Effective methods for extracting extracellular polymeric substances from *Shewanella oneidensis* MR-1

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ABSTRACT

Extracellular polymeric substances (EPS) play crucial roles in bio-aggregate formation and survival of bacterial cells. To develop an effective but harmless method for EPS extraction from *Shewanella oneidensis* MR-1, five extraction methods, i.e. centrifugation (control), heating (40, 45, 50, and 60 °C), and treatments with H_2SO_4, ethylenediaminetetraacetic acid (EDTA) and NaOH, were examined, respectively. Results from scanning electron microscope and flow cytometric analyses indicate that MR-1 cells were severely broken by H_2SO_4, NaOH and heating temperature $\geq$45 °C. Proteins and polysaccharides in EPS extracted by heating at 40 °C were 7.12 and 1.60 mg g^{-1} dry cell, respectively. Although EDTA treatment had a relatively lower yield of EPS (proteins and polysaccharides yields of 5.15 and 1.30 mg g^{-1} dry cell, respectively), cell lysis was barely found after EPS extraction. Three peaks were identified from the three-dimensional excitation–emission matrix spectrum of each EPS sample, suggesting the presence of protein-like substances. Furthermore, the peak intensity was in good accordance with protein concentration measured by the chemical analysis. In short, heating (40 °C) and EDTA treatments were found the most suitable methods for EPS extraction considering the cell lysis and EPS content, composition and functional groups together.

Key words | dissimilatory metal-reducing bacterium, extracellular polymeric substances, extraction, *Shewanella oneidensis*

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INTRODUCTION

Extracellular polymeric substances (EPS), mainly composed of proteins and polysaccharides, are sticky macromolecular materials excreted by microorganisms (Costerton *et al.* 1987; Yuan *et al.* 2010; Lee *et al.* 2013). EPS usually exist on external surfaces of cells as well as in microbial aggregates, forming a protective layer and thereby enabling microorganisms to adapt in extremely adverse environmental conditions (Liu & Fang 2002).

The basic step for EPS study is to find an efficient technique to extract EPS from target microorganisms. Many published works have investigated and compared different methods for EPS extraction from microbial cultures, including centrifugation, cation exchange resin, heating, alkaline, ethylenediaminetetraacetic acid (EDTA), sulfide and formaldehyde/NaOH methods (Liu & Fang 2003; Kunacheva & Stuckey 2014). For example, five methods – EDTA, NaOH, ion exchange resin, heating and centrifugation – were

compared by Tapia *et al.* to extract EPS from acidophilic bacterium *Acidiphilium 3.2Sup(5)*, and EDTA was identified to be the most effective method (Tapia *et al.* 2009). In another investigation, Zhu *et al.* found that EPS can be effectively extracted from microbial aggregates by ultrasound + formamide + NaOH and ultrasound + heat methods (Zhu *et al.* 2015). Previous studies also suggest that there is no universal method for EPS extraction from microorganisms or microbial aggregates (Sheng *et al.* 2010).

Shewanella oneidensis MR-1 is a model of dissimilatory metal-reducing bacteria and electrochemically active microorganisms. Owing to its respiratory versatility, it has been intensively studied for the biogeochemical cycle of metals and bioremediation of heavy metals (such as uranium and chromium). Three pathways for microbial extracellular electron transfer (EET) for *S. oneidensis* have been proposed: direct EET through membrane

cytochromes, indirect EET via electron shuttles and also the EET via appendages (Borole *et al.* 2011). However, these findings were based on the cells directly contacting the environment, without any substances covering their surface. Most bacteria cells were enveloped by a layer of heterogeneous EPS (Dohnalkova *et al.* 2011). Antibody recognition force microscopy showed that proteins MtrC and OmcA, the most important proteins on the outermost membrane, were present in EPS when Fe(III) served as the terminal electron acceptor (Lower *et al.* 2009). Furthermore, EPS extracted from *Shewanella* sp. HRCR-1 biofilms by EDTA contain both MtrC and OmcA (Cao *et al.* 2011). These studies indicated that EPS from *Shewanella* strains contain some important molecules for electron transfer. However, the potential function of EPS in EET has still received little attention.

Evaluation of an EPS extraction method should be based on three aspects: EPS yield, cell lysis, and EPS disruption (Sheng *et al.* 2010). Different methods yield varying amount and composition of EPS; for instance, eight different extraction methods extracted 21.0 (centrifugation method) to 318.0 (formamide + NaOH method) milligrams of EPS per gram of volatile suspended solids from activated sludge (Comte *et al.* 2006). Although chemical treatments usually produce higher quantity of EPS than that from physical treatments, chemical extracts may result in residual reagents and contamination in EPS (Comte *et al.* 2006). Cell lysis usually happens during EPS extraction, and the carbohydrate-to-protein ratios of EPS (Cao *et al.* 2011), nucleic acid content (Brown & Lester 1980) and the activity of glucose-6-phosphate dehydrogenase (Frølund *et al.* 1996) are regarded as indicators of cell lysis. However, little attention has been paid to morphological characterization of microbial cells after EPS extraction. Scanning electron microscopy (SEM), which can offer visual information about morphological specificity of microbial cells treated in certain environments, was considered to be another index to assess EPS extraction methods in this study.

