sludge by strengthening the separation of EPS acid groups and the repulsion between negatively charged EPS molecules [17]. With regard to the cation exchange resin (CER) method, the cations attached to the cell surface in EPS can be exchanged to depolymerize the microspheres and EPS are separated from the cell surface [18]. The heating method can loosen the sludge structure by heating, which is conducive to extraction [19]. The NaOH and CER heating methods are used as secondary extraction steps, respectively, to disperse the granular sludge from the microsphere level to the submicron level for another type of EPS separation.

According to the above-mentioned procedures, the four combination extraction procedures of NaOH-heating, ultrasonic-NaOH, ultrasonic-NaOH-heating, and ultrasonic-CER were adopted to achieve hierarchical extraction (Fig. 1).

3. Results and discussion

3.1. Effect of different extraction methods on particle size distribution

As shown in Figs. 2a and b, granules were formed in the SBR when acetic acid and propionic acid were respectively used as the carbon source. The organizational structure of the sludge exhibited a spherical or spheroid shape at the microscopic level. The average particle size of R1 and R2 was 585 and 768 μm, respectively. The particle growth rate

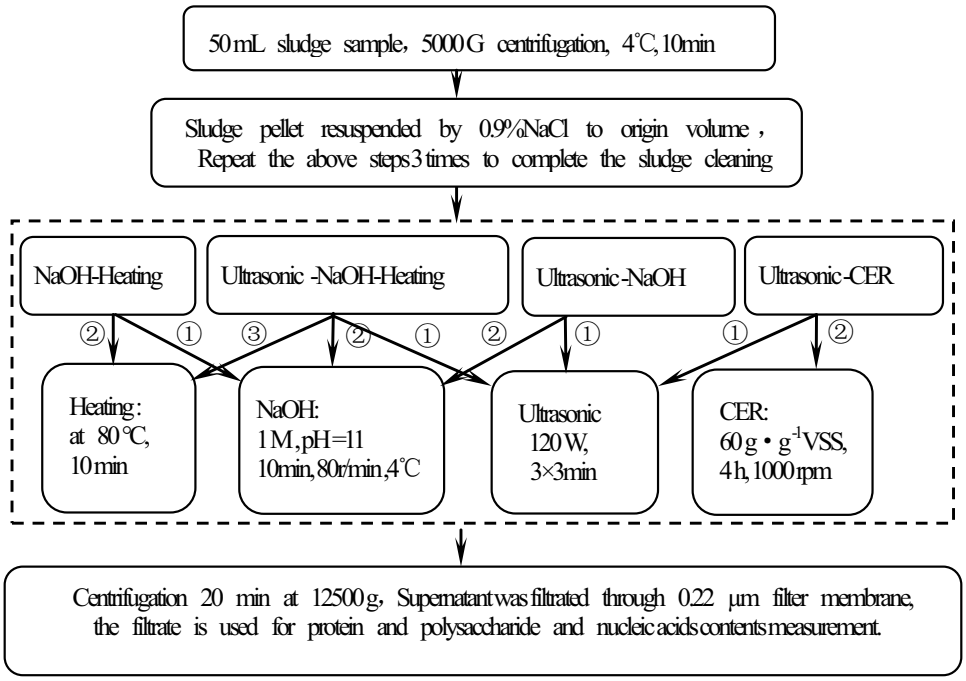

Fig. 1. Procedures for the EPS extraction protocols.

