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Extraction and characterization of stratified extracellular polymeric substances in *Geobacter* biofilms

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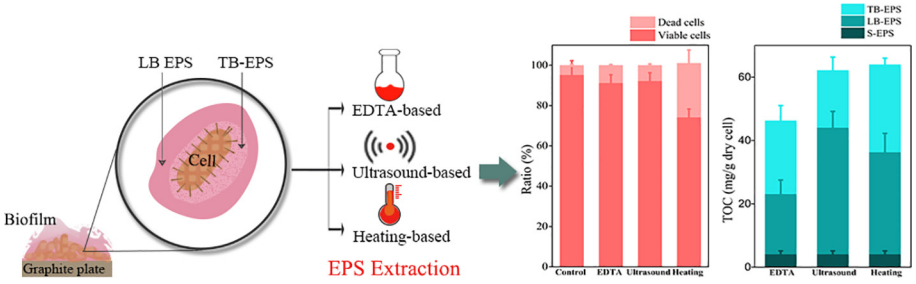
GRAPHICAL ABSTRACT



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ABSTRACT

Extracellular polymeric substances (EPS) play crucial roles in promoting biofilm formation and contribute to electrochemical activities of biofilms in bioelectrochemical systems (BES). In this study, three stratified EPS fractions were extracted from *Geobacter* biofilms using EDTA-, ultrasound- and heating-based protocols and characterized with chemical, spectral and electrochemical analyses. Results suggested that, for *Geobacter* biofilms, ultrasound-based extraction protocol was more effective in EPS yield (62.1–66.5 mg C/g dry cell) than EDTA method, and had less cell lysis than heating method. The extraction methods greatly affected the proteins composition in the extracted EPS, indicated by the varied ratios of tryptophan/tyrosine protein-like substances. Electrochemical measurements demonstrated a good correlation between protein concentration and extracellular electron transfer function for both tightly-bound EPS and total EPS. This is the first study to extract and characterize stratified EPS fractions from *Geobacter* biofilms, and helpful for better understanding the function of EPS in BESs predominated by *Geobacter*.

1. Introduction

Extracellular polymeric substances (EPS) are fundamental microbial components that govern the physiochemical properties of biofilm (Zhang et al., 2015). Almost every microbial cell in biofilms is surrounded by EPS, which assist biofilm formation, mass transfer and microbial protection from unfavorable environments (Lin et al., 2014). In biofilms, EPS is generally considered as a network of organic components which tightly attached to the cell surface as peripheral capsules (tightly bound EPS, TB-EPS), indirectly attached to the cell surface (loosely bound EPS, LB-EPS), or shed into the surrounding environment as slimes (slime EPS, S-EPS) (Lin et al., 2014; Jia et al., 2017; Teng et al., 2019). Although the stratified EPS fractions (S-EPS, LB-EPS and TB-EPS) are classified based on their degree of binding with cell surface, more practically, these EPS fractions are classified by the force strength required for separating EPS from the microbial cells (Hong

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et al., 2017).

To extract EPS fractions, multiple EPS extraction protocols, including the use of pH adjustment, formaldehyde, ethylenediaminetetraacetic acid (EDTA), ultrasound and heating, have been reported (Zhang et al., 2016; Keithley and Kirisits, 2018). During EPS extraction, the biofilm matrix has to be destabilized by chemical or physical methods, and the EPS extraction protocols should not be too harsh to avoid the significant cell lysis, since lysis would release intracellular polysaccharides and proteins to cause the contamination of EPS pool (Keithley and Kirisits, 2018). In addition, the extracting method employed greatly affects the EPS yield and composition, and there is no a universal extraction method for all possible EPS, especially the functional EPS components, in various biofilms dominated by different microorganisms (Pronk et al., 2017). For example, the quantity of EPS obtained can fluctuate from 165 mg (EDTA-based method) to 26 mg (centrifugation alone) of EPS per gram of sludge (Liu and Fang, 2002)); and acetic acid instead of the usually required alkaline extraction was more suitable to obtain a new acid soluble EPS from granules dominated by *Defluviicoccus* sp. (Pronk et al., 2017). Therefore, to effectively extract EPS from the specific biofilm, the EPS extraction methods should be carefully chosen based on its functional EPS components (Nouha et al., 2016).

In bioelectrochemical systems (BESs), electrochemically active microorganisms (EAMs) are capable of donating electrons to or accepting electrons from electrode surfaces (Hirose et al., 2018). Electroactive biofilms (EABs) generated by EAMs are important to many attractive environmental applications of BESs, such as bioelectricity, wastewater treatment and chemicals production (Kouzuma et al., 2018; Sevda et al., 2018). *Shewanella* and *Geobacter* are the most extensively studied EAMs due to their high extracellular electron transfer (EET) efficiency, annotated genome sequence, ease of cultivation and genetic manipulation, and EET capability without the need for exogenous mediators (Malvankar et al., 2012; Kumar et al., 2017).