The objective of this work is to conduct a comparative study on methods for EPS extraction from Gram-negative strain *S. oneidensis* MR-1 and sequentially propose the most applicable method. Cell lysis analysis of MR-1 after EPS extraction was characterized by SEM, flow cytometry and nucleic acids content. A high level of nucleic acids after EPS extraction indicates serious cell lysis, as the nucleic acids content in the exopolymer matrix is usually low. The main content of nucleic acids comes from the leaked intracellular materials (Sheng *et al.* 2010). The EPS

yields were determined by proteins, polysaccharides and nucleic acids measurements. Additionally, the spectrum characteristics of EPS extract were analyzed by three-dimensional excitation–emission matrix (EEM) and Fourier transform infrared (FTIR) techniques. While EEM fluorescence spectroscopy can offer the fluorescent characteristics and the chemical properties of EPS, FTIR analysis can show the functional groups of organic matter and predict the major components in EPS.

MATERIALS AND METHODS

Bacteria culture and EPS extraction

S. oneidensis MR-1 was cultured in Luria-Bertani broth containing (g L^{-1}) 10 tryptone, 5 yeast extract and 10 NaCl (pH 7.0) in a 250 mL Erlenmeyer flask at 25 °C in a rotary shaker (150 rpm). After 48 h, 40 mL of bacterial culture, with about 3 g dry cell L^{-1}, was collected by centrifugation with a rate of 5,000 g at 4 °C for 10 min and then washed twice with 0.9% NaCl solution.

The water-soluble EPS from MR-1 was extracted by applying five different methods: heating, H$_2$SO$_4$, EDTA, NaOH and centrifugation (as control) treatments, respectively. For heating method, four temperatures of 40, 45, 50 and 60 °C were selected to optimize the treating temperature. Figure 1 shows detailed procedures for different EPS extraction protocols.

For extraction with H$_2$SO$_4$, EDTA and NaOH, pellets of bacteria were re-suspended with 20 mL of 0.9% NaCl solution, followed by adding the three extractants, H$_2$SO$_4$
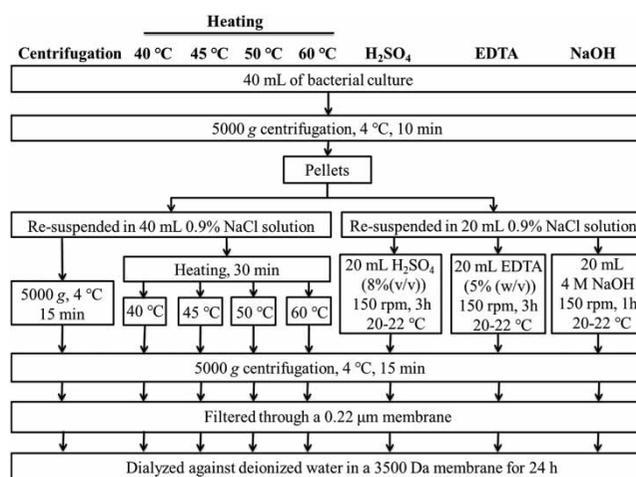

Figure 1 │ Procedure of different EPS extraction methods.

(8%, v/v), EDTA (5%, w/v) and NaOH (4 mM), to a final volume of 40 mL. The three mixtures were incubated in a shaker at 150 rpm and at 20–22 °C for 3, 3 and 1 h, respectively.

For the heating method, the bacteria pellets were re-suspended in 40 mL of 0.9% NaCl solution and then heated in water baths for 30 min at 40, 45, 50 and 60 °C, respectively.

Low speed centrifugation was regarded as a control because it was a less destructive method and a common process in EPS extraction procedures. Therefore, bacteria pellets were re-suspended in 40 mL of 0.9% NaCl solution and placed in 25 °C environment for 30 min.

Thereafter, all the treated cell suspensions were centrifuged with a rate of 5,000 g at 4 °C for 15 min, and then the supernatant, i.e. EPS solution, was filtered through a 0.22 μm membrane. The collected EPS solutions were dialyzed against deionized water in a 3,500 Da membrane for 24 h prior to chemical analysis. All experiments were conducted in triplicate.