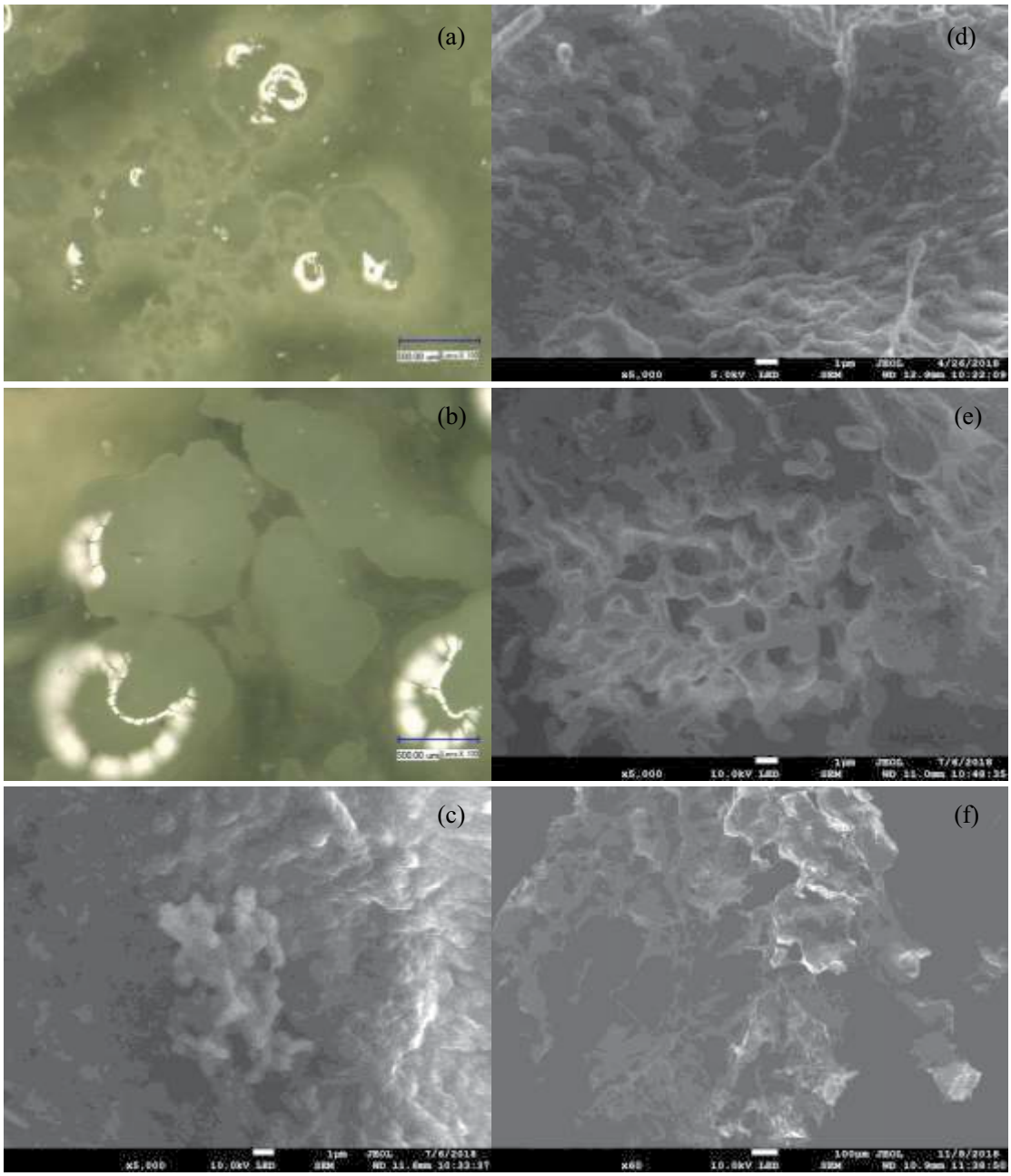

Fig. 2. Mature granular sludge morphologies in R1 and R2.

in the propionic acid system was faster, relevant to the relative ratio of phosphorus accumulating organisms (PAOs) in the granular sludge found in our earlier studies [20]. Fig. 2c shows SEM images of particle morphology, where white particles with a loose structure were generated in the anaerobic phase, the presence of which promoted the formation of granular sludge based on the "microsphere-collision-bonding" theory. SEM micrographs of EBPR granular sludge before and after EPS extraction and extracted EPS are shown in Figs. 2d–f. From Fig. 2d it can be seen that a large number of pellets were present on the surface of the formed granular sludge and these pellets are the microspheres mentioned above. The structural integrity was significantly disrupted into smaller pieces without appreciable destruction of bacterial cells after EPS extraction (Fig. 2e). In addition, the surface characterization of the lyophilized EPS had a gel-type cross-linked structure (Fig. 2f), which formed a matrix to capture and bind the cells together.

