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Influence of different extraction methods on the compositions and structural characteristics of extracellular polymeric substances in biofilms from biological aerated filters

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ABSTRACT

Extracellular polymeric substances (EPS) were extracted from biofilms in a continuously running biological aerated filter (BAF) reactor by five different methods, including heating treatment at 60°C, cation exchange resin (CER), EDTA, sodium hydroxide (NaOH), and sulfuric acid (H_2SO_4). High-speed centrifugation was used as a control method. The results showed that the extraction method played a great role on EPS yields and chemical compositions, whereas no remarkable difference in their structural characteristics was observed. Heating treatment was determined as the most effective protocol with the highest EPS recovery and lowest cell damage. The total amount of the extracted EPS was 131.6 mg/g volatile suspended solids (VSS), while the DNA content only accounted for 7.0%. The intensity of absorption bands in the UV-visible (UV-vis) spectra was in agreement with chemical compositions of the extracted EPS. In addition, the Fourier transform infrared (FT-IR) results indicated that no noticeable effect was found on EPS structures extracted by different methods. However, both UV-vis and FT-IR spectroscopic analyses revealed that chemical reagents used during extraction processes might contaminate the extracted EPS, and then interfered with the determination of proteins in EPS.

Keywords: Extracellular polymeric substance; Biofilm; Heating treatment; Cell lysis; UV-vis spectrum; Fourier transform infrared

1. Introduction

Extracellular polymeric substances (EPS) have a three-dimensional network structure [1], presenting an aqueous macromolecular gel matrix state. The main components of EPS consist of polysaccharides (PS), proteins (PN), humic acid substances, and small amounts of nucleic acids and lipids [2–4]. Due to the special distribution characteristics and physicochemical properties, EPS plays a determinant role on the biomass structural integrity, making the microbial cells cluster together for enhancing resistance to the harsh external environment. Thus, EPS have critical relevance to the morphology and structure of biomass aggregates in biological wastewater treatment [3], which can potentially be used as indicators for microbial survival ability and aggregate morphology [5]. Also, the chemical compositions in different fractions of EPS have been correlated with the sludge dewaterability [2]. As a result, the investigation on yields and chemical compositions of EPS can provide insights into the formation mechanisms of biomass morphology.

Normally, according to the combination of EPS and microbial cell surface, the EPS can be divided into two major fractions: soluble EPS (S-EPS) and bound EPS (B-EPS). B-EPS exhibit a dynamic double-layer-like structure, which can be further divided into (1) loosely bound EPS (LB-EPS) that can assemble clusters to form micro-colonies and flocs; (2) tightly bound EPS (TB-EPS), which are found on the cell surface and to bridge cells together in clusters. Since S-EPS bind to microbial cells weakly and are able to move freely, this fraction can be extracted using a centrifugation method [1]. As a consequence, a number of the reported researches were primarily focused on B-EPS extraction with various

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physical and/or chemical methods [6–14]. However, the quantities and compositions of the extracted EPS are strongly dependent on the biomass sample origins and extraction protocol used [3], and no standardized method is now available for extracting and distinguishing each fraction of EPS. In most cases, the results of EPS yields and compositions were often contradictory even when using similar biomass samples, because various factors (i.e., chemical compositions, microbial communities, and operating conditions of bioreactors) were able to affect the characteristics of biomass samples [14]. Therefore, a suitable approach to extract EPS in the specific biomass samples should be explored and assessed for achieving maximum EPS yields and minimum cell lysis.

The main objective of this work is to identify a favorable and feasible protocol for EPS extraction in biofilms in a continuously operating biological aerated filter (BAF) reactor. Five different methods including heating treating at 60°C, cation exchange resin (CER), EDTA, sodium hydroxide (NaOH), and sulfuric acid (H_2SO_4) were compared, while high-speed centrifugation was used as control method. Amounts and compositions of EPS extracted by different methods were determined. UV-visible (UV-vis) spectroscopic analysis was used to evaluate the degree of cell damage during extraction processes. Also, the structural characteristics of the extracted EPS were investigated with Fourier transform infrared (FTIR) analysis.

