A comparison of five extraction methods for extracellular polymeric substances (EPS) from biofilm by using three-dimensional excitation-emission matrix (3DEEM) fluorescence spectroscopy

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Abstract

Two physical methods (centrifugation and ultrasonication) and 3 chemical methods (extraction with EDTA, extraction with formaldehyde, and extraction with formaldehyde plus NaOH) for extraction of EPS from alga-bacteria biofilm were assessed. Pretreatment with ultrasound at low intensity doubled the EPS yield without significant modification of the composition of EPS. Extraction with EDTA or extraction with formaldehyde plus NaOH increased yield by about 1 order of magnitude compared with other methods. However, the protein and polysaccharide content in EPS prepared with EDTA or formaldehyde plus NaOH were low. Two fluorescence peaks belonging to protein-like peaks and 2 fluorescence peaks belonging to humic acid-like substances were found in 3DEEM fluorescence spectra of all the EPS samples prepared using different methods. Fulvic-like fluorescence was detected only in the EPS extracted with formaldehyde plus NaOH. Location of, and fluorescence intensity at, each peak were clearly affected by the extraction methods. Dialysis was also found to be an important factor influencing the yield, composition and fluorescence characteristics of EPS.

Keywords: biofilm, extracellular polymeric substances, 3DEEM fluorescence spectroscopy

Introduction

Extracellular polymeric substances (EPS) are secreted by microorganisms with their growth, and accumulate on cell surfaces (Liu and Fang, 2002a; Tsuneda et al., 2003). EPS is composed of some high molecular weight compounds, including polysaccharide, protein, nucleic acids, humic substances, and ionisable functional groups like carboxylic, phosphoric amino and hydroxyl groups (Tsuneda et al., 2003; Guibaud et al., 2008). EPS plays a key role in the formation of biofilm. It makes the biofilm stable and forms a barrier protecting the biofilm from harmful effects, as well as reducing water loss from the cell to environment (Laspidou and Rittmann, 2002). EPS can efficiently remove heavy metals and organic contaminants since EPS has a large number of negatively-charged functional groups (Zhang et al., 2006; Liu et al., 2001; Sheng et al., 2005; Bhaskar and Bhosle, 2006). EPS can form multiple complexes with many heavy metal ions and consequently has a great impact on geochemical behaviour, bioavailability and toxicity of heavy metal ions (Selck et al., 1999). EPS has been considered as one of the most important types of dissolved organic matter (DOM) influencing the fate of heavy metals in the aquatic environment (Bhaskar and Bhosle, 2006). In the past several decades EPS has been extensively studied with regard to its physicochemical properties, interaction with contaminants and role in biofilm formation (Laspidou and Rittmann, 2002; Bhaskar and Bhosle, 2006; Meisen et al., 2008).

Methods to extract EPS are important for studying its physicochemical properties and its impact on contaminants in the aquatic environment. Various physical and chemical methods have been reported to extract EPS associated with cells from different sources (e.g. biofilm, sludge and cell suspension). Common physical methods include centrifugation, ultrasonication and heating (Liu and Fang, 2002b; Comte et al., 2007). Chemical methods include extraction of EPS with chemical agents. Ethylenediamine tetraacetic acid (EDTA), formaldehyde, NaOH and NaOH-formamide are usually used as the extractants (Liu and Fang, 2002b; Adav and Lee, 2008). A review of the literature showed that extraction yield, composition and physicochemical properties of EPS varied greatly with the extraction method. Liu and Fang (2002b) reported that extracting with formaldehyde plus NaOH obtained more EPS from sludge than other methods including extraction with EDTA, cation exchange resin or formaldehyde. Comte et al. (2007), using high-pressure size exclusion chromatography (HPSEC), demonstrated that extraction of EPS by heating induced hydrolysis of EPS, and extraction with chemical reagents strongly affected the composition of EPS. Mcswain et al. (2005) showed that EPS was contaminated by DNA due to cell lysis during extraction of EPS by heating. The various EPS extraction methods in the literature led to uncertainty and non-comparability of results. Therefore, it is necessary to assess the various extraction methods from the perspective of extraction yield and composition.

