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Component Analysis of Microbial Extracellular Polymeric Substances Influencing Fluid Mud Anti-settlement Properties by Different Extraction Methods

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New findings on the application of microbial extracellular polymeric substances (EPS) in minimizing harbor siltation have been reported recently. However, the key components of EPS influencing fluid mud anti-settlement properties have not been well elucidated. The relationships between EPS contents and anti-settleability of mud were thereby investigated. Results showed that, after cultivation with nutrients, the settling velocity of mud increased with the increase of crude EPS contents, indicating anti-settleability of mud was positively correlated to EPS content. The yield and composition of EPS were analyzed by extraction with five different methods. It was shown that polysaccharide and protein were the main components of EPS with verification by Fourier transformed infrared spectroscopy, and polysaccharide was the dominant composition in bound EPS. Thus, it was speculated that polysaccharide in EPS was the key component influencing the anti-settlement properties of mud. Moreover, ultrasound process was the optimum protocol for extracting EPS from mud.

Key words: Extracellular polymeric substances (EPS); Component analysis; Fluid mud; Anti-settlement properties.

Extracellular polymeric substances (EPS), a complex high-molecular-weight mixture of polymers, are secreted by microorganisms during their growth to defend the severe environment, and have been found widely present in pure cultures, activated sludge, granular sludge, and biofilms[1,2]. EPS have been reported with a significant influence on the physicochemical properties of microbial aggregates e.g. structure, surface charge, flocculation, and adsorption ability[2-5], and extensively investigated in waste water treatment. Recently, there are new findings in the application of EPS in minimizing harbor siltation[6-8].

Within the last decades, siltation problems in navigations channels, ports and impoundments have been complex and severe, while the current strategy of dredging practices not only was very expensive but also could not solve these completely. Nautical Depth technology, i.e. keeping sediment navigable to create and perpetuate navigability, is one of the most effective measurements to solve the problem of fluid mud in the muddy harbor basin and channel[7-11]. But with the gradual consolidation, the mud would move as a viscous fluid named fluid mud, which in turn caused the decrease of available depth. Thus, a strategy by manual intervention the consolidation

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process of fluid mud in order to keep fluid mud navigable has been proposed[7,8]. During this, the microbial extracellular polymer substance (EPS) has been reported to play an important role in altering the characteristics of mud, reducing the settling velocity of suspended matter and finally delaying settling over periods up to several months.

Through the microscopic investigation of suspended particles from rivers, harbors and the marine environment, some aquatic bacteria were found attached to solid surfaces. The EPS produced by those settled bacteria were able to carry great volume of water and might function as glue to form a polymeric particle with microbial cells and solids, which therefore reduced the density and settling velocity of suspended matter[6]. Though the composition of these biopolymers seems to be variable and extremely dependent on the bacterial species involved and the existing environmental conditions, the main constituents were polysaccharides, proteins and nucleic acids. However, the key components of EPS in fluid mud are not well recognized, and the mechanism for the interaction between EPS and mud remains unclear. Most EPS characterization studies rely on the extraction of EPS components from samples and subsequent analysis of the recovered extracts[2]. The amounts and compositions of extracted EPS vary widely as a function of mud origin and also extraction conditions. Methods to extract EPS are crucial for studying its physicochemical properties[12]. Various physical and chemical methods have been reported to extract EPS from different sources of activated sludge. A review of the literature showed that physical extraction methods generally included centrifugation, heating, ultrasound and cation exchange resin. Meanwhile, chemical methods consisted of ethylenediamine tetraacetic acid (EDTA), formaldehyde, NaOH and formamide-NaOH[2,13-16]. However, the systematic study on comparison of EPS extraction methods from fluid mud was greatly lacking and no reports on the influence of extraction process on the composition and properties of EPS were available till now.

In this study, the fluid mud sampled from the Port of Lianyungang, China, was cultivated with nutrients to investigate the relationship between EPS contents and anti-settleability of mud. The yield and composition of EPS of cultivated mud were analyzed by extraction with five different methods including two chemical and three physical protocols. The key component of EPS influencing fluid mud anti-settlement properties was discussed in this study. Moreover, the efficacy of different protocols for extracting EPS from mud was also evaluated.