A granulometric distribution curve with a mean representative diameter (d_{50}) was obtained from the granulometric analysis, as shown in Fig. 3. The d_{50} of the original granular sludge in R1 was 585 μm (as seen in Fig. 3a), of which more than 42% was over 563 μm. The d_{50} of the original granular sludge in R2 was 768 μm (as seen in Fig. 3b), of which more than 48% was over 800 μm. When the NaOH-heating treatment was used, the granulometric distribution of treated

sludge was not significantly changed. Treatment disintegrated the 585 and 768 μm granules into 365 and 481 μm particles, respectively, in R1 and R2, which were still at the granular level. Mc Swain et al. [21] noted that due to the large size of the granules, EPS extraction required a mechanical breakup of aggregates before chemical extraction. Ultrasound is a popular pre-treatment method to disperse the granular sludge, which can play an important role in enhancing the extraction effect. Therefore, ultrasound was used as a pre-treatment method in the following three extraction methods.

The results showed that ultrasound results in extensive disintegration of granular sludge. In the R1 system, the granular sludge showed further disintegration after CER treatment. The d_{50} of treated sludge was approximately 33 μm (as shown in Fig. 3a), of which up to 68%–70% of the sludge particles were within the range of 10–100 μm (microsphere level particles) and 21%–26% were within the range of 0.5–5 μm (submicron level particles). These results showed that ultrasonic-CER treatment can effectively disperse the granular structure and microsphere structure, and partly disperse them to submicron particles. However, the d_{50} of sludge subsequently treated by the NaOH and NaOH-heating methods was approximately 88 and 63 μm, respectively, which only disintegrated the granules into microsphere level particles. Based on the granulometric distribution results of treated sludge, ultrasonic-CER was selected as the extraction method.

In the R2 system, three extraction methods were used and ultrasound was used as pre-treatment only to disintegrate the granules into microsphere level particles. The d_{50} of treated sludge using ultrasonic-CER was 79 μm (as seen in Fig. 3b), and 50%–56% of the sludge particles were in the range of 10–100 μm (microsphere level), and 30%–32% of the sludge particles were larger than 200 μm (granular level). Considering that the size of granular sludge in the R2 system was larger than that in the R1 system, in addition to the extraction mechanism, the extraction intensity was also important for the separation of EPS. Thus, the extraction intensity of the propionic acid system needs to be enhanced to achieve higher extraction.

3.2. Effects of different extraction methods on EPS production and composition

At present, there is no standard method for the extraction of EPS, which has led to differences in the results obtained in various studies on EPS to some extent [17,18,22–25], as shown in Table 1. The extraction efficiencies, based on TOC, of the four mentioned processes in this study are shown in Table 2. The efficiency of EPS extraction was as follows: ultrasonic-CER > ultrasonic-NaOH-heating > ultrasonic-NaOH > NaOH-heating, and showed the same trend for both types of granular sludge. As important structural components of granular sludge, proteins and polysaccharides can account for more than 70% of the total EPS in sludge. This is inconsistent with previous research which reported that the CER method had lower extraction efficiency than alkaline extraction in terms of organics such as TOC and VSS [22,23]. Following a comparative analysis, it was found that the extraction time for the alkaline extraction method in this study was 10 min, while in other studies, it was approximately 120 min when other parameters were unchanged, resulting in a low level of alkaline extraction in this study. In consideration of the pollution caused by the extraction reagent and its influence on the structure of EPS, the extraction time in the alkaline extraction method in this study was reduced to 10 min.

It was noted that the number of EPS extracted in R1 was more than that in R2, which is consistent with the particle size distribution before and after extraction of EPS in R2. Due to the larger particle size, the same extraction intensity only disintegrated the granules into microsphere level particles. Therefore, the number of EPS extracted was limited. Thus, the ultrasonic-CER method can achieve higher extraction of proteins and polysaccharides and a lower proportion of nucleic acids compared with the other three methods.