Three-dimensional excitation-emission matrix (3DEEM) fluorescence spectroscopy is well known for its speed, good

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selectiveness and high sensitivity in detecting fluorophore substances (Ni et al., 2009). The location of peaks on a 3DEEM fluorescence spectrum provide information on composition of organic matter, and the fluorescence intensity at the characteristic peaks gives information on the relative content of the fluorescent components (Chen et al., 2003; Sheng and Yu, 2006). Three-dimensional excitation-emission matrix fluorescence spectroscopy has been extensively used for studying dissolved organic matter (DOM) (Wu and Tanoue, 2001; Baker, 2002). Recently, 3DEEM fluorescence spectroscopy has also proven to be a useful tool for studying EPS (Sheng and Yu, 2006; Zhang et al., 2009). Limited studies have suggested that there are several peaks in 3DEEM of EPS extracted from activated sludge, being assigned to protein-like substances, humic-like substances and fulvic-like substances (Sheng and Yu, 2006; Adav and Lee, 2008; Ni et al., 2009).

In this study, 5 EPS extraction methods reported in the previous literature were compared in preparing EPS from alga-bacteria biofilm using 3DEEM fluorescence spectroscopy. The purpose of this study was to assess the pros and cons of these methods and their suitability in preparing EPS samples for scientific research.

Materials and methods

Samples

Alga-bacteria biofilm was collected from a pond at the Institute of Geochemistry, Chinese Academy of Sciences, Guiyang, China, using a sterile plastic spatula and placed in a 200 mℓ autoclaved beaker. Immediately after the biofilm sample was collected, it was washed with de-ionized water and dispersed into suspension by a stirrer. The cell suspension was then immediately subjected to EPS extraction by different methods.

Extraction methods of EPS

Three chemical methods and 2 physical methods were used to extract EPS from alga-bacteria biofilm. Figure 1 illustrates the processes of these 5 methods. The following 3 chemical methods were assessed for EPS extraction:

- Extraction with EDTA – the cell suspension was extracted with 2% EDTA for 3 h at 4°C.
- Extraction with formaldehyde – the cell suspension was extracted with formaldehyde (36.5%) for 1 h at 4°C.
- Extraction with formaldehyde plus NaOH – the cell suspension was first extracted with formaldehyde (36.5%) for 1 h at 4°C and then with NaOH (1M, 4°C, 3 h) (Liu and Fang, 2002b).
- All the samples treated with chemical agents were centrifuged at 20 000 r/min, 4°C for 20 min and the supernatants were filtered through a 0.22 μm membrane. The filtrates were used as EPS samples.

The following 2 physical methods were assessed for EPS extraction:

- **Extraction with high-speed centrifugation** – The cell suspension was centrifuged at 20 000 r/min, 4°C for 20 min. This method was believed not to cause cell lysis (Liu and Fang, 2002b). The supernatants were filtered through a 0.22 μm membrane. The filtrate was used as the EPS sample.
- **Extraction with ultrasonication** – The cell suspension was firstly subject to ultrasonication at 40W in an ice bath for 2 min (Comte et al., 2006). The ultrasound was generated by an ultrasound generator (Sonopuls JY88-II, Ningbo, China). The sonicated cell suspension was then centrifuged at 20 000 r/min, 4°C for 20 min. The supernatants were filtered through a 0.22 μm membrane. The filtrate was used as the EPS sample.