MATERIALS AND METHODS

Fluid mud samples and cultivation

The mud used in this study was sampled from the Port of Lianyungang, China. The mud was adjusted to concentration at 1000g/L and cultivated with the nutrient medium containing glucose (25g/L), $MgSO_4.7H_2O$ (0.2 g/L), $Na_2HPO_4.12H_2O$ (2g/L), KH_2PO_4 (1g/L), NH_4Cl (1g/L) in 50 L glass tank. The nutrients were supplied twice during cultivation. Mud samples were collected for subsequent analysis of settling velocity (SV) and EPS contents. The SV was determined by calculating the percentage of the height of the settled bed in the 1 L glass-column after 30 min settling[17]. The content of crude EPS was determined by weighing the freeze-dried residue from alcohol precipitation of supernatant of mud separated by centrifugation.

Extraction of EPS by different methods

EPS of the fluid mud samples were extracted according to five conditions illustrated in Fig. 1. The detailed procedures for each extraction process are summarized in Fig.1.

Two extraction methods based on the use of chemical reagents[13]: 2% EDTA for 3h at 4°C, 36.5% formaldehyde for 1 h at 4°C then 1M NaOH for 3h at 4°C.

Three extraction methods were based on physical processes: centrifugation at 12000 rpm for 20 min at 4 °C, sonication at 60 W for 2.5 min using Scientz II D ultrasonic crusher (Ningbo Scientz Biotechnology Co., Ltd), heating at 70°C for 1h [13,14].

Finally, all extracted EPS were purified in a dialysis membrane placed in 1500 ml of ultra-pure water for 24 h at 4 °C and freeze-dried for subsequent analysis. After lyophilization, the total quantity of extracted EPS was measured by the weight of solids to compare the extraction efficiency of different methods.

Chemical analysis of extracted EPS

The yield of EPS was measured by weighing the dry weight. The polysaccharide contents in EPS were measured by the phenol-sulfuric acid method with glucose as the standard[18]. The contents of protein in EPS were determined by Bradford method[19] using bovine serum albumin as standard. Besides, the nucleic acids contents in EPS were analyzed by NanoPhotometer (Implen Corp, Germany).

Fourier transformed infrared (FT-IR) spectroscopy

The IR spectra were characterized using a Tensor 27 Fourier transform infrared spectrometer (Bruker Corporation, Germany). Freeze-dried EPS powder was mixed with KBr, compacted to form pellets and then scanned at wavenumbers from 4000 to 400 cm^{-1}.

RESULTS AND DISCUSSION

The variations of SV and EPS contents of mud during cultivation

In this study, the fluid mud was sampled from Port of Lianyungang in China and cultivated with nutrients to increase the EPS contents, and the changes of SV and EPS content during cultivation were monitored to determine the relationship between EPS contents and anti-settlement properties of mud. The settling velocity is used to characterize the anti-settleability of mud, with a high SV value denoting good anti-settleability. The variations of these indexes during cultivation were shown in Fig. 2.

Before cultivation, the SV of mud was 52.3%; after cultivation, the SV value increased to 74.3% on days 13 and continued to increase to 80.4% on day 26. For crude EPS contents, before cultivation, no EPS was detected in the mud. During cultivation, the EPS contents increased with time; the EPS amounts was 1.78 g/L on day 13 and 4.42 g/L on day 26. Thus, a positive correlation between EPS contents and SV value was observed, i.e. the settlement of mud delayed with the increase of EPS contents, and therefore it could be inferred that EPS was a key factor influencing the anti-settlement properties of fluid mud.

The yields of cultivated mud EPS extracted by different methods

To analyze the key component of EPS responsible for the anti-settlement properties of fluid mud, the characterization of mud EPS before and after cultivation was necessary, which mainly relies on the extraction of EPS components from samples and subsequent analysis of the recovered extracts[2]. In this study, as no EPS was detected in mud before cultivation, thus the mud EPS after 13 days of cultivation as a typical state was characterized.