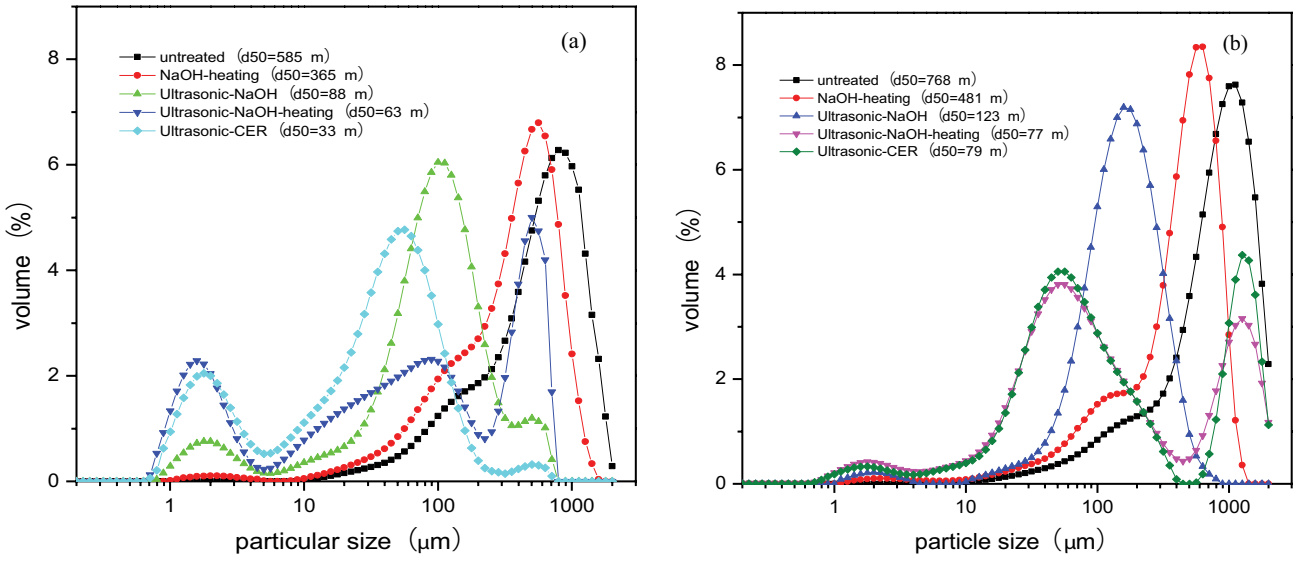

Fig. 3. Granulometric distribution by volume of untreated and four extracted sludge in R1 and R2.

Therefore, the ultrasonic-CER method was selected as the ideal extraction method for subsequent investigations. In the first extraction step, ultrasound mainly extracted GEPS, and CER mainly extracted MEPS during the secondary extraction step.

In this study, the SBR system was operated in an alternate anaerobic/aerobic mode. In order to analyze the periodic variation of proteins and polysaccharides in EPS, the ultrasonic-CER method was used to extract the EPS in the anaerobic initial-stage, anaerobic and aerobic end-stage of the SBR systems in a step-wise manner. As shown in Fig. 4, the content of PN in EPS at the end of the aerobic period was higher than that of PN at the end of the anaerobic period; thus, it can be seen that when microorganisms are starved, they mainly secrete proteins to change their cell surface properties to make them stick together. Polysaccharides at the beginning of the anaerobic period were higher than polysaccharides at the end of the aerobic period, probably because the anaerobic period is rich in carbon sources. When microorganisms use these carbon sources, they secrete extracellular polysaccharides. In the aerobic period, microorganisms will use these extracellular polysaccharides to maintain their energy demands.

The results also showed that the contents of protein and polysaccharide in the secondary extraction were much higher than those in the first extraction in R1 and accounted for approximately 33% and 67% of the total EPS in granular sludge, respectively. Therefore, these findings showed that more exopolymers were produced within the microspheres. The value of the PN/PS ratio was 1.51 in the granular level and 2.88 in the microsphere level, respectively. This indicated that more proteins were produced inside the microspheres. Similarly, the contents of protein and polysaccharide in the secondary extraction were higher than those in the first extraction in R2 and accounted for approximately 40% and 60% of the total EPS, respectively. The value of the PN/PS ratio was 1.27 in the granular level and 3.28 in the microsphere level, respectively. The increased PN/PS ratio in EPS contributes to the formation of sludge particles [26,27]. The positive charge carried by amino groups in proteins can neutralize the negative charge generated by carboxylic acid and other groups in polysaccharides, which can reduce the negative charge on the surface of sludge. In this way, the electrostatic repulsion between cells can be reduced, which is advantageous for the aggregation of granular sludge. The ratio of protein to polysaccharide represents the result of the neutralization of positive and negative charges on the sludge surface.

