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Isolation and characterization of extracellular polysaccharides produced by *Pseudomonas fluorescens* Biovar II

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Abstract

Extracellular polysaccharides (EPS) were isolated from *Pseudomonas fluorescens* Biovar II through repeated ethanol precipitations. The neutral monosaccharides in the EPS that were determined by GC–MS consisted of rhamnose, fucose, ribose, arabinose, xylose, mannose, galactose and glucose. The acidic groups in the EPS were mainly composed of carboxylic acid and other minor polyanionic groups, e.g. sulphate and phosphate. Up to 70% of total carbohydrates were uronic acids, and total carbohydrates made up 26–31% of organic carbon. Besides the neutral and acidic sugars in the EPS, EPS also contained 2% of proteins in terms of carbon. The characterization of the main components of the EPS should increase our understanding of the potential binding sites for trace metals and radionuclides to these exopolymeric biosorbents.

Keywords: Polysaccharides; Extracellular polysaccharides; Uronic acids; GC–MS; *Pseudomonas fluorescens* Biovar II

1. Introduction

Extracellular polysaccharides, or exopolymeric substances (EPS) are produced by bacteria and have an important function in the removal of heavy metals and radionuclides from wastewater and natural waters. For instance, EPS produced by specific bacteria have been used as biosorbents for toxic metals (Dhami et al., 1998; Tolley & Macaskie, 1993; Volesky & Holan, 1995; Wilhelmi & Duncan, 1995). In the past 50 years, heavy metal and radionuclide, e.g. actinide, contamination has been widespread in the surface soil and subsurface environments throughout the Department of Energy (DOE, USA) complex. Choppin (1992) concluded that organic material plays an important role in Pu mobilization and immobilization. Recently, Santschi, Roberts, and Guo (2002) found that bacterial exopolymers (e.g. alginic acid and xanthan gum) lowered the tendency for actinide (e.g. Pu) remobilization in soils and fostered the binding of soil particles into aggregates that resist erosion. Also, Chen et al., (2002) reported that calcium alginate-conditioned sludge was found to be the most appropriate sorbent for copper removal from wastewater. An attractive avenue would thus be to use bioremediation to stabilize actinides and trace metals in the surface and subsurface environment, i.e. to enhance metal immobility in soil and aquatic environments. Besides metal sequestration, other important applications of microbially produced exopolymers are in inducing and controlling flocculation of colloidal materials in natural and engineered systems (e.g. Santschi, Burd, Gaillard, & Lazarides, 2005, chap. 9, and references therein), as well as initiating gel formation (Verdugo et al., 2004, and references therein) and biofilm and marine or lake snow formation (Leppard, 1995, 1997, and references therein) in aquatic environments. All these applications require experimentation with well characterized and purified extracellular polymers harvested from microbial cultures. The characterization of potential binding sites for metals and radionuclides in exopolymeric

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material can then lead to improved actinide and heavy metal sequestering agents.

Here, we used *Pseudomonas fluorescens* Biovar II, isolated from a DOE site, to investigate the isolation procedures, and to characterize the extracellular polysaccharides.

2. Materials and methods

2.1. Isolation and purification of extracellular polysaccharides from bacteria

A bacterial sample from *Ps. fluorescens* Biovar II, (ATCC no. 55421), was inoculated into a 1 l of soy broth (30 g of soy broth into 1000 ml of distilled water) within 24 h after it had been autoclaved at 121 °C for 15 min. After letting it grow overnight, an aliquot from the bacterial culture was taken and centrifuged at 3500 rpm for 30 min. After centrifugation, the culture contained two layers: a pellet and a supernatant. Extracellular polysaccharides were then isolated from the pellet according to a procedure based on the method of Kushner et al. (1992). To the supernatant, a proteinase solution (final conc. = 0.5 mg/L) was added to hydrolyze proteins of the soy broth, and the solution was incubated at 37 °C for 12 h at 70 rpm. After incubation, four volumes of 95% ethanol and 5% methanol were added to the solution, which was then placed in the refrigerator for 12 h to form a precipitate. The precipitate was removed by filtration through a 0.22 μm Millipore membrane. The precipitate was re-dissolved in pure water and NaCl was added to result in a final NaCl concentration of 30 g/L. Four volumes of an alcohol mixture (95% of ethanol and 5% of methanol) were then added to one volume of aqueous solution. The resulting precipitate was filtered through a 0.22 μm Millipore membrane. This separation procedure was repeated again three times. The clear final solution was dialyzed for 5 days using a 6–8 kDa membrane, and resulted in an EPS fraction called here the 'dissolved EPS'.