2. Materials and methods

2.1. BAF configuration and operation

A lab-scale BAF was continuously operated for almost 3 months to achieve steady performance. The reactor is made of transparent plexiglass with a height of 1500 mm and an inner diameter of 80 mm. The working volume was about 6 L. The granular media for biofilm attachment were ceramic particles having an average size of 3 mm and a specific surface area of 4.0 m^2/g, with a packing density of 1650 kg/m^3. An air diffuser was installed at the bottom of BAF reactor to keep the dissolved oxygen (DO) concentration in the range of 2–4 mg/L, and the flow rate was controlled using an airflow meter. The reactor was inoculated with activated sludge and domesticated with synthetic wastewater. The detailed compositions (mg/L) of wastewater are summarized as follows: CH_3COONa 200, $(NH_4)_2SO_4$ 35, $K_2HPO_4{\cdot}3H_2O$ 10, $CaCl_2$ 10, $MgSO_4$ 0.42, $CoCl_2{\cdot}6H_2O$ 1, $FeCl_3{\cdot}6H_2O$ 1.15, $MnSO_4{\cdot}H_2O$ 0.21, H_3BO_3 0.02, $ZnCl_2$ 0.02, $Na_2MoO_4{\cdot}2H_2O$ 0.06, $CuSO_4{\cdot}5H_2O$ 0.02, and EDTA 0.05, resulting in raw influent qualities with COD_{Cr} of 180–200 mg/L and NH_4^+-N of 10–15 mg/L. The BAF was operated in an up-flow mode at pH of 7.0 ± 0.2 and ambient temperature (20 ± 5°C).

2.2. Biofilm EPS extraction

Detailed extraction procedures are given in Fig. 1. As indicated, a portion of ceramic particles was first collected from the bottom 20 cm of BAF reactor due to its higher amount of biomass in contrast to upper positions [15]. The attached biofilms were scraped with a sterilized blade and placed in a Teflon centrifuge tube. Deionized water was added into the tube for obtaining homogenous suspension, which was pretreated with ultrasound at 30 W for 5 min. Then the suspension was centrifuged at 4°C and 2000 rpm for 20 min, and the supernatant was collected as soluble EPS. The residual bottom biomass was re-suspended using deionized water to the original volume and oscillated for 1 h