3.3. MW distribution of protein EPS

In order to determine the average MW of EPS as well as the percentage of each MW fraction with the ultrasonic-CER extraction method, HP-SEC measurements were performed. Table 3 shows the MW distribution in each extraction step using the ultrasonic-CER method. As shown in Table 3, only a small fraction consisting of high MW compounds with 1,312 ≤ MW ≤ 3,650 kDa was seen in R1 and R2. The extracellular proteins at the granular level were extracted in the first extraction step by ultrasound, which was in the low MW range (1.3 ≤ MW ≤ 10 kDa). After the secondary extraction using cation-exchange resin, the extracellular proteins at the microsphere level were extracted and low MW (10 ≤ MW ≤ 21.7 kDa) EPS were dominant in R1. These results indicated that the proteins in the two types of EPS were different and the proteins at the microsphere level contained a higher MW distribution. The proteins with a larger MW may easily form a gel network to act as structural proteins.

The type of carbon source seemed to influence the extracellular protein sizes in the first and secondary extraction. EPS were composed of almost the same amount of low MW (1.3 ≤ MW ≤ 10 kDa) and (10 ≤ MW ≤ 21.7 kDa) compounds in the first extraction; however, moderate MW compounds (21.7 ≤ MW ≤ 48.8 kDa) in the secondary extraction were dominant in R2. These macromolecules increased the whole protein MW distribution. It is speculated that macromolecular proteins can provide more ionic binding sites or

Table 1
Comparison of EPS extracted from different sludge samples by various extraction methods in the past studies

Extraction method	Sample	EPS	Polysaccharides	Proteins	Nucleic acids	PN/PS	Study
NaOH-heating	AS	–	32.94	293.47	–	8.91	Wu et al. [22]
	AS	–	46.00	186.00	4.80	4.04	Hong et al. [23]
	AS	195.83	100.63	78.89	16.31	0.78	Yang et al. [25]
	GS	–	25.0	190.0	–	7.60	Mc Swain et al. [21]
NaOH	AS	152.31	61.10	77.00	14.21	1.26	Yang et al. [25]
Ultrasonic-NaOH	AS	175.50	78.83	81.98	14.69	1.04	Yang et al. [25]
Ultrasonic	GS	–	104.00	126.00	–	1.21	D'Abzac et al. [17]
Ultrasonic-NaOH-heating	AS	227.33	121.03	86.67	19.64	0.72	Yang et al. [25]
CER	GS	–	108.00	140.00	–	1.30	D'Abzac et al. [17]
CER	AS	–	48.00	243.00	–	5.06	Bo et al. [18]
Ultrasonic-CER	AS	100.47	57.03	30.40	13.05	0.53	Yang et al. [25]
	GS	398.50	168.20	230.30	–	1.40	Mahendran et al. [24]
	GS	325.40	120.00	200.50	–	1.67	D'Abzac et al. [17]

Note: AS: Activated sludge; GS: Granular sludge; Data inside brackets are in mg g^{-1} VSS-sludge

Table 2
EPS components of biological phosphorus removal granules by various extraction methods in R1 and R2

Extraction method	R1 system(expressed in mg g^{-1} VSS)					R2 system(expressed in mg g^{-1} VSS)				
	TOC	Polysaccharides	Proteins	Nucleic acids	PN/PS	TOC	Polysaccharides	Proteins	Nucleic acids	PN/PS
NaOH-heating	273.40 ± 14.65	95.27 ± 5.81	172.12 ± 8.20	0.087 ± 0.033	1.81	160.00 ± 11.05	57.54 ± 6.90	70.69 ± 4.42	0.056 ± 0.020	1.23
Ultrasonic-NaOH	298.40 ± 6.90	126.98 ± 6.64	137.85 ± 4.72	0.047 ± 0.023	1.09	155.10 ± 9.60	50.05 ± 5.41	63.69 ± 6.94	0.056 ± 0.032	1.27
Ultrasonic-NaOH-heating	334.00 ± 6.90	122.37 ± 8.23	177.58 ± 5.24	0.082 ± 0.030	2.27	199.10 ± 10.55	58.38 ± 4.34	77.28 ± 10.21	0.113 ± 0.080	1.32
Ultrasonic-CER	340.90 ± 10.90	96.18 ± 5.92	277.41 ± 9.80	0.056 ± 0.021	2.88	283.10 ± 7.35	56.88 ± 2.95	186.57 ± 7.10	0.062 ± 0.020	3.28

polymer interaction points, thus promoting microbial flocculation [27,28].