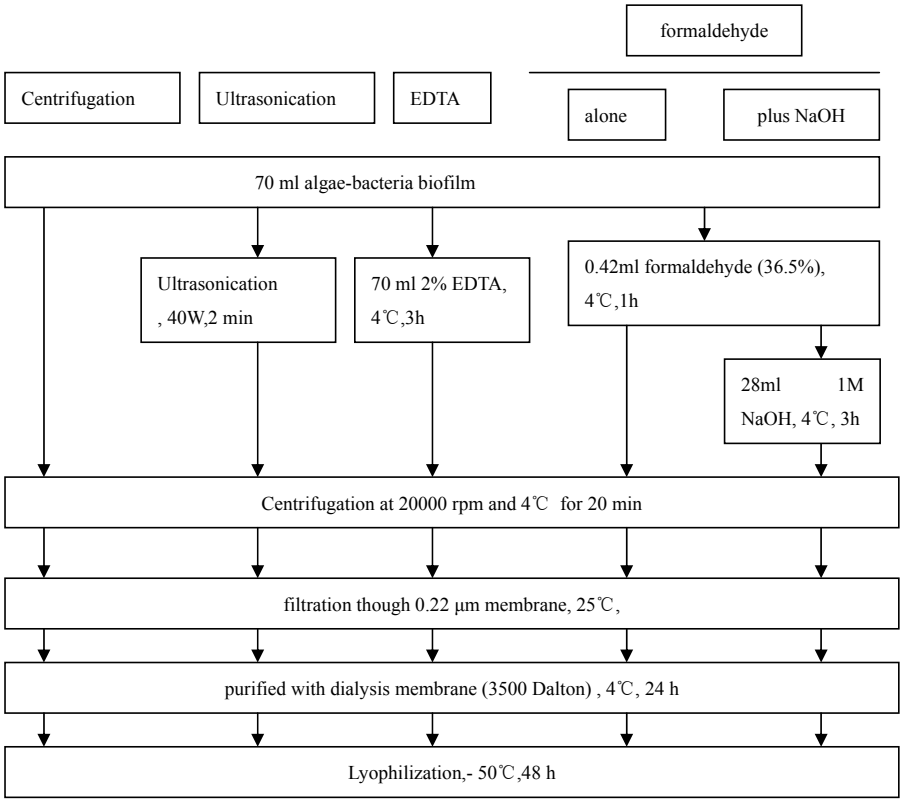

Figure 1
Flow chart of EPS extraction processes

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Table 1
EPS yield extracted by different methods and its composition before dialysis and after dialysis

Extraction Method	EPS content before dialysis (mg/g dry biofilm)	Composition of EPS after dialysis			
		Carbohydrate (mg/g EPS)		Protein (mg/g EPS)	
		BD	AD	BD	AD
Centrifugation	7.20	57.00	38.70	--[***]	--[***]
Ultrasonication	12.70	56.60	34.90	22.30	20.39
EDTA	164.50	3.20	1.80	1.70	0.71
Formaldehyde	14.20	25.20	14.42	8.00	2.57
Formaldehyde-NaOH	114.70	13.30	10.60	17.07	13.64

BD- before dialysis, AD- after dialysis, --- undetected

Chemical analysis of EPS

Polysaccharide content in EPS was determined by the phenol-sulphuric acid method, according to Dubois and Gilles (Dubois et al., 1956), with glucose as the standard. The protein content of EPS was determined by the Bradford method (1976) with bovine serum albumin as the standard.

3DEEM fluorescence spectroscopy

All of the EPS samples, before or after dialysis with 3500 Dalton molecular weight cutoff membranes, were characterised with 3DEEM fluorescence by using fluorescence spectrometry (Hitachi, F-4500, Japan). Three-dimensional excitation-emission matrix spectra were determined with scanning emission spectra from 250 nm to 600 nm at 2 nm increments by varying the excitation wavelength from 200 nm to 450 nm at 5 nm increments, and with a speed of 1 200 nm/min. The blank spectrum was recorded with double-distilled water. The data were analysed with Sigmaplot 10 (Systat Software Inc., San Jose, USA).