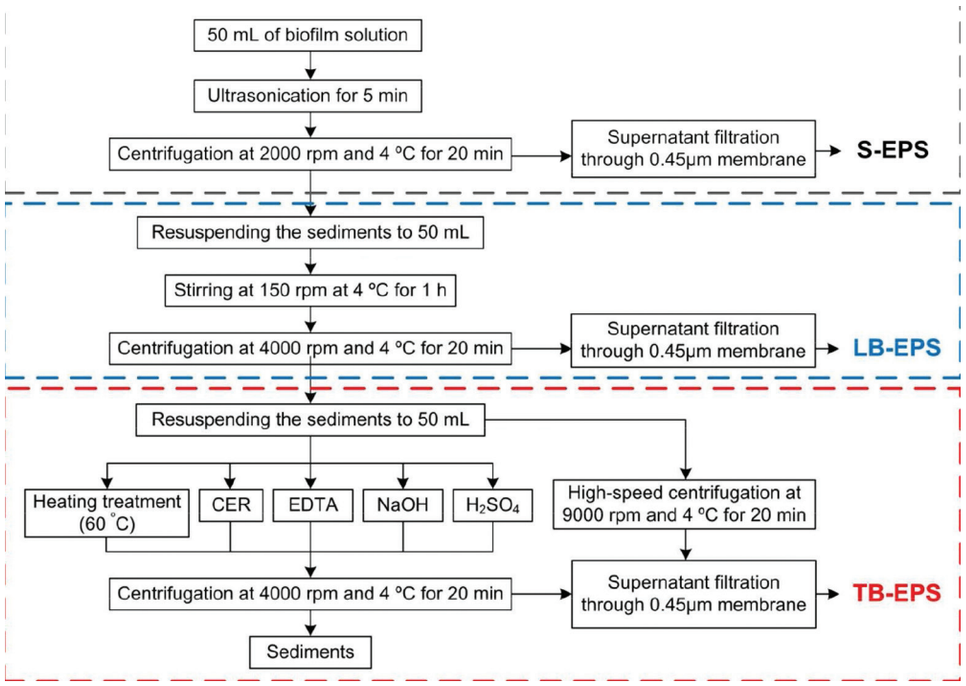

Fig. 1. Detailed procedures for biofilm EPS extraction.

in an orbital incubator at 150 rpm. The biomass mixture was centrifuged at 4°C and 4000 rpm for 20 min, and the obtained supernatant was collected as LB-EPS. Prior to TB-EPS extraction, the residual biomass was re-suspended again with deionized water to the origin volume. Five different methods were used and compared for the treatment of the suspension, including thermal treatment, CER, EDTA, NaOH, and H_2SO_4. Briefly, the extraction procedures for the above individual method included: (1) heating at 60°C in a water bath for 1 h; (2) adding 70 g/g VSS of gel CER (20~50 mesh, Na^+ form, Sinopharm Chemical Reagent Co., Ltd, China) and mixing at 4°C and 150 rpm for 1 h using an orbital shaker; (3) adding 50 mL of 2% EDTA and mixing at 4°C and 150 rpm for 1 h; (4) adding 1 mol/L NaOH for adjusting the suspension pH to 11; (5) adding 8% H_2SO_4 for adjusting the suspension pH to 3. The final supernatant was centrifuged at 4°C and 4000 rpm for 20 min and collected as TB-EPS. Besides, high-speed centrifugation at 4°C and 9000 rpm for 20 min was used as a control method without any further treatment. All suspensions mentioned above were filtered through a syringe filter (polytetrafluoroethylene, 0.45 μm pore size), and the filtrate was collected and stored at –20°C for subsequent analysis.

2.3. Chemical analysis

In this work, the total amount of biofilm TB-EPS was determined by the sum of PS, PN and DNA concentration, although other components like humic-like substances were also contained in EPS. The PS content in EPS was determined by the anthrone method using glucose as standard [16]. The PN content in EPS was measured using the Lowry method with bovine serum albumin as standard [17]. The DNA content of EPS was used as an indicator of cell lysis [18], and quantification of DNA in EPS was carried out using the diphenylamine colorimetric method [19] with calf thymus DNA as standard. The volatile suspended solids (VSS) were measured according to standard methods [20]. All chemicals were of analytical grade, and used without further purification.

2.4. UV-vis and FT-IR spectra

Absorption spectra of the extracted EPS were determined using a Persee TU-1810 UV-visible spectrophotometer (Beijing, China). The scanning wavenumber ranged from 200 nm to 700 nm at an optical path of 1 cm. The functional groups in the extracted EPS were measured with a Bruker TENSOR 27 FT-IR spectrometer (Germany). Prior to measurement, the freeze-dried samples were grounded into powders and mixed with KBr to prepare disks. The spectrum was scanned and recorded in wavenumber ranging from 4000 cm^{-1} to 400 cm^{-1} with a resolution of 4 cm^{-1}.