Previous studies have demonstrated that EPS from EABs are redox-active or (semi) conductive, and are able to immobilize and reduce heavy metals, and synthesize metal nanoparticles (Cologgi et al., 2014; Xiao et al., 2017). The abundant extracellular redox-active proteins, such as *c*-type cytochromes (*c*-Cyts), are the primary functional EPS components of EABs. They confer redox-active properties to the EAB-sourced EPS and mediate the EET between EAMs with electrodes or other microorganisms, and thereby influence the performance of BESs (Steidl et al., 2016; Liu et al., 2018). To date, protocols based on EDTA, heating, and pH adjustment have been used to extract EPS from *Shewanella*, and the EDTA and heating methods were demonstrated to be the most suitable methods (Cao et al., 2011; Dai et al., 2016). The establishment of EPS extraction method enhanced the study of the characterization and function of EPS of *Shewanella* biofilms (Xiao et al., 2017).

Although *Geobacter* biofilms are predominant in many BESs, there is no study to date reported EPS extraction protocols for *Geobacter* biofilms. Only few genetic studies reported the genes encoding some extracellular *c*-Cyts and the biofilm exopolysaccharides that anchors extracellular *c*-Cyts in *Geobacter sulfurreducens*, the model representative of the genus *Geobacter* (Rollefson et al., 2011; Steidl et al., 2016). The study of elucidating the composition of EPS in *Geobacter* biofilms and the link between EPS components and function is important to explain the fundamental ecology and performance of *Geobacter*-dominated BESs. Therefore, the current study was aimed to explore the suitable EPS extraction protocols for *Geobacter* biofilms attached on electrode, measure the EPS components in *Geobacter* biofilms, and reveal the correlation between EPS composition and their EET function.

2. Materials and methods

2.1. Bacterial strains and biofilm formation

Geobacter sulfurreducens PCA and *Geobacter soli* GSS01were selected as representative species of the genus *Geobacter*. *Geobacter sulfurreducens* PCA (DSM 12127) was obtained from the German Collection of Microorganisms and Cell Cultures. *Geobacter soli* GSS01 was previously isolated in our laboratory, and have been deposited in two publicly accessible culture collection with accession number of KCTC 4545 and MCCC 1K00269 (Zhou et al., 2014). Both strains were cultured under anaerobic conditions (N_2:CO_2, 80:20) in freshwater medium containing acetate (16 mM) as the electron donor, and Fe(III)-citrate (56 mM; for GSS01) or fumarate (40 mM; for PCA) as the electron acceptor.

Single-chamber BESs with a liquid volume of 250 ml were set up. Graphite plates were used as the working and counter electrodes, and a saturated calomel reference electrode (SCE) was used as the reference electrode. Electrolyte was freshwater medium containing 10 mM acetate as electron donor. All reactors and electrolyte were autoclaved at 121 °C for 20 min and bubbled with N_2:CO_2 (80:20) for 30 min to maintain anoxic conditions, and then the log-phase cultures of strain PCA or GSS01 were inoculated (10%) into the chamber. The reactors were connected to a workstation (CHI1000C, Chenhua, China) with the working electrode potentials poised at 0 V. Current production was collected by CHI1000C every 40 s. All potentials in this study were determined relative to the SCE electrode. All BESs were operated in fed-batch mode at 30 °C.

2.2. EPS extraction protocols

The electrolyte in the working chamber was sampled, centrifuged (7500 rpm, 15 min), filtered through 0.22 μm membrane filter, and defined as S-EPS. The biofilm on the electrode was peeled and collected for the extraction of LB-EPS and TB-EPS using three different extraction protocols (EDTA, ultrasound and heating).

The EDTA treatment for EPS extraction and preparation of both LB-EPS and TB-EPS fractions were carried out by following a previously reported protocol (Cao et al., 2011; Castro et al., 2014; Praveen and Loh, 2016). The cells were suspended in 5 ml of 0.9% NaCl solution, centrifuged at 5000*g* for 15 min. The supernatant was collected and the precipitate was further washed two times with 1 ml of 0.9% NaCl solution. All supernatants were combined, filtered through 0.22 μm membrane filter, and defined as the LB-EPS. To harvest the TB-EPS, the precipitated cells were re-suspended in 2 ml of 0.9% NaCl solution, mixed with an equal volume of 2% Na_2-EDTA (53.7 mM), and shaken horizontally at 150 rpm (4 °C) for 4 h. The cells were pelleted by centrifugation with 5000*g* for 20 min and washed two times using 1 ml of 0.9% NaCl solution. The supernatant was collected after each centrifugation, combined together, filtered through 0.22 μm membrane filter, and regarded as the TB-EPS.

EPS extraction using heating was performed using a modified protocol (Li and Yang, 2007; Han et al., 2018; Du et al., 2019). The bacterial pellets were re-suspended in 4 ml of 0.9% NaCl solution, and the suspension was further diluted with 3 ml of 0.9% NaCl solution that was pre-heated to 70 °C. This provided that the suspension reached an immediate warm temperature of 35 °C. The suspension was then sheared by a vortex mixer for 2 min (Rollefson et al., 2011), followed by centrifugation at 5000*g* for 15 min. The supernatant was filtered through 0.22 μm membrane filter, and the organic matter in the supernatant was regarded as the LB-EPS of the biomass. For the extraction of the TB-EPS, the bacterial pellet left in the centrifuge tube was re-suspended in 0.9% NaCl solution to a volume of 4 ml. The suspension was then heated in water bathes for 30 min at 40 °C (Xiao et al., 2017), centrifuged at 5000 g for 20 min and washed two times using 1 ml of 0.9% NaCl solution. The supernatant was collected, filtered through 0.22 μm membrane filter, and was regarded as the TB-EPS.