Results and discussion

Protein and polysaccharide in EPS

Table 1 summarises the yields and protein and polysaccharide contents of EPS samples prepared with the 5 methods. EPS yield varied significantly with the extraction method. Generally, more EPS was extracted with the chemical methods than with the physical methods. This result was in accordance with some previous studies (Liu and Fang, 2002b; Adav and Lee, 2008). The least EPS was extracted with centrifugation. Ultrasonication at 40 W slightly increased the amount of EPS extracted. EDTA seemed to be most effective in extracting EPS. There are 2 possible reasons for this. One explanation is that EDTA could increase the solubility of EPS and thus increase the yield (Comte et al., 2006). The other explanation is that EPS extracted by EDTA may be overestimated since EDTA may bind with some components in EPS and form complexes that could not be removed by dialysis (Liu and Fang, 2002b). Extraction with formaldehyde plus NaOH was also found to be an effective method for EPS extraction. Comte et al. (2006) explained that an increase in pH due to the presence of NaOH resulted in separation between acidic groups in EPS and thus more EPS were extracted. In Liu's study (Liu and Fang, 2002b), treatment with formaldehyde plus NaOH was shown to be the most efficient method.

Composition of EPS was also significantly affected by the extraction method (Table 1). Protein was undetected in EPS samples prepared with centrifugation alone, indicating that little protein was extracted by this method. The protein content was highest in the EPS sample prepared with ultrasonication. The possible reason for this is that ultrasonication may promote the shifts of protein and carbohydrate from the inner layer to the outer layer (Yu et al., 2008). Interestingly, extraction with EDTA gave the highest yield of EPS but the protein and carbohydrate contents were low. This might be explained by the fact that EDTA, carbohydrate and protein form complexes that were not removed by dialysis, resulting in overestimated content of EPS but lower proportions of carbohydrate and protein (Liu and Fang, 2002b).

3DEEM fluorescence spectra of EPS

Four or five peaks were found in each 3DEEM fluorescence spectrum of EPS samples prepared by different methods (Fig. 2). Peak A was at excitation/emission wavelengths (Ex/Em) of 225 to 230 nm/340 to 372 nm, Peak B at Ex/Em of 275 to 285 nm/346 to 368 nm, Peak C at Ex/Em of 255 to 270 nm/442to 464 nm, Peak D at Ex/Em of 330 to 360 nm/390 to 464 nm, and Peak E at Ex/Em of 325to 330 nm/390 nm. Peaks A, B, C and D were found in spectra of all the EPS samples. Peak E was only found in the EPS sample prepared with formaldehyde plus NaOH. Peak A and B were described as protein-like peaks, at which the fluorescence is associated with the aromatic amino acids and tryptophan (Wu and Tanoue, 2001; Baker, 2002; Sheng and Yu, 2006; Adav and Lee, 2008). Fluorescence at Peaks C and D was attributed to the presence of humic acid-like substances (Wu and Tanoue, 2001; Baker, 2002). Peaks A, B, D and E were also reported to be found in the EPS from sludge (Sheng and Yu, 2006; Adav and Lee, 2008; Ni et al., 2009). Peak C was not found in EPS samples in the previous studies. As far as the originality of fluorescence at Peak E was concerned, different studies had different interpretations. Most studies held that fluorescence at Peak E originated from humic acid-like substances (e.g., Wu and Tanoue, 2001; Baker, 2002), but several recent studies demonstrated that it belonged to fulvic acid-like fluorescence (Chen et al., 2003; Ni et al., 2009).