3. Results and discussion

3.1. EPS compositions and extraction efficiency of different methods

There is a particular challenge in EPS extraction protocols aiming for the maximal yields (mg/g VSS) with a minimal contribution to the cell lysis products. Taking into account the recovery obtained for TB-EPS extraction by the five different methods, it is possible to compare and analyze their efficiencies and determine the most appropriate one. Fig. 2 shows the contents of different components in EPS matrix extracted from biofilms with various extraction methods. For comparison, the EPS extraction results in other literatures were summarized (Table 1). As indicated, the total amounts of the extracted EPS and the proportion of each component were strongly related to the extraction methods. PN and PS were the main fractions of the extracted EPS in biofilms. Thus, their proportions were subsequently used to assess the distinction in the extraction efficiency among different methods. As a control, high-speed centrifugation extraction yielded the least quantity of total EPS (23.4 mg/g VSS) with corresponding contents of PS, PN, and DNA of 14.5, 5.2, and 3.7 mg/g VSS, respectively, indicating that any chemical reagent was able to facilitate the extraction of EPS from biofilms. By contrast, heating treatment method yielded the highest amount of EPS (131.6 mg/g VSS) with corresponding contents of PS, PN, and DNA of 108.1, 14.3, and 9.2 mg/g VSS, respectively. This quantity was twice more than those extracted by other methods, and also much higher than the results reported previously (Table 1). One possible explanation is that rising temperature was able to increase the separation and diffusion rates of EPS components, and enhanced the dissolution of EPS from biofilms [21]. H_2SO_4 was found to be the lowest efficient method for EPS extraction in biofilms (42.0 mg/g VSS). Similar results were also observed by other researchers [8,22]. The extraction efficiency by EDTA method (65.8 mg/g VSS) was higher than others except heating treatment. It was reported that EDTA was able to chelate Ca^{2+} in sludge flocs [23], resulting in the reduction of floc strength and enhancement of the EPS solubility for extraction [24,25]. In addition, the total EPS extraction yield using CER method (54.1 mg/g VSS) was higher than that extracted by NaOH (45.6 mg/g VSS) and H_2SO_4 methods, and the quantity was quite similar to the results reported previously (Table 1).

In this study, the amounts of the extracted EPS using chemical methods (i.e., NaOH and H_2SO_4) were lower

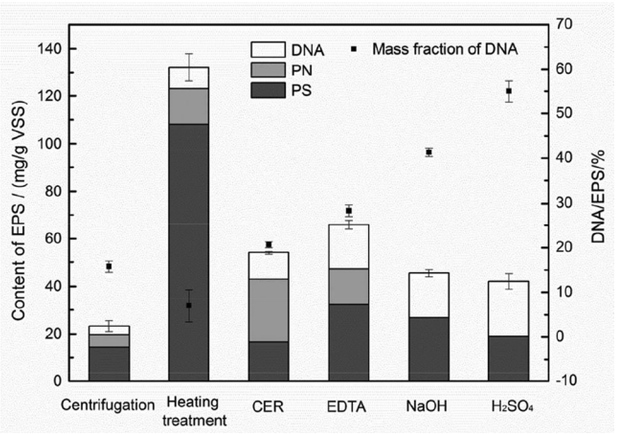

Fig. 2. TB-EPS contents and cell lysis in biofilms with different extraction methods.

Table 1
Comparison of EPS extraction efficiencies by different methods with previous studies

Extraction method	Yield (mg/g VSS)	Analytical method			Reference
		PS	PN	DNA	
NaOH (1 mol/L, pH = 11)	61.3	Lowry	Anthrone	Picogreen	[3]
EDTA (3 h, 4°C)	95.6				
CER (70 g/g VSS)	53.2				
Heating treatment (80°C, 1 h)	61.2	Bradford	Anthrone	Diphenylamine	[13]
EDTA (4°C, 3 h)	70.3	Lowry	Anthrone	UV spectrophotometer	[24]
Heating treatment (70°C, 1 h)	71.6				
H_2SO_4 (8%, 4°C)	21.4				
Heating treatment (80°C, 0.5 h)	92.7	Lowry	Anthrone	Diphenylamine	[26]
CER (70 g/g VSS)	14.8	Lowry	Anthrone	/	[27]

than physical methods (i.e., heating treatment and CER). It is worth noting that PN was not found in the extracted EPS with NaOH and H_2SO_4 methods, suggesting that the presence of chemical reagents used for EPS extraction may unfortunately cause protein denaturation and/or interfere chemical analysis. Similar results were also found that the extraction yield of EPS and protein contents by H_2SO_4 method were lower than physical methods [24,28].