An ultrasound extraction method was modified from the method extracting different EPS fractions for activated sludge and bacterial cells (Yu et al., 2008; Zhang et al., 2015, 2016). Briefly, the bacteria pellets were re-suspended in 7 ml of 0.9% NaCl solution and then the tubes were sonicated at 20 kHz for 2 min, shaken horizontally at 150 rpm for 10 min and sonicated again for additional 2 min. The suspension was centrifuged at 5000*g* for 15 min to separate bacterial pellet and supernatant. The collected supernatant was filtered through 0.22 μm membrane filter and regarded as the LB-EPS. The organic compounds in the supernatant were regarded as the LB-EPS. After that, the residues left in the tube were re-suspended in 0.9% NaCl solution again to a volume of 4 ml, sonicated for 10 min, centrifuged at 5000*g* for 20 min and washed two times using 1 ml of 0.9% NaCl solution. All supernatant was collected, filtered through 0.2 μm membrane filter and regarded as the TB-EPS.

2.3. Cell lysis assessment

The extent of cell lysis caused by the EPS extraction processes was assessed. The morphology of the bacterial cells was observed using a Zeiss Ultra 5 field-emission SEM (FE-SEM). Flow cytometry and fluorescence microscopy were applied to evaluate the cell viability affected by the EPS extraction protocol (Xiao et al., 2017). For flow cytometry, cells were fluorescence-stained with propidium iodide, and about 10,000 cells were measured by BD-FACSCanto flow cytometer. For fluorescence microscopy, cells were stained using a LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen, CA), and examined by inverted fluorescence microscope (IX53, Olympus).

2.4. EPS composition measurements and electrochemical activity analysis

EPS was quantified by measuring total organic carbon (TOC) using a TOC analyzer (TOC-l, Shimadzu). The bulk concentration of polysaccharide in EPS extractant was measured using a modified phenol-sulfuric acid colorimetric method with glucose as the standard (Masuko et al., 2005). Proteins were measured using the Pierce bicinchoninic acid (BCA) kit (Thermo Scientific Pierce) with bovine serum albumin (BSA) as the standard. Nucleic acids were determined using a Qubit 3.0 fluorimeter (Thermo Fischer Scientific) and the associated kit. The concentrations of TOC, polysaccharide, protein and nucleic acid were normalized to the biomass of biofilms as mg/g dry cells.

Three-dimensional excitation-emission matrix (3D-EEM) spectra of EPS were obtained using a fluorescence spectrophotometer (F-7000, Hitachi). The EEM spectra were collected with subsequent scanning emission spectra from 200 to 600 nm by varying the excitation wavelength from 200 to 450 nm. The PARAFAC was used to model the EEM fluorescence data, which was conducted in MATLAB R2013a (MathWorks, Natick, MA). And the scores of PARAFAC components were applied to indicate the relative concentration of fluorophore (Ye et al., 2018).

The electrochemical activity (i.e., the function of EET) of EPS was characterized by electron transfer capacity (Q_{ETC}) and cyclic voltammetry (CV). The CV was conducted with a CHI660D workstation (Chenhua, China) at a scan rate of 5 mV/s. The Q_{ETC} includes electron accepting capacity (Q_{EAC}) and electron donating capacity (Q_{EDC}), both of which can be determined through chronoamperometry measurement (Yu et al., 2015).

2.5. Statistical analysis

All tests were conducted in triplicates, and data are presented as the mean ± SD. Comparison of the measurement data were performed with Student's *t*-test. The statistical analysis was done using SPSS software (version 16.0). A P value < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Cell lysis assessment

Two stable electrical current generation cycles were completed with ultimate current densities of 1.9 A/m^2 for *G. sulfurreducens* and 2.1 A/m^2 for *G. soli*, which indicated that the typically electrochemical active and mature *G. sulfurreducens* and *G. soli* biofilms were successfully developed on the anode surface. Then, the biofilms were peeled and collected for EPS extraction using three different protocols. To ensure the rationality of the downstream analysis and data interpretation that might be interfered by cell lysis during EPS extraction processes (Keithley and Kirisits, 2018), here we first conducted cell lysis assessment.

FE-SEM was used to observe the bacterial cell EPS and to visually evaluate the efficacy of EPS removal (Ishii et al., 2004; Zhao et al., 2015). The intact *Geobacter* cell surfaces were rough due to the covering of EPS layers, and the cells were cross-linked by filamentous structures formed by EPS. It is hardly possible to observe similar morphology of bacterial cells after EPS extraction. After EPS removal using EDTA and ultrasound methods, no cracking was found on cell surfaces, indicating that these two methods led to slight cell lysis. However, certain residual EPS can still be observed on the cell surfaces after EPS extraction using EDTA method, indicating that EDTA method was not very efficient for EPS removal. By contrast, the cell surface was smooth and covered with very little EPS after extraction using ultrasound method. Although heating method with a temperature of 40 °C has been reported to be mild for EPS extraction from *Shewanella*, *Bacillus* and *Pichia* cells (Dai et al., 2016; Xiao et al., 2017), this temperature caused most severe cellular disruption for *Geobacter* cells. This result indicated that *Geobacter* was susceptible to temperature, and heating method might be not suitable for EPS extraction from *Geobacter* biofilms.