EPS is classified in 2 categories: bound EPS, bound to the external cell surface and gluing clusters to form micro-colonies and flocs; and soluble EPS, dissolved in the bulk liquid and involved in surface conditioning. Compared with soluble EPS, bound EPS is more difficult to extract, thus the amounts and compositions of extracted EPS vary as extraction conditions. Therefore five protocols for EPS extraction were compared in this study.

Table 1. Main functional groups observed from IR spectra of EPS extracted by different methods

Wave number (cm^{-1})	Vibration type	Functional type
Wave number (cm^{-1})	Vibration type	Functional type
3200–3400	Stretching vibration of OH	Polymeric compounds
2987	Symmetric stretching of CH_2	
2936	Asymmetric stretching of CH_2	
1720–1730	Stretching vibration of C=O	Carboxylic acids
1555-1600	Antisymmetric stretching vibration of COO^-	Proteins
1384	Deformation vibrations of CH	
1310-1240	Amide III	Protein
1061-1124	Stretching vibrations of lipids of C-C	Lipids
	Stretching vibrations of C-N	Protein
1040-1080	Stretching vibration of OH, “fingerprint” zone	Polysaccharides

Results for EPS extraction yields of five methods were summarized in Fig. 3. The least amount of EPS was obtained by centrifugation with the yield of 1.98 mg/ml mud, which was due to that only soluble EPS could be extracted by this method. The protocols of ultrasound, heat and formaldehyde-NaOH increased the amounts of extracted EPS, compared with centrifugation, with the yields of 2.86, 2.46, 2.64 mg/ml mud respectively, indicating that some bound EPS had been extracted besides soluble fraction. The method of EDTA obtained most EPS with 8.67 mg/ml mud, which was consistent with previous studies[14]. It might be explained by the complexes formed between EDTA and EPS[13,14]. However, this protocol was not the best method since EDTA would also complex some other non-EPS components like metal ions and reduce the purity of extract (the deduction can be further verified by Fig. 4). Thus the ultrasound method was most suitable for extraction of EPS considering the extraction efficiency, and it could be further estimated that the bound and soluble fractions accounted for 30% and 70% of total EPS respectively.

The components of cultivated mud EPS extracted by different methods

The components of EPS extracted by five different methods were subsequently analyzed, the result of which was shown in Fig. 4. EPS has been reported to be 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[20,21]. In this

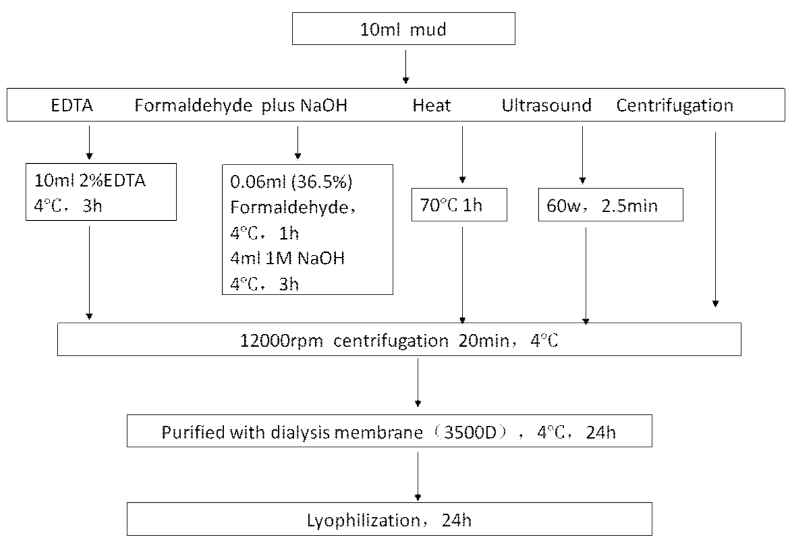
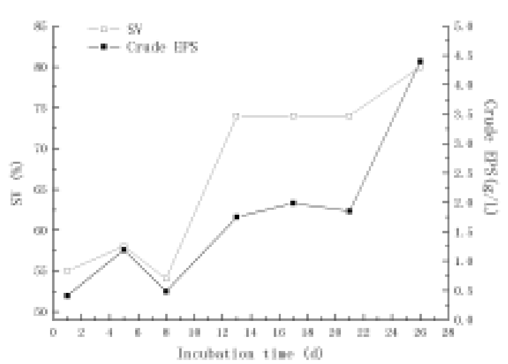