It is generally accepted that a favorable extraction method shall have a sufficient high EPS yield with minimal loss in cell viability, ensuring the limited release of intracellular components into the extraction buffer. As a result, the relative content of DNA was used to evaluate the degree of cell lysis by different extraction methods. As shown in Fig. 2, each method yielded different quantities of DNA. Chemical methods were more effective in extracting DNA in biofilms

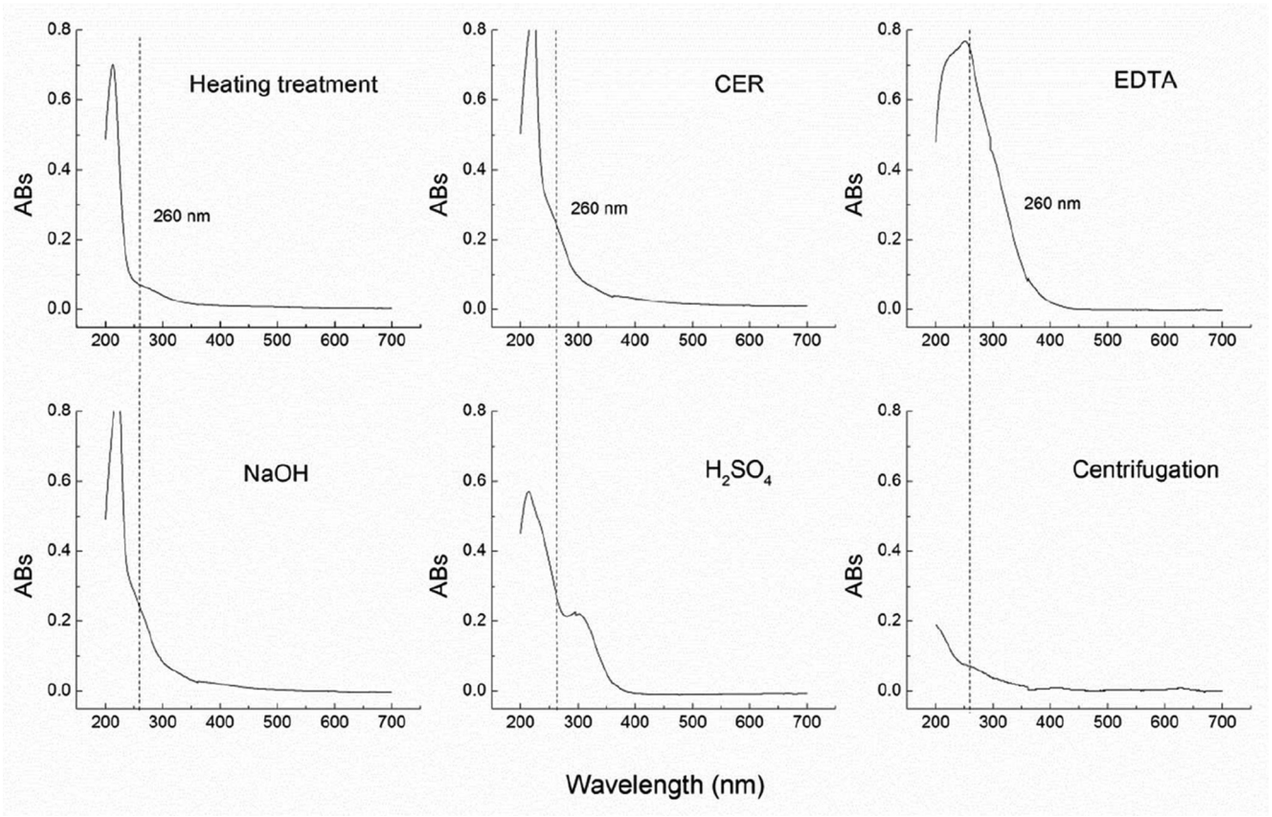

Fig. 3. UV-vis spectra of TB-EPS extracted by different methods.

than physical methods, suggesting that a higher degree of cellular destruction occurred. The overall percentage of DNA contents in EPS for the used five extraction methods was ranked in the following order: H_2SO_4 (55.0%) > NaOH (41.4%) > EDTA (28.3%) > CER (20.2%) > centrifugation (15.7%) > heating treatment (7.0%). Liao et al. [30] reported that the DNA content extracted in EPS ranging from 2% to 15% was reasonable. In contrast to other methods, the amount of DNA extracted by heat treatment was much less. These results demonstrated that heating treatment was potentially the best option for EPS extraction in biofilms, yielding the least DNA content.