SEM

The morphology of the bacteria cells was observed using SEM (S-4200 FE-SEM, Hitachi, Tokyo, Japan). Treatment of the samples for SEM observation was performed according to a previous report (Wang *et al.* 2014).

Flow cytometry analysis of cell viability

Flow cytometry (Quanta SC, Beckman Coulter, USA) analysis was employed to quantify the proportion of viable cells in the cell suspension after EPS extraction. Bacteria cells before and after EPS extraction were collected and then re-suspended with 0.9% NaCl solution to an OD$_{600}$ of about 0.02. The bacteria suspension was filtered through a 200 mesh filter screen to separate bacteria from large particles (Berney *et al.* 2007) and then immediately stained with propidium iodide (PI) (7.48 mM) in the dark at room temperature for 15 min (Falcioni *et al.* 2008). Bacterial spontaneous fluorescence and PI fluorescence were detected in the green (FL2) and red (FL3) cytometric channels, respectively. The samples were fixed in cold ethanol (70%) for 30 min and sequentially stained with PI to set the threshold of live and dead cells. A dot plot of green versus red fluorescence allowed differentiation of the 'live' cell cluster (cells with intact membranes) from the 'dead' cell cluster (cells with permeable membranes of non-viable cells).

Chemical analysis of EPS

Proteins in EPS extract were determined by the modified Bradford method using bovine serum albumin (Sangon, Shanghai, China) as a standard (Frølund *et al.* 1996). The polysaccharides content in EPS was measured using the anthrone method with glucose as a standard (Yuan *et al.* 2010). Nucleic acids were determined by the diphenylamine colorimetric method with calf thymus DNA (Sigma) as the standard (Burton 1956). All the measurements were completed on a UV-1200 spectrophotometer (Mapada, Shanghai, China).

Three-dimensional EEM spectroscopy

EEM fluorescence spectra of EPS extract were measured on an F-4600 fluorescence spectrometry (Hitachi, Tokyo, Japan). The parameters of EEM analysis were the same as a previous report (Wang *et al.* 2014). The peak intensity showed a linear relationship with low EPS concentration (e.g. <10 mg) (Sheng & Yu 2006). All the samples were diluted 40 times with 0.9% NaCl solution before EEM spectroscopy analysis. The spectrum of 0.9% NaCl solution was recorded as blank.

FTIR spectroscopy

EPS extracts were freeze-dried (Labconco Inc., USA), mixed with KBr at a ratio of 1:100 and ground in an agate grinder. The mixtures were then compressed and analyzed using a Nicolet™ iS™ 10 FTIR spectrometer (Thermo Fisher Scientific, USA). The spectra of samples were recorded on a KBr disk in scan region of 4,000 to 400 cm^{-1} at a resolution of 4 cm^{-1}.