Distilled water of 100 ml was added to the pellet precipitate and 3 g of NaCl was added to the solution. The particulate polysaccharides were then extracted by stirring for 1 h. After that, the solution was centrifuged at 3500 rpm for 30 min. EPS from the supernatant were precipitated by the slow addition of alcohol (95% of ethanol and 5% of methanol). This procedure was repeated three times, and the remainder of the extraction procedure was the same as that for 'dissolved EPS'. The extracellular polysaccharides obtained from this fraction is defined here as 'particulate EPS'. Both dissolved and particulate EPS fractions were freeze-dried for storage and analysis.

2.2. Analysis of extracellular polysaccharides

2.2.1. Measurement of carbohydrates, proteins and acidic sugars

Total carbohydrates in the EPS were measured by a spectrophotometric method (Myklestad, Skanoy, & Hestamann, 1997), as modified by Hung and Santschi (2001). Basically, about 1 mg of EPS sample was placed into a 10 ml glass container with 4 ml of DW and 0.4 ml of 1 N hydrochloric acid. The sealed ampoules were then placed in an oven at 150 °C for 1 h. After hydrolysis, NaOH was used to neutralize the solution to a pH of 7. Then, 1 ml of the hydrolysate was added to a dark glass bottle containing 1 ml of 0.7 mM potassium ferricyanide, and the well-mixed solution was placed in a boiling water bath for 10 min. One millilitre of 2 mM ferric chloride solution and 2 ml of 2.5 mM TPTZ (2,4,6-tripyridyl-s-triazine, Sigma) solution in 3 M acetic acid were immediately added and mixed on a Vortex mixer. After 30 min, the absorbance was read at 595 nm. The concentration of total carbohydrates (TCHO) is expressed as μM-C.

The concentration of protein in 0.5 mg of freeze–dried EPS was measured by the method of bicinchoninic acid by colorimetric detection (Smith et al., 1985). Bovine serum albumin (BSA) was used as a standard to quantitatively calculate the protein content.

The procedures for the determination of different acidic functional groups, e.g. phosphate, sulphate in the EPS are as follows. Phosphate and sulphate in the lyophilized EPS were determined based on the pyrolysis method of Silvestri, Hurst, Simpson, and Settine (1982) and Grotjan, Padrnos-Hicks, and Keel (1986). The EPS samples were dissolved in distilled water in glass tubes and the solution was pyrolyzed. The dry residue was then dissolved in 2 ml of ultra-pure distilled water for the analysis by ion chromatography. A mixed solution of Na_2CO_3 and $NaHCO_3$ was used as an eluant at a flow rate of 2 ml/min. The anions of phosphate and sulphate were separated by an Ion Pac AS4A analytical Anion Exchange Column and detected by a Dionex Conductivity detector-II. A calibration curve of phosphate and sulphate was used to compute the concentrations of both phosphate and sulphate in the EPS samples.

The concentration of uronic acids, i.e. sugars containing carboxylic acids, in the EPS was analyzed according to Filisetti-Cozzi and Carpita (1991), as modified by Hung and Santschi (2001). In short, about 0.5–1.0 mg of freeze–dried EPS samples were placed into a vial and 0.4 ml of Nanopure water was added. Then, 40 μl of 2 M sulfamic acid was added, and the solution was stirred on a vortexer. Subsequently, 2.4 ml of 75 nM sodium tetraborate in concentrated sulfuric acid solution was added to the vial and heated at 100 °C for 10 min in a boiling water bath. After cooling, 30 μl of 0.5% m-hydroxydiphenyl was added and the absorbance was measured at 525 nm. Glucuronic acid was used as a standard for calculating the total uronic

acid concentration. The concentration of total uronic acids (URA) is expressed as μM-C.

2.2.2. Characterization of polysaccharides by GC–EI–MS

The analysis of neutral monosaccharides was based on the methods of Chen et al. (2003) and Osborn, Lochey, Mosley, and Read (1999). Briefly, about 1 mg of freeze–dried EPS and 50 nmol of inositol (as an internal standard) were dissolved in a hydrolysis tube containing 1 ml of 4 M trifluoroacetic acid (TFA). The sample was hydrolyzed at 100 °C for 2 h. After hydrolysis, TFA was removed by a stream of nitrogen gas. Neutral monosaccharides were reduced to alditol using 10 mg of sodium borohydride with 0.5 ml of 0.05 M NaOH solution at 60 °C for 1 h. Acetic acid was added to the solution to decompose excess sodium borohydride until bubble formation stopped. A stream of nitrogen gas was used to dry the solution and 1 ml of methanol was added to remove borohydrate, and the procedure repeated four times. The residue was then dried at 100 °C for 30 min. After that, 0.5 ml of pyridine and 0.5 ml of acetic anhydride were added to the residue, and the mixture was reacted at 100 °C for 1 h. A stream of nitrogen gas was used to remove the solvents and 1 ml of distilled water was added to dissolve the residue. Finally, a 2 ml aliquot of dichloromethane was added to extract the sample, and the concentrated volume was injected into a GC–MS (PolarisQ GC/MS, Thermo Finnigan) with a J&W Scientific column (DB 1701, 0.25 mm ID, 30 m). The temperature of the GC program was set up as follows: the initial temperature was set to 120 °C. After 1 min, the column was heated at 10 °C/min to 180 °C, and held for another 1 min. Then the column was heated at 0.3 °C/min to 190 °C and held for another 1 min. Finally, the column was heated at 10 °C/min to 210 °C. Peaks of neutral monosaccharides in the EPS were identified by comparison of retention time and mass spectral fragmentation patterns with monosaccharide standards (Fig. 1). The amount of individual neutral monosaccharides was calculated by comparison of peak areas to the peak area of inositol.