Fig. 1. Flow chart of five EPS extraction processes



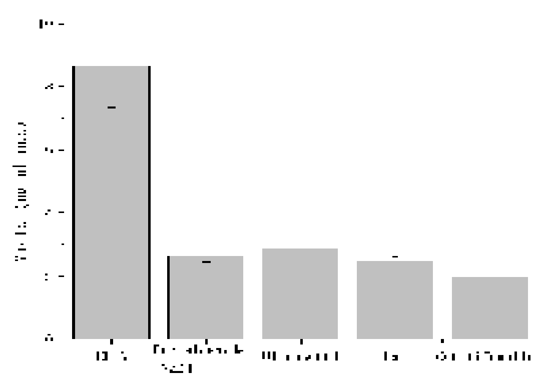


Fig. 2. The variations of settling velocity (SV) and EPS contents of mud during cultivation with nutrients

Fig. 3. The yields of mud EPS after 13 days of cultivation with nutrients from different extraction methods

study, the components of polysaccharide, protein, and nucleic acid were mainly analyzed. For the EPS extracted by centrifugation, polysaccharide, protein, and nucleic acids accounted for 14.8%, 9.1%, and 5.1% respectively. For the protocols of ultrasound and heat, the proportions of polysaccharide increased to 43.2% and 34.3% respectively but the ratios of protein both decreased to 4%, which suggested that the proportion of polysaccharide in bound EPS was much higher than that of protein compared with soluble fraction. The formaldehyde-NaOH method could extract considerable amount of EPS, but the extraction efficiencies on polysaccharide and protein was lower, which was not a suitable protocol for EPS analysis. As for EDTA method, the significantly lower contents of polysaccharide and protein than other four methods combined with the highest yield of EPS indicated that lots of contaminants existing in the extracted EPS. This result also verified the previous deduction on the high yields of EPS from EDTA. The nucleic acids content was detected as the determination of DNA contamination in EPS. Results showed that the ratio of nucleic acids in EPS ranged from 2.5% to 5.8%, in accordance with other studies[14,21]. According to the previous results, the ultrasound process yielded highest amount of EPS and showed highest efficiency on polysaccharide and proteins, which therefore was the optimum protocol for extracting EPS from mud.

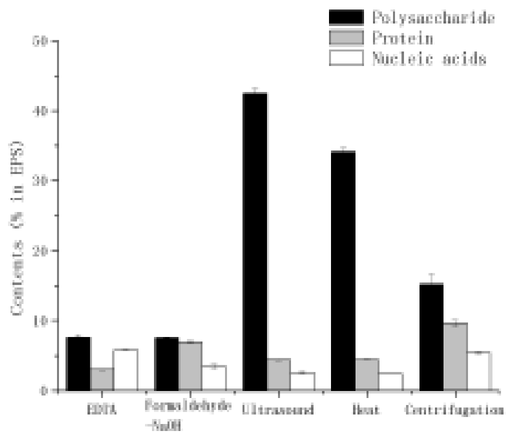


Fig. 4. The compositions of mud EPS after 13 days of cultivation with nutrients from different extraction methods