Cell viability is an important index for evaluating EPS extraction methods, since the bacterial cells, after EPS removal, are often used for further reactive studies. Before EPS extraction, the percentage of dead cells was 3.4 ± 0.1% for *G. sulfurreducens* and 4.7 ± 0.4% for *G. soli*. After the EDTA and ultrasound treatment, a proportion of 7.4–9.4% for *G. sulfurreducens* and 7.7–8.7% for *G. soli* were identified as dead cells, which was slightly higher than that of the intact cells. These results indicated that the EDTA and ultrasound methods produced little harm to *Geobacter* cells, and this was in accordance with the FE-SEM results. For the heating method, 18.4 ± 4.2% and 26.7 ± 6.6% of dead cells were detected for *G. sulfurreducens* and *G. soli*, respectively. These results, together with FE-SEM images, indicated that the heating method in this study (40 °C for 30 min) damaged cell membranes and was not suitable for EPS extraction from *Geobacter* cells.

3.2. EPS extraction yield and chemical quantification of EPS components

The highest TOC of EPS was extracted using heating method (63.9–71.5 mg C/g dry cell), followed by ultrasound (62.1–66.5 mg C/g dry cell) and EDTA method (46.2–59.5 mg C/g dry cell) (Fig. 1A). The polysaccharides and proteins were the major components in all EPS fractions (Fig. 1B–C). Among the three protocols, heating-based method yielded higher amounts of proteins (71.6 ± 10.9 and 65.9 ± 2.1 mg/g dry cell for *G. sulfurreducens* and *G. soli*, respectively) than EDTA (52.7 ± 3.6 and 54.3 ± 6.7 mg/g dry cell for *G. sulfurreducens* and *G. soli*, respectively) or ultrasound (65.1 ± 4.8 and 55.0 ± 4.3 mg/g dry cell for *G. sulfurreducens* and *G. soli*, respectively) methods. Meanwhile, more polysaccharides were yielded using ultrasound method (*G. sulfurreducens*: 71.4 ± 6.7 mg/g dry cell; *G. soli*: 65.8 ± 3.7 mg/g dry cell) than using heating (*G. sulfurreducens*: 63.2 ± 3.1 mg/g dry cell; *G. soli*: 56.2 ± 4.2 mg/g dry cell) or EDTA (*G. sulfurreducens*: 62.1 ± 4.3 mg/g dry cell; *G. soli*: 56.9 ± 5.8 mg/g dry cell) methods. This result was consistent with previous reports that, although EDTA-based method is widely suitable for various biofilms and activated

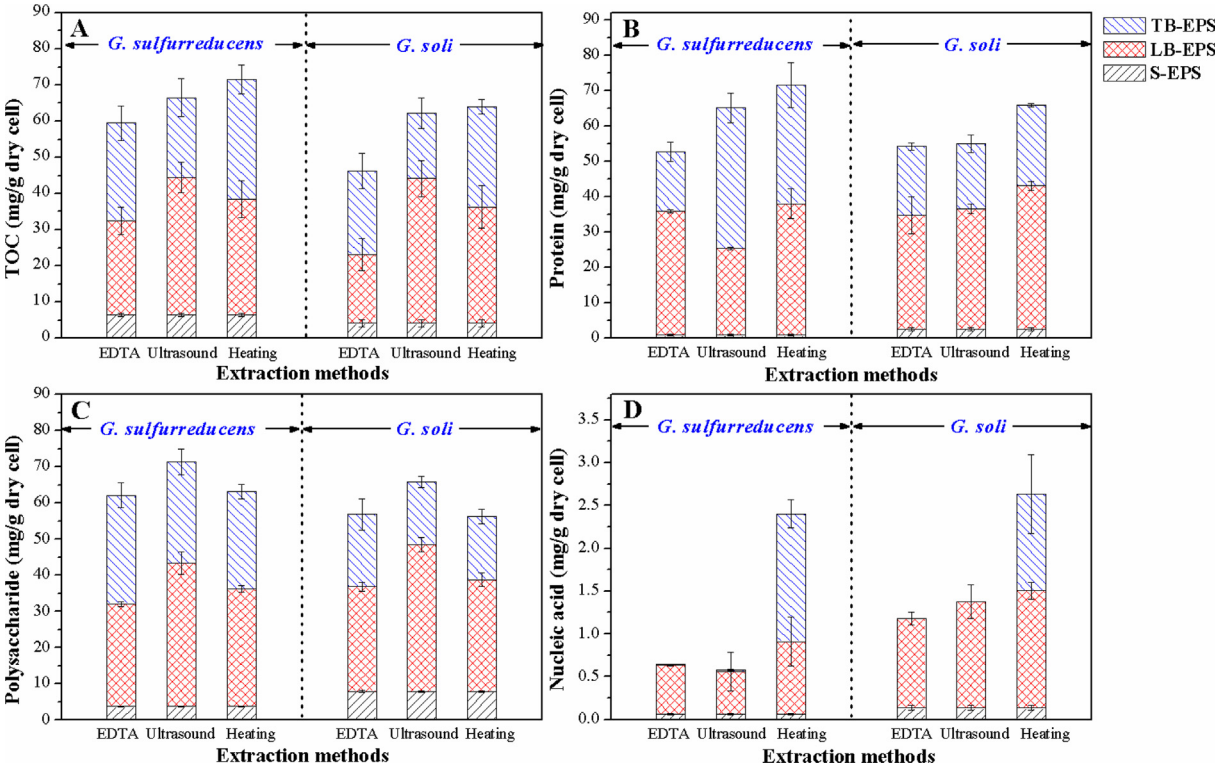

Fig. 1. The chemical characterization of EPS components extracted using different methods, TOC (A), protein (B), polysaccharide (C), and nucleic acid (D).

sludges, it yielded relatively less EPS (TOC, protein and polysaccharide) compared with other extraction methods (Cao et al., 2011; Dai et al., 2016).