3.4. FTIR analysis

As shown in Fig. 5, the positions and number of FTIR peaks in the sludge samples initially appeared to be quite close, suggesting that the types of chemical groups in these fractions were similar. Several strong frequency bands associated with proteins and polysaccharides were observed in the sludge components, including the stretching vibration of N–H and O–H (hydrogen-bonded), the stretching vibration of C–H (–CH_2 and –CH_3 groups), the COO– stretches possibly associated with uronic acid, the C=O stretch (amide I) associated with proteins, the bending vibration of N–H (amide II) associated with proteins, the C–O possibly associated with polysaccharides, and the C–O–C stretching vibration of polysaccharides. These findings were consistent with previous reports [29,30].

However, a much closer inspection of the intensity of the respective peaks illustrated that the relative contents of chemical groups were distinctly different. First, the bands at 3,300–3,200 cm^{-1} attributed to the N–H and O–H stretching vibrations and the peak at 2,930 cm^{-1} corresponded to C–H, where a relatively higher intensity pattern was clearly observed for MEPS than for GEPS in R1 and R2, as shown in Fig. 5. Secondly, strong bands at 1,650 and 1,533 cm^{-1} were detected in the FTIR analysis of MEPS in R1, similarly strong bands at 1,641 and 1,529 cm^{-1} were detected in the FTIR analysis of MEPS in R2, indicating the relatively higher number of C=O and N–H in MEPS than in GEPS in the two different sludge samples. Third, the corresponding position in the FTIR analysis of MEPS was covered by wide bands from 1,451 to 1,383 cm^{-1} in R1 and R2, indicating that the COO– containing compounds in MEPS of the two different sludge samples were both more associated with amino acids. The distinguishable FTIR spectral features of MEPS compared with GEPS revealed that hydrophobic groups, especially protein-related –CO–NH– and amino acids associated COO– were present in a greater proportion in MEPS. These findings, which explained the specificity of MEPS in the chemical structure, were consistent with the aforementioned data of the higher content of proteins in MEPS.

4. Conclusion

The current study determined the impact of the substrate type and extraction technique on chemical compositions and functional groups of EPS in EBPR systems. The main findings of this study are summarized as follows:

- EPS in EBPR granular sludge were divided into the microsphere level and granule level based on the formation mechanism of granular sludge, which provided theoretical support for a selection of the EPS extraction method in granular sludge.
- In the granular sludge, the protein and polysaccharide content and the PN/PS ratio at the microsphere level were much higher than those at the granule level. Also, the EPS at the microsphere level contained higher MW proteins than the granule level. The variety and size of