3.2. UV-vis spectra of TB-EPS extracted by different methods

It is generally known that DNA molecules contain conjugated double bonds, which have the nature of ultraviolet absorption at a wavelength of about 260 nm [13,24]. Thus, UV-vis spectra can be used to characterize the degree of cell damage in the EPS extraction process, which has been demonstrated as an easy and rapid technique [24]. As depicted in Fig. 3, the intensity of the band at 260 nm was very strong in the UV-vis spectrum of TB-EPS extracted with EDTA method, implying that a high degree of cellular destruction occurred. For other methods except heating treatment, the bands at 260 nm were similar, and were much weaker than that by EDTA method. However, this band was not observed in the spectrum of EPS extracted with heating treatment, which was almost identical with the result by centrifugation method. These results are consistent with the chemical analysis as mentioned above (Fig. 2). Similarly, Sheng et al. [24] also found that the TB-EPS extracted from sludge with NaOH method caused a high cellular destruction. Nevertheless, an additional peak was observed at about 296 nm for the EPS extracted with H_2SO_4 method. This is possibly resulted from the formation of byproducts from the interaction between DNA molecules and chemical residues, which have absorption at this wavelength. Therefore, heating treatment was determined as the most favorable method for TB-EPS extraction from biofilms with a high product yield and a low degree of cell lysis.

In addition, ultraviolet scanning at wavelengths ranging from 200 nm to 220 nm can be used to interpret the absorbance of PN with concentrations of 20–100 μg/mL, which are caused by peptide bonds [29]. Fig. 3 clearly showed that the intensity of bands at 220 nm was very remarkable in the spectra of TB-EPS extracted using heating treatment, CER, EDTA and NaOH methods, whereas the absorption peak for TB-EPS extracted with H_2SO_4 method was a little weaker. Similar results were also found by Chen et al. [13]. However, these results were inconsistent with those of chemical measurement of PN contents as discussed above (Fig. 2), which suggested that PN was not found in the extracted EPS with NaOH and H_2SO_4 methods. The possible explanation is that the use of chemical reagents for EPS extraction probably unfortunately caused protein denaturation, but the peptide bonds still remained, resulting in the differences between the results of chemical and spectral analyses.

3.3. FT-IR spectral analysis

The FT-IR spectra of TB-EPS extracted with different methods are shown in Fig. 4. Also, the functional groups corresponding to the absorption bands found on FT-IR spectra are summarized in Table 2. As indicated, the primary bands were observed in the wavenumber ranges of 3500–3200 cm^{-1}, 1660–1640 cm^{-1}, and 1150–1030 cm^{-1}. The broad absorption band at around 3300 cm^{-1} was assigned to the O-H stretching vibration of polymeric compounds such as PN and PS [30]. The visible weak peak at a wavelength of about 2920 cm^{-1} was attributed to the H-C-H asymmetric stretching vibration [26], which was associated with the aliphatic chains of proteins, carbohydrates, lipids, and humic acids. Besides, several absorption regions were related to the biochemical compounds. As an example, the band at around 1660 cm^{-1} was assigned to amide I: C=O and C=N stretching vibration [31], while the peak at 1543 cm^{-1} was attributed to amide II: C-N stretching vibration and N-H deformation vibration. Furthermore, the band at about 1080 cm^{-1} was assigned to the C-O-C stretching vibration in the PS structures [24,27]. The bands near 1390 cm^{-1} were in connection with the C-H deformation vibrations in the methyl groups [26]. The absorption bands at wavelengths