Location of, and fluorescence intensity at, each peak were clearly affected by the extraction methods (Fig. 2). The location of, and fluorescence intensity at, each peak are listed in Table 2. It was found that fluorescence intensity at Peaks A and B for EPS prepared with centrifugation was much weaker than that for EPS samples prepared with the other 4 methods, indicating that only a small amount of protein-like substances was extracted by centrifugation. This result confirmed the result of undetected protein in the EPS sample prepared with centrifugation (Table 1). Since Coomassie Brilliant Blue has

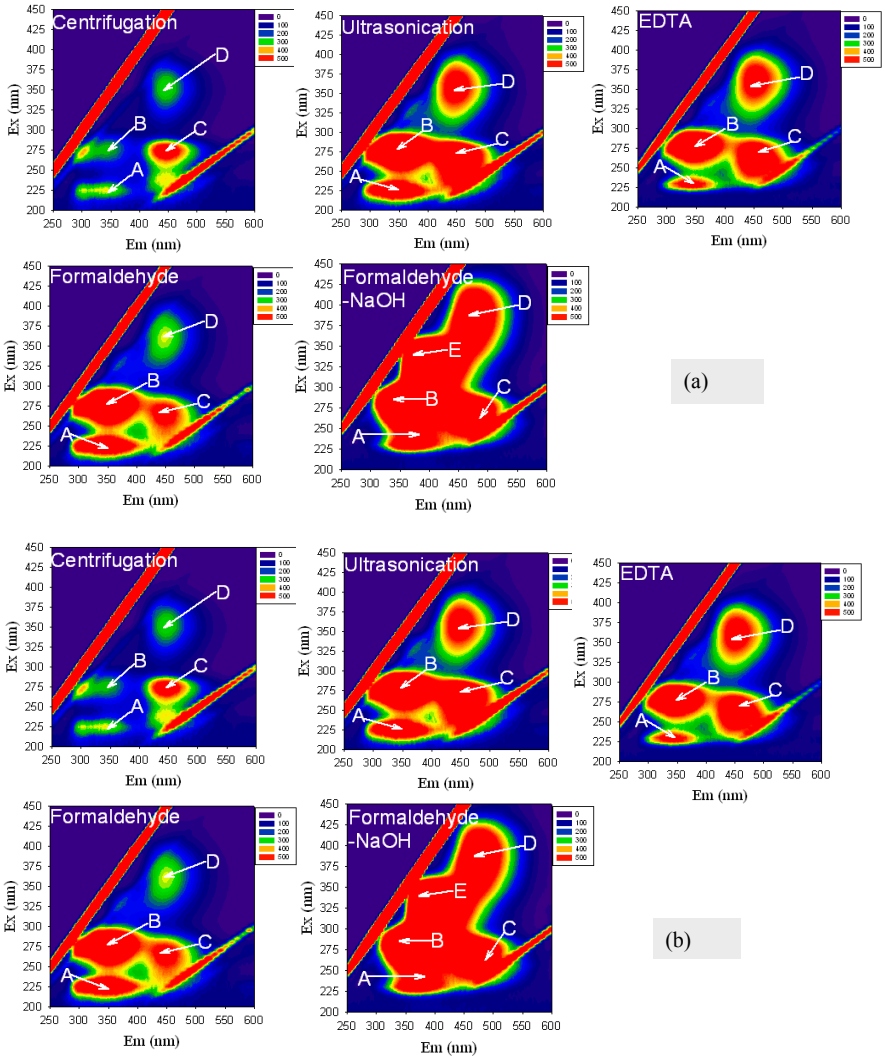

Figure 2
The EEM fluorescence spectra of EPS extracted by using different methods (control, ultrasonication, EDTA, formaldehyde and formaldehyde plus NaOH), (a):before dialysis (b): after dialysis

Table 2
Location of and fluorescence intensity at peaks 3DEEM of EPS samples prepared with different methods