The ratio of protein to polysaccharides (PN/PS) in total EPS extracted from *G. sulfurreducens* and *G. soli* biofilms using heating method was 1.2 and 1.3, respectively, which was higher than that of using EDTA (0.9 for *G. sulfurreducens*; 1.1 for *G. soli*) and ultrasound (1.0 for *G. sulfurreducens*; 0.9 for *G. soli*) methods. For EPS, the ratio of PN/PS is typically within the range of 0.2–5.0 for various microbial biofilms and planktonic cultures (Jiao et al., 2010; Cao et al., 2011). The high PN/PS ratios of EPS can be indicative of contamination by cell lysis, and also indicative of inefficient extraction of EPS (Gong et al., 2009). Thus, the higher ratios of PN/PS in total EPS extracted using heating method in this study suggested that the EPS samples yielded by heating method might be contaminated by lysed cell materials. In addition, heating method yielded significantly higher amounts of nucleic acids (2.4 ± 0.5 and 2.6 ± 0.6 mg/g dry cell for *G. sulfurreducens* and *G. soli*, respectively) than EDTA (0.6 ± 0.1 and 1.2 ± 0.1 mg/g dry cell for *G. sulfurreducens* and *G. soli*, respectively) or ultrasound (0.6 ± 0.2 and 1.4 ± 0.2 mg/g dry cell for *G. sulfurreducens* and *G. soli*, respectively) methods (Fig. 1D). Although extracellular nucleic acids (e-NA) has been reported as a EPS component of biofilms, a high level of e-NA is commonly considered as a remnant of lysed cells (Flemming et al., 2007; Sheng et al., 2010). The significantly higher concentrations of nucleic acids in EPS samples (especially TB-EPS) extracted using heating method supported the speculation that the EPS samples yielded by heating method might be contaminated by cell lysis.

For all protocols, the fraction of S-EPS (4.1–6.5 mg C/g dry cell) was minor, and the LB-EPS (19.1–40.3 mg C/g dry cell) and TB-EPS (18.5–33.4 mg C/g dry cell) were the major components of total EPS (Fig. 1A), which was consistent with the previous findings for other biofilms (Lin et al., 2014; Jia et al., 2017). In addition, in the EPS extracted by heating and ultrasound methods, the PN/PS ratio in TB-EPS (1.1–1.4) was higher than in LB-EPS (0.6–1.3), which was in agreement with a previous report for *Shewanella* biofilms (Cao et al., 2011). It has been reported that, polysaccharides has hydroxyl and carboxyl groups capable of binding various heavy metals, such as Cr(VI), As (III), As(V) and U(VI) (Huang et al., 2011; Cologgi et al., 2014; Dobrowolski et al., 2017). As TB-EPS are directly attached to cell surface while LB-EPS are distributed in the surrounding environment of the cells, the lower PN/PS ratio in the LB-EPS (i.e., higher content of polysaccharides) could offer the first barrier that protect cells against harmful effects of toxic chemicals such as heavy metals.

3.3. Characterization of EPS components using 3D-EEM-PARAFAC

The 3D-EEM can sensitively detect and accurately characterize the micro-view of fluorescence characteristics in EPS (Qian et al., 2017). Three main peaks were recorded from the EEM spectra of EPS at Ex/Em of (225, 275)/335 nm, (205, 255–285)/305 nm and (215, 245, 290–315)/395 nm (Fig. 2). Using contrastive analysis of the excitation and emission loading data with OpenFluor database, the first two peaks were easily identified to be tryptophan protein-like substance (Component 1, C1) and tyrosine aromatic protein-like substance (Component 2, C2) (Jia et al., 2017; Qian et al., 2017). However, the third peak was rarely reported in previous studies, and only matched with a fluorescent component of EPS extracted from aggregated anammox bacteria (Jia et al., 2017), which was tentatively regarded as heme-like substances (Component 3, C3). Nevertheless, the identification of C3 is needed to be studied further. The *Geobacter* biofilms anchored an amount of extracellular multiheme *c*-Cyts to mediate sequential electron transfer reactions, and it was thereby reasonable to detect fluorescence of heme-like substances. It is notable that, the C3 or C3-like spectra was not observed in EPS analysis of *Shewanella* spp. (Dai et al., 2016). The fluorescence characteristics of the functional groups of heme (porphyrin compounds) can be influenced by their molecular configurations and the interaction in the heme with proteins (Sheng and Yu, 2006; Hirsch, 2002). The distinct difference of *c*-Cyts types in *Shewanella* spp. from that in *Geobacter* spp. might attribute to the absence of heme-like substances spectra in 3D-EEM analysis of EPS extracted from *Shewanella* spp.