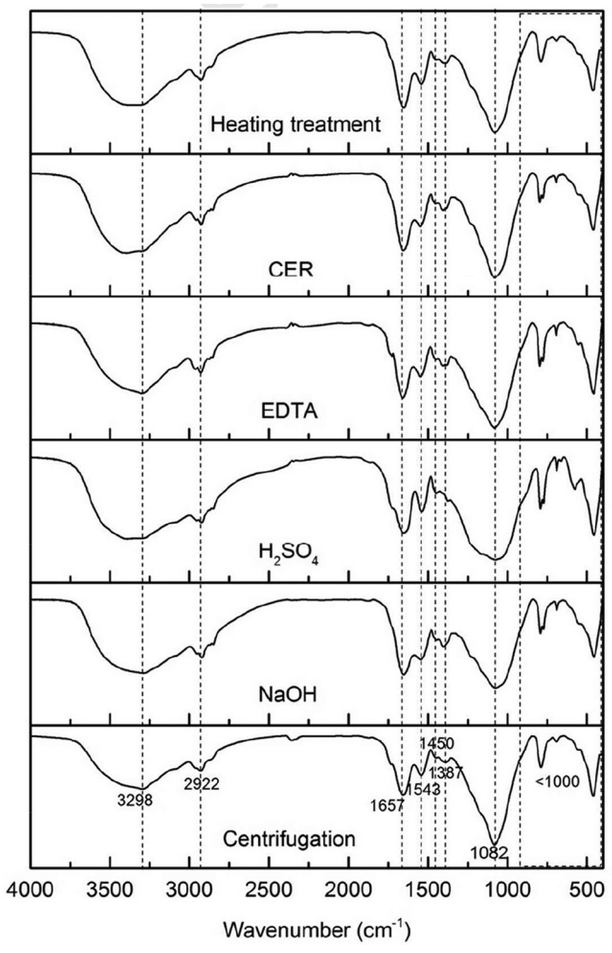

Fig. 4. FT-IR spectra of TB-EPS extracted by different methods.

Table 2
Main functional groups observed from the infrared spectra of TB-EPS extracted by different methods

Wave number (cm^{-1})	Vibration type	Functional group
3300	O-H stretching vibration	OH into polymeric compounds
2920	H-C-H asymmetrical stretching, symmetrical stretching	Aliphatic groups
1660	Amide I: C=O and C-N stretching vibration	PN (peptidic bond)
1540	Amide II: C-N stretching vibration and N-H deformation vibration	PN (peptidic bond)
1450	H-C-H deformation vibration	Aliphatic chains
1390	C-H deformation vibration	Aliphatic CH2-group
1080	C-O-C stretching vibration	PS
<1000	Several bands visible	Phosphate or sulphur-containing functional groups

lower than 1000 cm^{-1} were formed by the unsaturated bonds [32], which were probably assigned to the phosphate and/or sulfur functional groups.

Generally, no noticeable difference was observed in the infrared spectra of TB-EPS extracted by different methods (Fig. 4). However, there was an intense newly added peak at around 600 cm^{-1} for TB-EPS extracted by H_2SO_4 method. This difference was probably owing to the interference by the chemical residues. These results might explain the difficulties in determining PN contents in EPS using the Lowry method.

4. Conclusions

In this work, the compositions and structural characteristics of EPS extracted from biofilms with five different methods were analyzed and compared. The cell lysis degree and structural characterization were evaluated using UV-vis and FT-IR spectral analyses. Among these methods, heating treatment at 60°C was found to be the most efficient method for EPS extraction in biofilms, yielding as high as 131.6 mg/g VSS. Also, the degree of cell damage caused by heat treatment was the lowest, as the DNA contents only accounted for 7.0% of the total EPS quantity. The results of UV-vis and FT-IR spectral analyses were almost in agreement with the chemical measurements of EPS components. However, the residues of chemical reagents used for EPS extraction were able to affect the spectroscopic analysis of PN in biofilm EPS.

Acknowledgments

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