RESULTS AND DISCUSSION

SEM analysis

Centrifugation generated a gravitational field to accelerate the sedimentation velocity of bacteria and then separated EPS from the cell surfaces by fluid traction (Brown & Lester 1980). As shown in Figure 2(a), EPS with gel-forming properties between bacteria cells were observed, and a colloid structure of EPS (insert in Figure 2(a)) was also observed, in agreement with Tapia *et al.* (2009). However, it is hardly possible to observe a similar phenomenon on cells in the other extraction methods. Moreover, no cracking was found on cell surfaces, indicating that the centrifugation
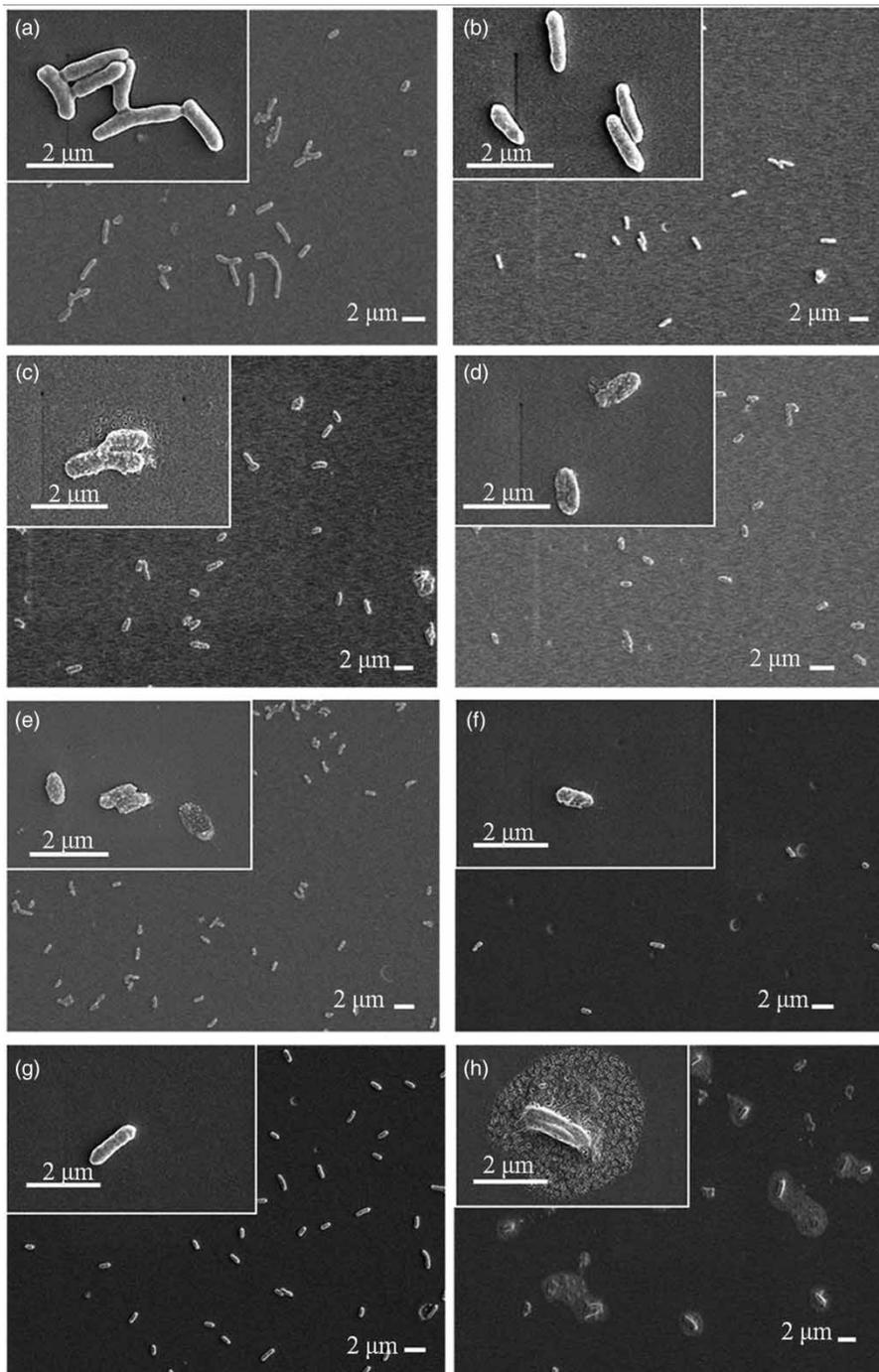
Figure 2 | SEM micrographs of *Shewanella oneidensis* MR-1 after EPS extracted by different treatments: (a) control; (b) heating at 40 °C; (c) heating at 45 °C; (d) heating at 50 °C; (e) heating at 60 °C; (f) H₂SO₄; (g) EDTA; (h) NaOH; and the insert shows the detail of each micrograph.

method led to little cell lysis during extraction and failed to efficiently remove EPS. Therefore, the centrifugation method was set as a control in this study.

Compared with other methods, heating extraction which does not use any additional chemical reagents is faster and easier to operate. Li & Yang (2007) reported a modified heating method which extracted both tightly bound and loosely bound EPS from activated sludge with no significant cell lysis. In heating treatment, higher temperature increases EPS yield by improving the solubility of EPS (Sun *et al.* 2012). Thus, temperature optimization was performed in the present study, and a temperature gradient

was adapted from previous reports (Sheng *et al.* 2005; Li & Yang 2007; Sun *et al.* 2012). SEM images showed serious cracks on the cells after heating at 45, 50 and 60 °C, whereas damage barely happened on cells being heated at 40 °C. The results indicated that MR-1 was susceptible to changes in temperature. Therefore, 40 °C, the lowest temperature compared to previous reports (Comte *et al.* 2006; Li & Yang 2007; Wang *et al.* 2014), may be the best temperature for the heating method to treat MR-1 in the present study.

Fewest cells were observed from the samples with H_2SO_4 treatment (Figure 2(f)). Furthermore, the cell in Figure 2(f) (insert) presented an incomplete cellular structure which was induced by H_2SO_4 reagent. EDTA releases EPS from bacteria by chelating and removing divalent cations which play an important role to crosslink the charged compounds in the EPS matrix (Sheng *et al.* 2010). Although EDTA was reported to be harmful to bacteria (Liu & Fang 2002), in the present study the bacterial cells treated with EDTA were as intact as those in the control group (Figure 2(g)), which may be mainly attributed to the mild process at pH 7.0. Since MR-1 is a multifunctional strain, EPS from MR-1 may contain more surface functional groups, which can bind with EDTA to protect MR-1 from being destroyed. The most significant cellular disruption was observed in NaOH extraction (Figure 2(h)), which may be caused by dramatic pH change in bacteria suspension, sequentially leading to cellular membrane destruction and release of intracellular substances (Tapia *et al.* 2009).