Extraction method		Peak A	Peak B	Peak C	Peak D	Peak E
		Ex/Em intensity	Ex/Em intensity	Ex/Em intensity	Ex/Em intensity	Ex/Em intensity
Centrifugation	BD	225/346 324.7	275/352 438.7	270/448 921.6	360/446 440.5	--
	AD	225/342 277.1	275/338 219.1	275/448 669.7	350/446 282.0	--
Ultrasonication	BD	225/350 906.4	280/350 1552.0	265/450 1158.0	360/448 660.4	--
	AD	225/336 1063.0	280/340 1090.0	275/446 962.2	350/446 414.1	--
EDTA	BD	230/340 570.3	280/346 192.0	255/452 967.9	360/456 644.5	--
	AD	230/338 829.7	280/334 1090.0	275/446 606.3	350/446 282.0	--
Formaldehyde	BD	225/350 909.6	275/348 1242.0	265/442 628.0	360/448 326.2	--
	AD	225/338 702.7	275/340 737.0	275/442 513.4	350/449 284.6	--
Formaldehyde -NaOH	BD	230/372 775.4	285/368 4381.0	255/464 1195.0	385/470 929.5	330/390 3067
	AD	230/346 1897.0	280/352 5432.0	255/462 1675	370/464 890.6	325/390 1840

BD - before dialysis; AD - after dialysis; -- - undetected

varying sensitivity to different species of amino acid (Compton and Jones, 1985) and the Bradford assay usually gives lower protein values (Berges et al., 1993), low protein content in the EPS sample could not be detected by the Bradford method. However, 3DEEM fluorescence was found to be a more sensitive method in detecting low content of protein or protein-like substances, which might not be detected by the Bradford method. The weak fluorescence at Peak D for EPS samples prepared with centrifugation alone or formaldehyde alone indicated that a lower proportion of humic-like substances was extracted by these 2 methods compared with the other 3 methods.

Fluorescence intensity was strongest for the EPS sample extracted with formaldehyde-NaOH, indicating that more

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fluorescent components were extracted by this method. The location and shape of peaks for alkali-extracted EPS also differed greatly from the other samples, which might be the result of the high pH during extraction in the presence of NaOH.

Dialysis was also an important procedure affecting the characteristics of fluorescence spectra of EPS. In most cases, fluorescence intensity at each peak was significantly reduced in the EPS sample dialysed with a 3 500 Dalton molecular cutoff dialysis membrane (Table 2). For instance, fluorescence intensity at Peak D in the EPS sample prepared with EDTA decreased by 56% after dialysis, indicating that a considerable amount of humic acid-like substances with molecular weight of less than 3 500 Dalton was removed by dialysis. It was also found that dialysis resulted in a shift of the location of some peaks. Both changes in fluorescence intensity and location indicated that the components with molecular weight of less than 3 500 Dalton contained substantive amounts of fluorophores which were lost after dialysis. In some cases, fluorescence intensities were enhanced after dialysis. For example, fluorescence intensities at Peaks A, B and C for NaOH-formaldehyde increased by 144.65%, 23.98% and 40.17%, respectively, after dialysis. This might be explained as follows. Fluorescence of these peaks was quenched due to interaction of the fluorophores with chemicals (e.g., Na^+ or EDTA) during extraction. Some chemicals bound to EPS were released to water again during dialysis and thus fluorescence intensities of these peaks recovered. Fluorescence of Peak A for EPS extracted with ultrasonication after dialysis also increased a little. This might be explained by the fact that sonolysis led to water dissociation or degradation of some components of EPS and some ions such as H^+ were produced. These ions might be binding to some fluorophores in EPS during extraction and may be released again during dialysis. Our study suggests that dialysis should be taken into account fully during the preparation of EPS samples.

Conclusions

The yield of EPS from biofilm varied greatly with extraction method. Pre-treatment with ultrasound of gentle intensity doubled the extraction yield without significant modification of the composition of EPS. Compared with the physical methods, chemical methods significantly increased the extraction yield. However, contamination of the added extractants (e.g. EDTA), interaction of extractants with EPS, and possible release of intracellular components due to the extractant could affect the composition of the EPS considerably. Therefore, appropriate physical methods (for example, ultrasonication at low intensity) for enhancing EPS yields are recommended in research of EPS, and great caution must be taken when chemical methods are used for EPS extraction.

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