The PARAFAC is a three-way method that is used to model the EEM

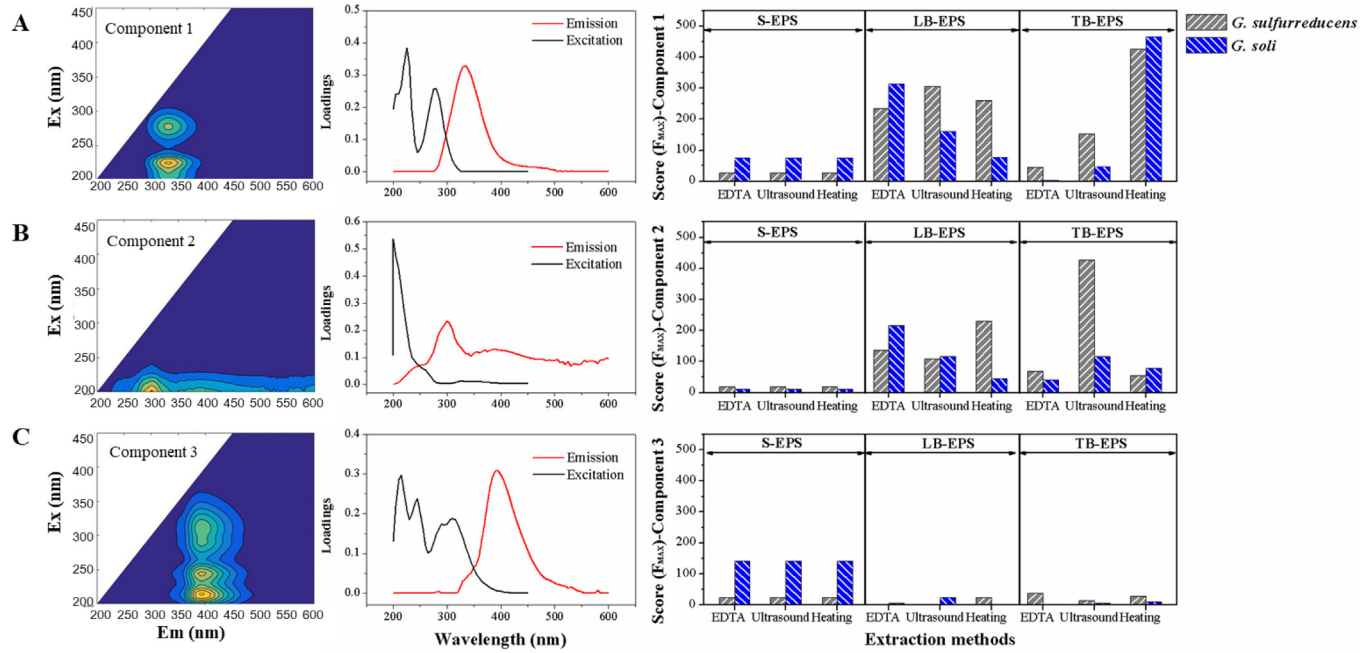

Fig. 2. EEM contours of three components, namely, tryptophan protein-like substance (Component 1, C1) (A), tyrosine aromatic protein-like substance (Component 2, C2) (B) and heme-like substances (Component 3, C3) (C), decomposed using the PARAFAC approach.

spectra (Jia et al., 2017). To avoid the impact on the component score caused by different EPS concentrations, the EEMs here were normalized by dividing the spectra by the corresponding TOC concentrations. As shown in Fig. 2, the tryptophan (C1) and tyrosine protein-like (C2) substances were mainly detected in LB-EPS and TB-EPS, which might be due to the higher protein content in LB-EPS and TB-EPS than in S-EPS (Fig. 1). By comparison, the higher fluorescence intensity of heme-like substances (C3) in S-EPS was inconsistent with the results of protein content, which might be caused by extremely different molecular configurations of different proteins in three spatial scales (Sheng and Yu, 2006; Cao et al., 2011). The intensity ratio of C1/C2 can reflect the protein composition of EPS. The great difference of C1/C2 ratio indicated the different protein composition in three EPS fractions. Unlike LB-EPS which showed similar C1/C2 ratios for both *G. sulfurreducens* (1.2–2.8) and *G. soli* (1.4–1.7) cells using three protocols, there were wide variations in C1/C2 ratios in TB-EPS extracted from *G. sulfurreducens* (EDTA: 0.7; ultrasound: 0.4; heating: 7.7) or extracted from *G. soli* cells (EDTA: 0.1; ultrasound: 0.4; heating: 5.9). The higher C1/C2 ratios of TB-EPS extracted using heating method indicated the distinctive protein composition, which might be due to the contamination of intracellular proteins caused by cell lysis. To sum up, the extraction methods affected not only extraction efficiency but also protein composition of EPS, which might further affect the electron transfer function of extracted EPS.

3.4. EPS components linked to the electron transfer function of EPS

Considering the spatial distance, electrons generated by active cells cannot be directly transferred to the electrodes when a thick layer of EPS covers the cell surface (Xiao et al., 2017). Fortunately, EPS have been proved to store redox-active substances, such as *c*-Cyts, which enables EPS-enveloped cells to transfer electrons to extracellular acceptors. Therefore, it is important to evaluate the electron transfer function of EPS from the biofilms.