Flow cytometric assays

Cell viability is of major importance for evaluating the response to distinct extraction methods. This is because an ideal EPS extraction method should induce the minimal cell lysis. PI was used as an indicator to distinguish 'live' cells (cells with intact membranes and PI impermeable) from the 'dead' cells (cells with damaged membranes and PI permeable). Dead cells demonstrated strong red fluorescence because PI combined with double stranded DNA, whereas the live cells displayed weak red fluorescence and strong green fluorescence as PI was excluded from cells. As shown in Figure 3, dot plots of green (under the gray background) versus red fluorescence (in online version of this figure; shown as dark gray and black, respectively, in print version) were shown to demonstrate the cell viability in different EPS extraction methods.

In control, the percentage of dead cells was $1.17 \pm 0.12\%$. The flow cytometric analysis showed that 10.26 ± 1.97, 52.46 ± 1.38, 50.68 ± 1.10 and $48.88 \pm 1.10\%$ of total

cells in 40, 45, 50 and 60 °C heating treatment were PI-stained dead cells, respectively. Fivefold more dead cells were found in the 45 °C treatment compared to that of 40 °C treatment.

In EDTA treatment, a proportion of $1.57 \pm 0.24\%$ was identified as dead cells, which was close to that of the control and indicated that EDTA treatment was harmless to MR-1 cells. The result was also in accordance with the SEM photomicrograph (Figure 2(g)). For the H_2SO_4 and NaOH methods, $40.27 \pm 1.54\%$ and $49.10 \pm 1.12\%$ of cells were PI-stained, respectively. The result indicated that both acid and alkali treatments significantly damaged cell membranes and were not suitable for EPS extraction from MR-1.

Comparison of EPS yield

The composition and yields of EPS extracted by H_2SO_4, EDTA, NaOH, 40 °C heating, and control are shown in Figure 4. The EPS of MR-1 mainly contain proteins and polysaccharides, and the amount of each biochemical compound varied in a wide range among different methods. However, all methods extracted more proteins than polysaccharides.

The lowest EPS yield (proteins and polysaccharides of 0.53 and 0.24 mg g^{-1} dry cell, respectively) was obtained from the control group, indicating that centrifugation was not an effective method for EPS extraction. In contrast, proteins and polysaccharides of 7.12 and 1.60 mg g^{-1} dry cell were produced from the heating treatment at 40 °C, respectively. Comparing with the heating treatment, H_2SO_4 extraction obtained less protein but more polysaccharide. Dramatic cells lysis occurred in H_2SO_4 treatment due to the destruction of native cell structures, which was shown in SEM images (Figure 2(f)). Further results from flow cytometric analysis showed that $40.27 \pm 1.54\%$ of cells were PI-stained dead cells after H_2SO_4 treatment. These results indicated that H_2SO_4 treatment significantly damaged cell membranes and was not suitable for EPS extraction from MR-1.

In the EDTA extraction, corresponding yields of proteins and polysaccharides were 5.15 and 1.30 mg g^{-1} dry cell, respectively. Although the amount of EPS extracted by EDTA was lower than other chemical and heating methods, less cell lysis was induced by EDTA extraction. The result agreed with a previous report from Zhang and co-workers (Zhang *et al.* 1999) who recommended EDTA treatment as a good choice for EPS extraction from biofilms.