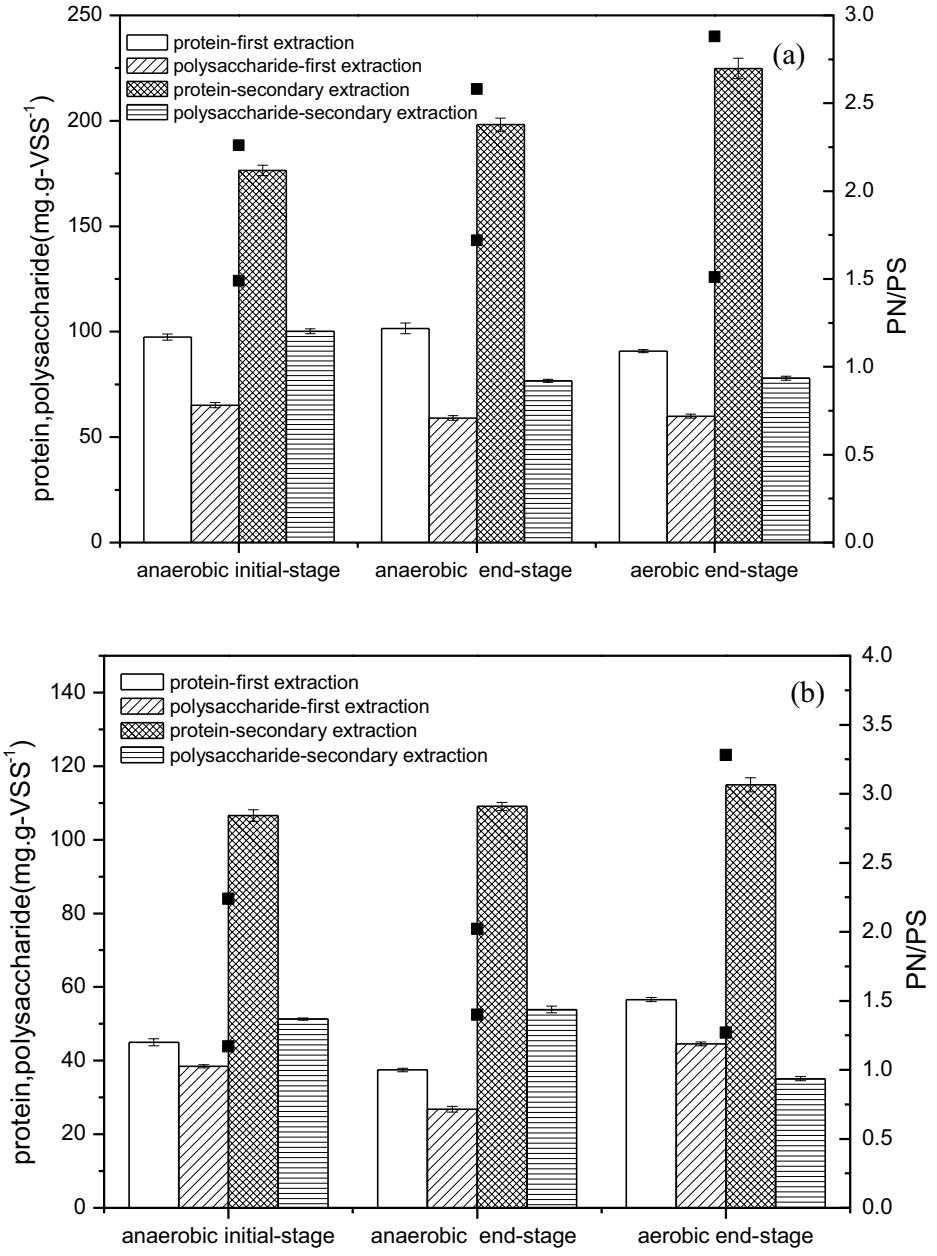

Fig. 4. Comparison of extraction quantity of EPS components by different steps in R1 and R2.

Table 3
Characterization of protein EPS in terms of MWs in R1 and R2 sludge (average values)

MW range of proteins (kDa)	% Distribution of proteins in R1 sludge						% Distribution of proteins in R2 sludge					
	GEPS			MEPS			GEPS			MEPS		
	A1	A2	O2	A1	A2	O2	A1	A2	O2	A1	A2	O2
1,312 ≤ EPS ≤ 3650	2	3	1	4	11	1	4	4	5		2	2
21.7 ≤ EPS ≤ 48.8										77	72	69
10 ≤ EPS ≤ 21.7				72	51	74	42	45	47			
1.3 ≤ EPS ≤ 10	98	97	99	24	38	26	54	51	48	23	26	28

Note: A1: anaerobic initial-stage of SBR systems; A2: anaerobic end-stage of SBR systems; O2: aerobic end-stage of SBR systems under anaerobic/aerobic operation.