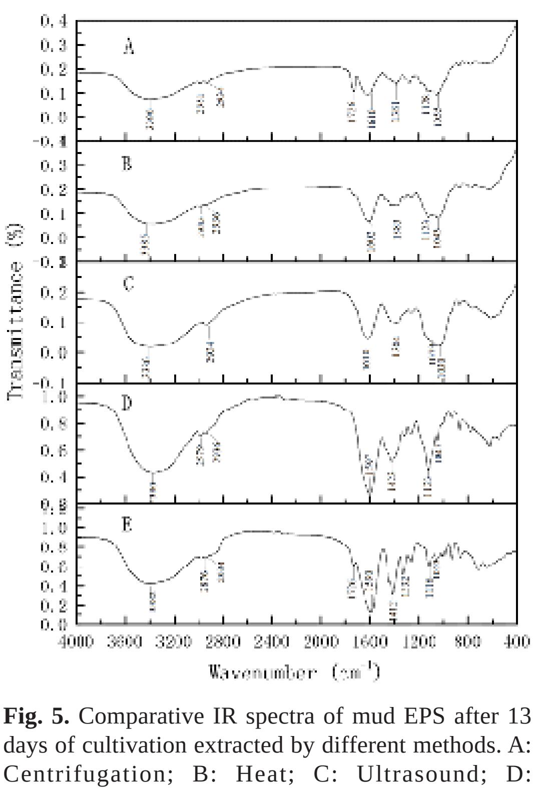


Fig. 5. Comparative IR spectra of mud EPS after 13 days of cultivation extracted by different methods. A: Centrifugation; B: Heat; C: Ultrasound; D: Formaldehyde-NaOH; E: EDTA

From the above results, the following could be summarized. After cultivation, the ratio of polysaccharide in EPS was higher than that of protein, especially in bound EPS; the bound EPS was mainly composed of polysaccharide. Based on the observation that the SV value of mud decreased with the increase of EPS content, it could be speculated that polysaccharide in EPS other than protein was the key component influencing the settlement of mud. And the increase of polysaccharide in EPS had a positive effect on the anti-settlement properties of fluid mud.

Recently, EPS has been proved to be responsible for bacterial adhesion onto solid surface[20]. As the increase of EPS contents, the polymeric interaction due to the EPS covering on the cell surface will overcome electrostatic barriers and promote cell adhesion onto suspended particle, which therefore promotes the formation of polymeric particles between microbial cells and solids, and thereby reduces the density and settling velocity of suspended matter. As the components of EPS are very complex, the specific role of each component in the above process remains unclear. However, according to the results in this study, it could be inferred that polysaccharide played a more important role during the above mentioned process than protein.

FT-IR analysis of cultivated mud EPS extracted by different methods

The EPS of cultivated mud with nutrients extracted by five different methods was characterized by FT-IR, the results of which were presented in Fig. 5. Though the IR spectra obtained from the different extracting methods had various shapes, EPS extracted with physical methods displayed similar IR spectra whereas EPS extracted using chemical methods displayed different IR spectra, due to the presence of chemicals used to extract the EPS from the mud, as also found in other studies with different sources of sludge[14,22].

For EPS extracted by physical methods like centrifugation, ultrasonication, and heat, the same characteristic bands can be observed on every EPS IR spectra except the peak around 1725 cm^{-1} attributed to carboxylic acids, which only existed in the EPS spectra from centrifugation method. While the IR spectra of chemical methods including EDTA and Formaldehyde-NaOH, showed some particular bands compared to the physical methods. The IR spectra obtained from EDTA and Formaldehyde-NaOH methods showed bands around 1123, 1420 cm^{-1}, which did not appear for the EPS extracted by the physical methods. However, the band around 1055 cm^{-1} presented in the EPS extracted by physical methods, disappeared in the spectra of EPS from chemical methods. Those changes in the IR spectra could be interpreted as the result of a formaldehyde and EPS reaction and contamination of EPS by the chemical reagent EDTA.

The predominant peaks of IR spectra were summarized in Table 1. According to Table 1, the main characteristic bands were attributed to, for example, hydroxyl groups of polysaccharides, amide groups of proteins, indicating polysaccharide and protein as main components of EPS, which were in accordance with the EPS composition observed for these treatments.

CONCLUSIONS

The relationship between EPS contents and anti-settleability of mud was investigated by cultivating mud with nutrients. Results showed that anti-settleability of mud were positively correlated to EPS contents. Through the analysis on the yield and composition of EPS by five extraction methods, it was shown that polysaccharide and protein were the main components of EPS, and polysaccharide was found to be the dominant composition in bound EPS. Thus, it could be speculated that polysaccharide in EPS other than protein was the key component influencing the anti-settlement properties of mud. Moreover, ultrasound process was the optimum protocol for extracting EPS from mud.

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