The highest yields of proteins and polysaccharides coupled with a large amount of nucleic acids were
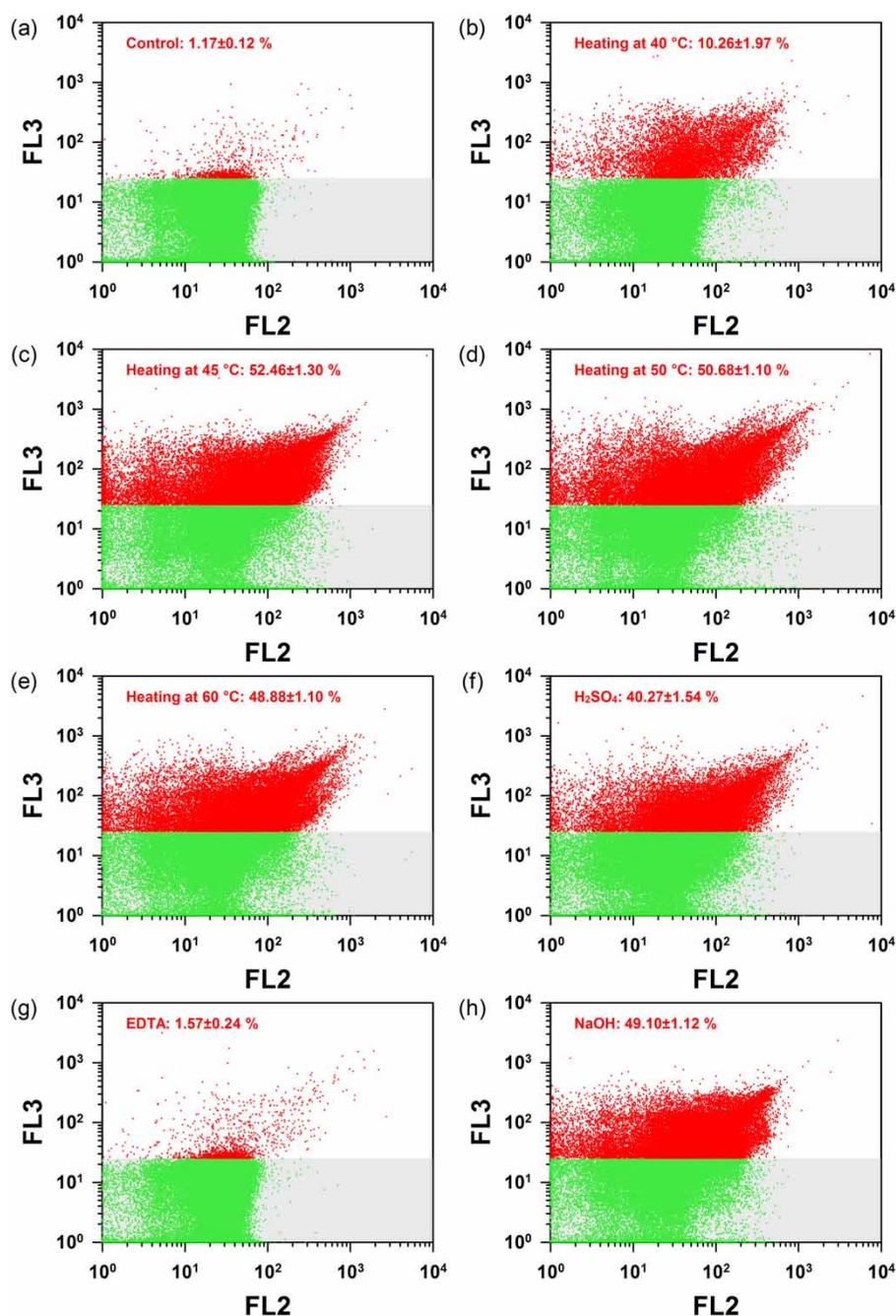
Figure 3 │ Flow cytometric analysis of *S. oneidensis* MR-1 treated with various methods for EPS extraction. Percentages reflect the ratio of the PI-stained cells. The full colour version of this figure is available in the online version of this paper: http://dx.doi.org/10.2166/wst.2016.473

measured in NaOH extract, whereas no nucleic acids were detected in the other methods. Since a high level of nucleic acids in extracted EPS was regarded as an indicator of serious cell lysis (Sheng *et al.* 2010), this result indicated that NaOH treatment was not an appropriate method for EPS extraction. The obviously disrupted cells indicated by the yield of nucleic acids was also

consistent with the severe cellular disruption depicted in Figure 2(h).

EEM fluorescence spectra

EPS contain large quantities of aromatic structures with a variety of functional groups, which have unique
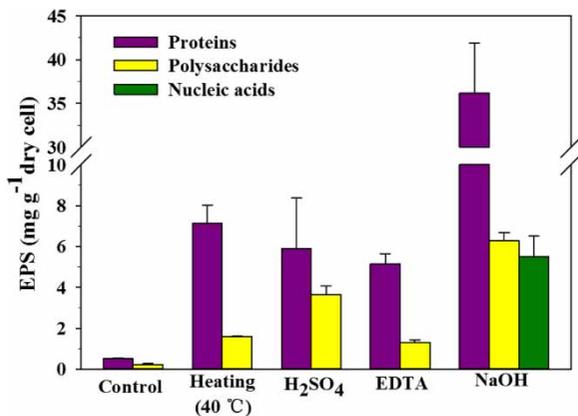
Figure 4 │ Chemical composition and amounts of EPS extracted from different methods. Error bars represent standard deviations obtained from experiments in triplicate.

fluorescence characteristics. EEM fluorescence spectroscopy is a non-destructive technique to rapidly identify a sample's fluorescent characteristics with high sensitivity and has been widely used for studying the chemical and physical properties of EPS (Sheng & Yu 2006).