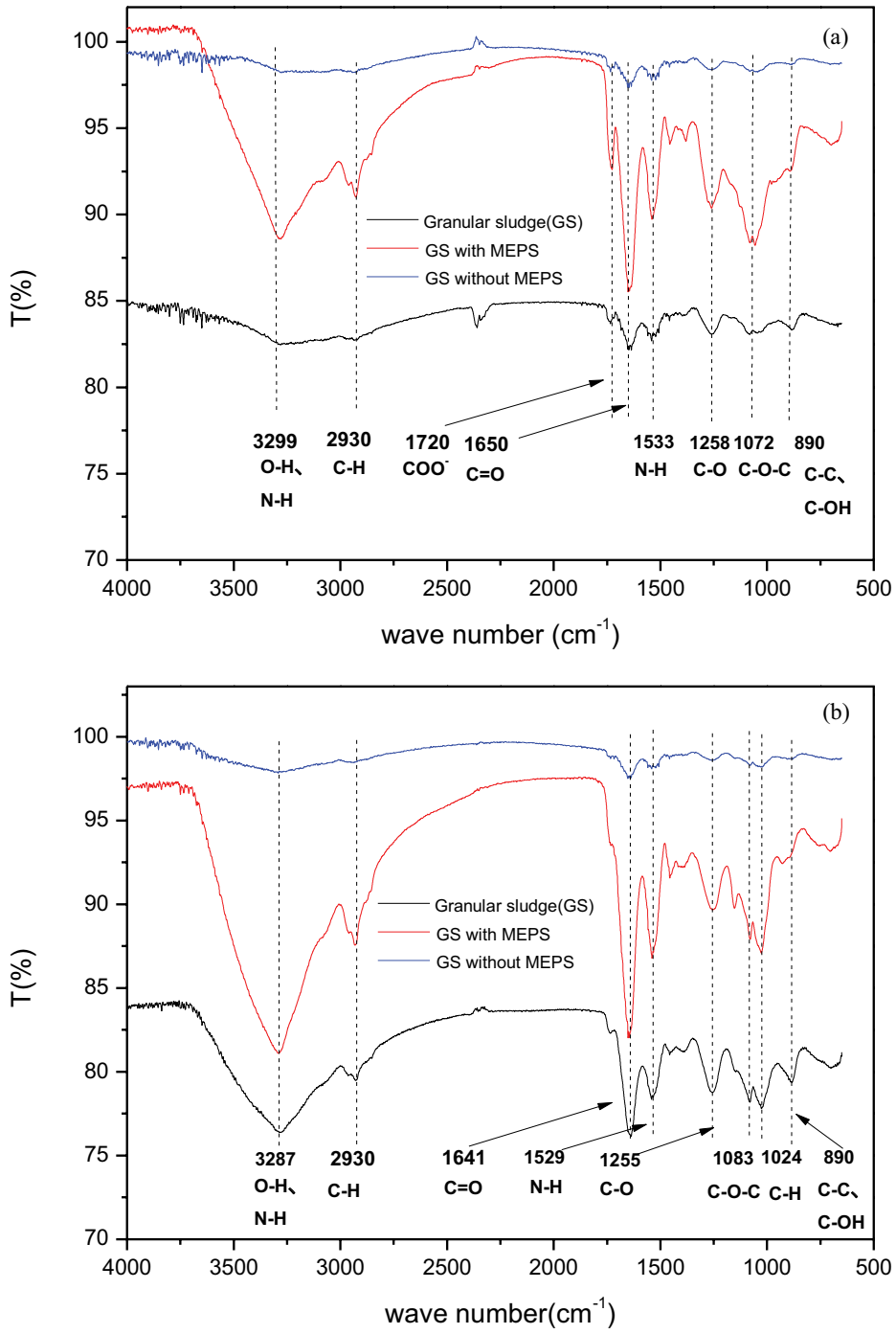

Fig. 5. Different Fourier-transform infrared spectra of granular sludge (GS), GS with MEPS and GS without MEPS in R1 and R2. *Note*: GS with MEPS was the granular sludge after the ultrasonic extraction, GS without MEPS was the granular sludge after ultrasonic-CER extraction.

proteins were clearly different in the case of different carbon source feeds.

- The spectroscopic characterization revealed the dominance of hydrocarbons, proteins, polysaccharides, and aromatic associated bonds in all sludge samples. However, the protein associated bonds (such as –CO–NH–, C=O, N–H) increased significantly in the MEPS, which indicated the higher content of proteins at the microsphere level.

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