The Q_{EDC} and Q_{EAC} of S-EPS, LB-EPS and TB-EPS fractions were measured, and the results demonstrated that all the three EPS fractions possessed electron transfer abilities (Fig. 3). Although *Geobacter* species do not appear to produce extracellular small molecular redox matters as electron transfer shuttles, some *c*-Cyts such as PgcA and OmcZ can be released into the electrolyte (Smith et al., 2014), which might contribute to the redox ability of S-EPS. It is well known that a higher concentration of *c*-Cyts is beneficial to the electron transfer. The contribution of TB-EPS and LB-EPS to the electron transfer capacity was greater than that of S-EPS in both *Geobacter* biofilms, which was consistent with previous studies that the extracellular *c*-Cyts were mainly located in LB-EPS and TB-EPS of EABs or aggregates (Ye et al., 2018). The Q_{EDC} and Q_{EAC} of each EPS fraction were counted up to calculate the Q_{EDC} and Q_{EAC} of total EPS of the biofilms, respectively. As shown in Fig. 3A, the EPS extracted from both *Geobacter* biofilms using heating method had the maximum Q_{EDC} (14.7 ± 1.4 and 12.9 ± 1.5 μmol e^-/g dry cell for *G. sulfurreducens* and *G. soli*, respectively), followed by EPS extracted using ultrasound method (13.5 ± 1.3 and 10.4 ± 1.6 μmol e^-/g dry cell for *G. sulfurreducens* and *G. soli*, respectively) and EDTA method (8.1 ± 1.1 and 7.1 ± 0.9 μmol e^-/g dry cell for *G. sulfurreducens* and *G. soli*, respectively). A similar trend was observed for the Q_{EAC} analysis of EPS extracted from *G. soli* biofilms (Fig. 3B).

In addition, both Q_{EDC} and Q_{EAC} of TB-EPS had a good linear correlation with the concentration of proteins ($R^2 > 0.64$, $P < 0.05$) and with protein/polysaccharide ratio ($R^2 > 0.44$, $P < 0.05$), and the Q_{EDC} and Q_{EAC} of total EPS also showed a positive correlation with their protein concentrations (Q_{EDC}: $R^2 = 0.87$, $P < 0.05$; Q_{EAC}: $R^2 = 0.47$, $P < 0.05$). Unlike TB-EPS and total EPS, Q_{ETC} of LB-EPS showed poor correlation with the protein concentration or protein/polysaccharide ratio. A similar correlation between Q_{EDC} and Q_{EAC} of total EPS and the concentration of proteins was also found in methanogenic communities, but the effect of polysaccharide was not studied (Ye et al., 2018). Outer membrane and extracellular redox-active proteins take charge of electron transport in EABs (Kumar et al., 2017) while electrically non-conductive polysaccharides would interfere with electron transfer (Kouzuma et al., 2010; Kitayama et al., 2017). Therefore, it is necessary to consider both protein and polysaccharide in analyzing the EET function of EPS for EAB.

The redox properties of EPS fractions were further characterized by the CV experiments. It has been reported that the more rectangular the CV shape, the better the capacitive behavior of samples was (Liu et al., 2006). Therefore, the EPS extracted from *G. soli* exhibited better capacitive behavior than that from *G. sulfurreducens* (Fig. 4), which might be a potential reason for the superior electrical current generation by *G.*

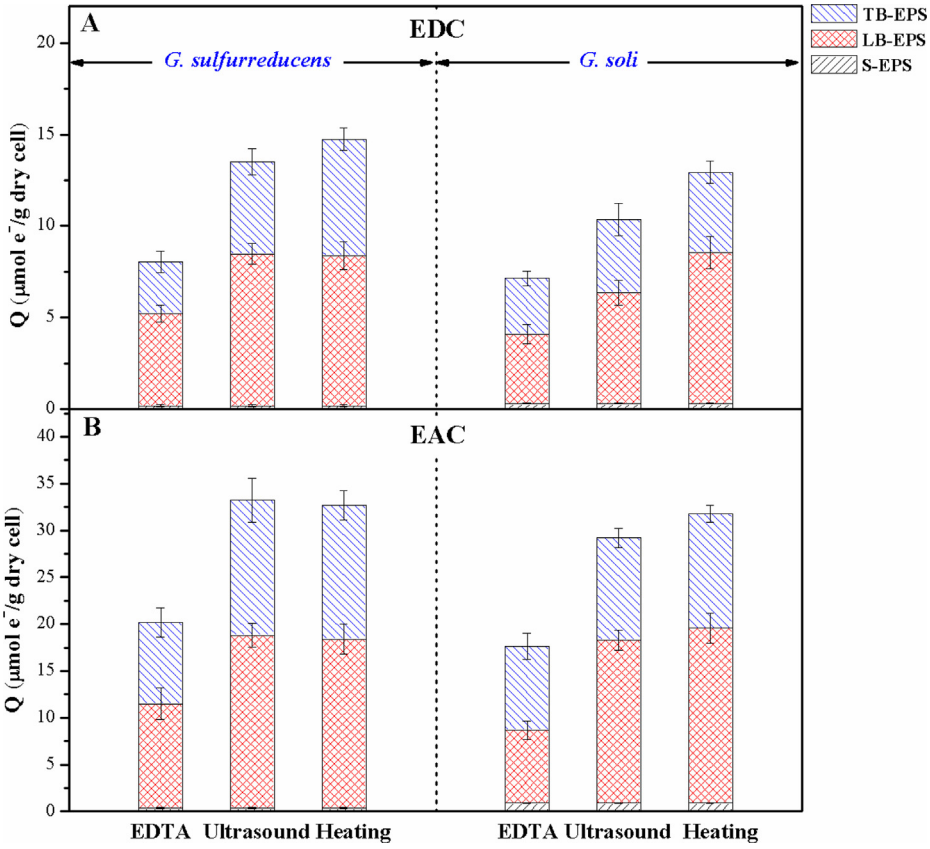

Fig. 3. The effects of extraction methods on Q_{EDC} (A) and Q_{EAC} (B) of the extracted EPS components from *G. sulfurreducens* and *G. soli*.