The EEM fluorescence spectra of EPS extracted by four methods are given in Figure 5. Two prominent peaks, peak A at excitation/emission (Ex/Em) of 275/325–340 nm and peak B at Ex/Em of 225/315–340 nm, were identified in all samples. The peaks A and B suggested the presence of tryptophan and other protein-like substances rich in aromatic amino acids, respectively (Zhu *et al.* 2015). The locations of the two peaks agreed with previous reports (Liu *et al.* 2014; Yu *et al.* 2014). A weaker fluorescence peak C at Ex/Em of 215/285–290 nm could be ascribed to simple aromatic amino acids like tyrosine and was easily identified from the EPS extracted by H_2SO_4 and EDTA methods (Wang *et al.* 2014).

The location and intensity of each spectrum are listed in Table 1. Compared with the locations of peak A and B identified from EPS extracted with heating (40 °C) method, both peak A and B in EPS extracted by H_2SO_4 method exhibit red shifts of 15 nm along the Em axis. However, peak A and B of EPS extracted by NaOH were blue-shifted by 20 and 10 nm along the Em axis, respectively. The red shifts might be due to the existence of hydroxyl, carboxyl and
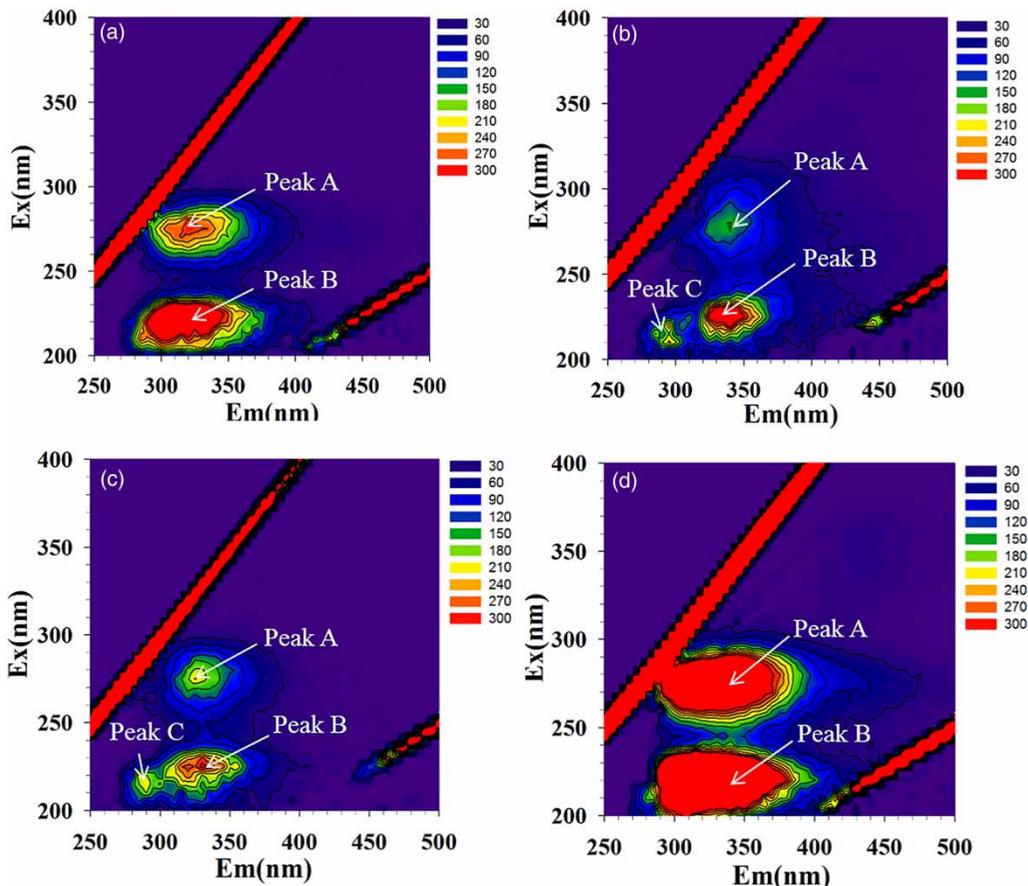

Figure 5 │ EEM fluorescence spectra of EPS extracted by (a) heating method (40 °C), (b) H_2SO_4 method, (c) EDTA method and (d) NaOH method.

amino constituents (Wang *et al.* 2014), and the blue shifts might come from the decrease of aromatic rings and conjugated bonds in a chain structure. As illustrated in Table 1, the peak intensity was in good accordance with the protein concentrations in EPS. We also found that the intensity of peak B was always higher than that of peak A in all extraction methods. The results indicated that protein-like substances rich in aromatic amino acids were relatively abundant in EPS.