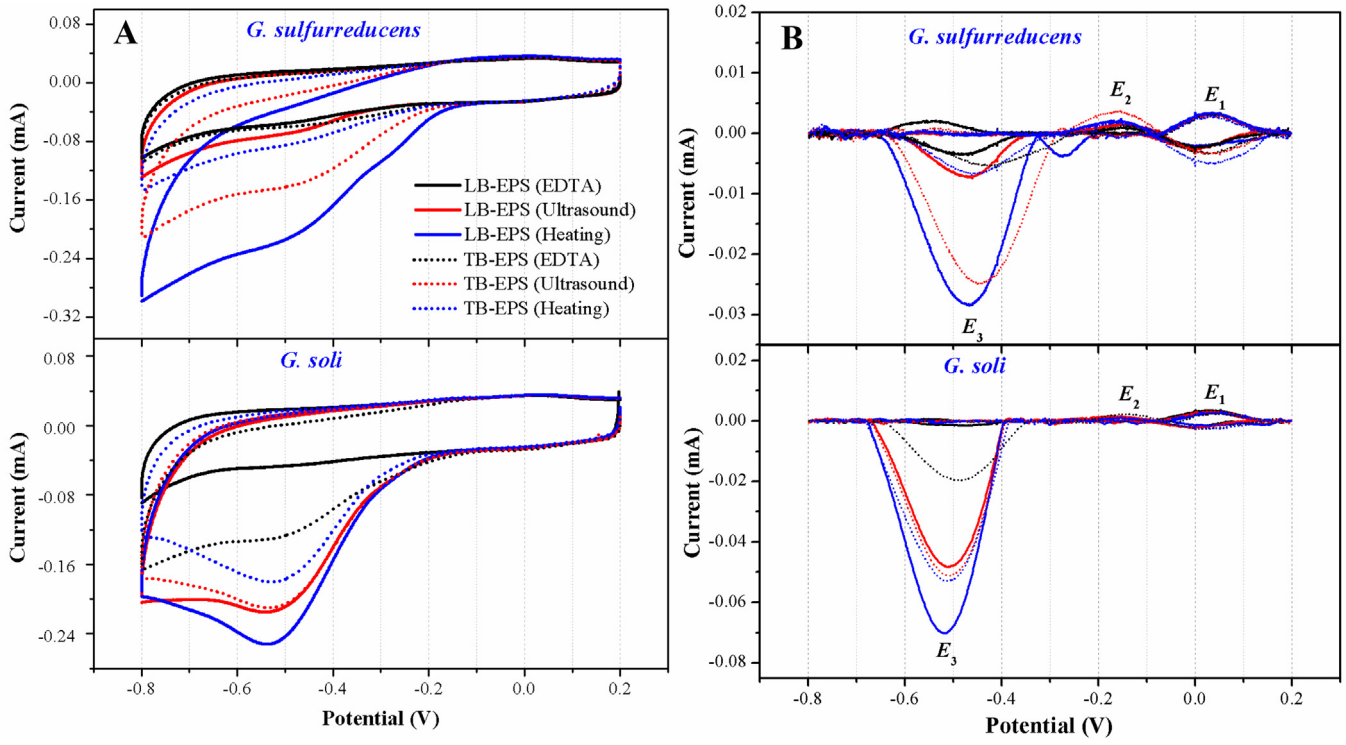

Fig. 4. The original (A) and baseline-substrated (B) CV spectra of EPS fractions extracted from *G. sulfurreducens* and *G. soli* using different methods.

soli (Cai et al., 2018). EPS from both *Geobacter* cells had three redox peaks, with a major peak at around −0.45 V (E_3) and two minor peaks at approximately −0.14 V (E_2) and 0.04 V (E_1). This finding was in line with previous reports that the main extracellular *c*-Cyts, such as OmcZ and OmcS, had a midpoint redox potential around −0.45 V (Qian et al., 2011; Yang et al., 2017). The peak currents at around −0.45 V for EPS samples extracted using ultrasound and heating methods were higher than that extracted using EDTA method, which indicated that a great amount of *c*-Cyts might be obtained by ultrasound and heating methods. Considering the heating method would result in cell lysis, the ultrasound method was proposed as a better method to extract extracellular redox-active proteins such as *c*-Cyts, the most important functional EPS component, from *Geobacter* biofilms.

4. Conclusions

This study used EDTA-, ultrasound- and heating-based methods to extract EPS from *Geobacter* biofilms. The extraction methods affected not only EPS yield but also composition and EET function of extracted EPS components. Heating-based protocol yielded the highest EPS concentrations but with the maximum cell lysis. Ultrasound and ETDA methods were both suitable for EPS extraction of *Geobacter* biofilm, of which ultrasound method was more effective in extracting redox-active proteins that was important to EET function of *Geobacter*. As a first trial to extract and characterize EPS from *Geobacter* biofilm, this study revealed the relationship between EPS fraction and electrochemical activities.

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Declaration of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2018.12.100.

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