FTIR spectra

FTIR spectroscopy is usually used to determine the functional groups of organic matter and predict the major components in samples as it can generate an infrared spectrum of a sample over a wide spectral range absorption (Kunacheva & Stuckey 2014).

In this study, FTIR spectroscopy was used to determine the functional groups in EPS and investigate whether the EPS was contaminated by extractants. As shown in Figure 6,

Table 1 | Location and intensity of each spectrum from the EEM fluorescence analysis on EPS.

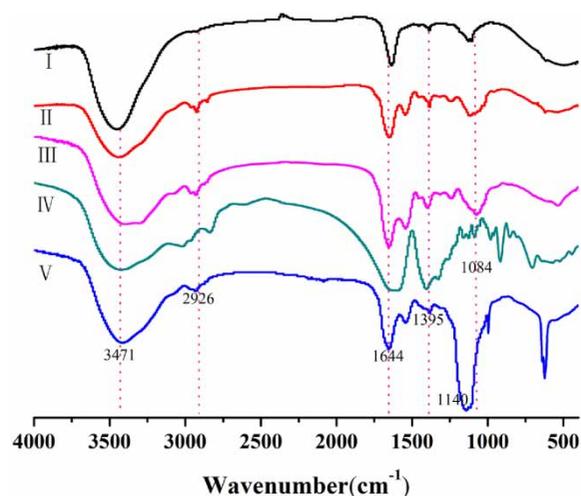| Sample	Peak A		Peak B		Peak C	
	Ex/Em	intensity	Ex/Em	intensity	Ex/Em	intensity
Heating (40 °C)	275/325	299	225/325	473	–	–
H_2SO_4	275/340	150	225/340	470	215/285	185
EDTA	275/325	213	225/330	314	215/290	209
NaOH	275/305	688	225/315	1175	–	–

Figure 6 | FTIR spectra of EPS extracted by (I) control, (II) heating at 40 °C, (III) NaOH, (IV) EDTA and (V) H_2SO_4 methods.

five major characteristic absorption bands around 3,471, 2,926, 1,644, 1,395 and 1,084 cm^{-1} can be observed in most EPS samples except that from H_2SO_4 method. These bands also agreed well with the results from *Rhodopseudomonas acidophila* (Sheng *et al.* 2005). The absorption band at 3,471 cm^{-1} comes from O—H symmetric stretching vibration in polymeric compounds, which may be from proteins, polysaccharides or the residual water in the samples (Tapia *et al.* 2009). The band at 2,926 cm^{-1} was assigned to C—H stretching vibration mode in hydrocarbon chains. The absorption bands near 1,644 and 1,395 cm^{-1} were consistent with the stretching vibrations of C=O in amide I and C—N stretching vibrations of proteins, respectively (Abzac *et al.* 2009; Castro *et al.* 2014). The characteristic band around 1,084 cm^{-1} was a composite of the C—O and C—O—C stretching vibrations linking to polysaccharides (Elzinga *et al.* 2012).

FTIR spectrum of the EPS extracted by EDTA (line IV in Figure 6) showed an obvious band in the range of 1,644 to 1,570 cm^{-1}, indicating the formation of EDTA–EPS complexes. Liu & Fang (2002) illustrated that EDTA can combine with EPS and EDTA-EPS complexes could not be removed completely by dialysis. However, we still can find the functional groups of hydroxyl, carboxyl and amino in this IR spectrum. This result means that the contamination from EDTA–EPS complexes can be ignored and the EDTA treatment is a favorable method for EPS extraction.

The distinctive and large band at 1,140 cm^{-1} could be assigned to H_2SO_4 and was observed on the FTIR spectrum of EPS from H_2SO_4 treatment (line V in Figure 6) (Roman & Winter 2004). Furthermore, the peak at 1,084 cm^{-1} which was attributed to polysaccharides groups disappeared from this spectrum. These results indicated serious contamination from H_2SO_4.

CONCLUSIONS

A comparative study on different methods for EPS extraction from MR-1 was conducted, and findings revealed that MR-1 cells were very sensitive to temperature. Heating treatment at 40 °C with the relatively high yield of EPS extraction was free from contamination by intracellular materials. The NaOH methods induced severe cell lysis and led to contamination of intracellular substances in EPS. For acid treatment, EPS were contaminated by H_2SO_4. The EDTA treatment was harmless to MR-1 cells, and the contamination from EDTA–EPS complexes was ignorable, though

the EPS yield was relatively low. Hence, heating treatment at 40 °C and EDTA methods are recommended as effective EPS extraction methods for MR-1.

ACKNOWLEDGEMENTS

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