Sub-samples of the EPS were analyzed for total organic carbon based on the procedures of Guo and Santschi (1997).

2.2.3. Testing of the sensitivity of the TPTZ method for carbohydrate analysis

Myklestad et al. (1997) examined the response of other monosaccharides (other than glucose), URA and BSA standards with regards to their sensitivities in the TPTZ method. Most of the monosaccharides (e.g. arabinose, mannose, galactose and glucuronic acid) give a response that is only slightly lower than the absorbance of glucose, i.e. 88–96% relative to glucose. We also tested the response of glucuronic acid and proteins such as BSA for their response in the TPTZ method. The results of these tests showed that the absorbance of glucuronic acid and BSA accounted for 97 and 15% of glucose, respectively, which is similar to that reported for glucuronic acid by Myklestad et al. (1997), considering that BSA likely was not hydrolyzed in the procedure of Myklestad et al. (1997). Thus, the protein content (of about 9% of OC, see Results section) in the EPS may result in an overestimate of the total carbohydrate content (26–31% of OC, see Results section) in the EPS by about 4 and 5% (i.e. 1–1.5% of OC) of the total carbohydrates content of dissolved and particulate EPS, respectively, while the different response of the uronic acids and non-glucose monosaccharide compounds would result in an underestimate by about the same amount.

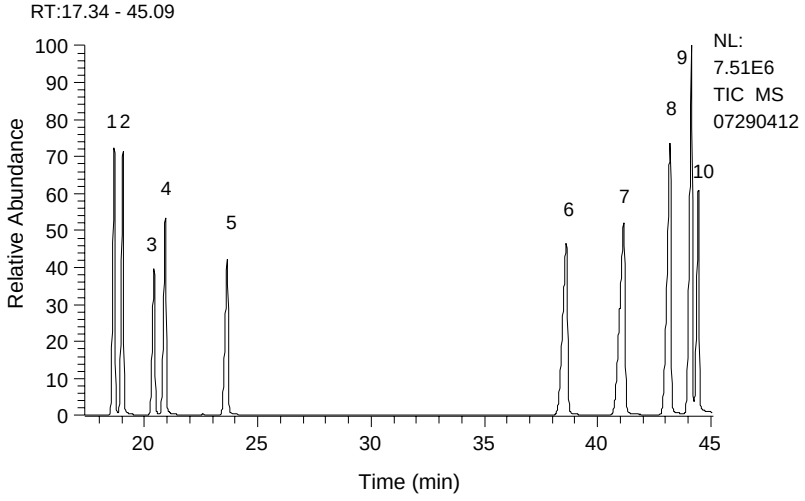

Fig. 1. Gas chromatograms of nine neutral monosaccharides and inositol. (1) Rhamnose, (2) fucose, (3) arabinose, (4) D-ribose, (5) xylose, (6) allose, (7) mannose, (8) galactose, (9) glucose, (10) inositol.

Table 1
Major composition of dissolved and particulate extracellular polysaccharides (EPS) produced by *Pseudomonas Fluorescens Biovar II*

(A) Type	OC wt(%, C)	TCHO wt(%, C)	N-CHO wt(%, C)	Protein wt(%, C)*	URA wt(%, C)	SO_4^{-2} wt(%, SO_4)	PO_4^{-3} wt(%, PO_4)
Diss.	24.5	7.6	2.8	2.2	3.4	0.008	0.024
Part.	23.4	5.4	1.9	2.0	3.9	0.015	0.011

(B) Type	TCHO/OC (%)	N-CHO/OC (%)	Protein/OC (%)	URA/OC (%)	C/SO_4^{-2} mole ratio	C/PO_4^{-3} mole ratio
Diss.	31.2	11.3	8.9	13.9	7722	5504
Part.	25.6	8	8.7	18.5	2953	1758

TCHO: total carbohydrates; N-CHO: summation of eight neutral monosaccharides. (A) OC: organic carbon. wt(%) represents the percent of dry weight for individual parameter in (A). *assuming 1 mg of protein containing 0.33 mg carbon. (B) All parameters are normalized to organic carbon (OC) except for SO_4^{-2} and PO_4^{-3} (expressing by mole ratio to C (carbon)).

3. Results

3.1. Composition of EPS

Particulate and dissolved EPS produced by *Ps. Biovar II* is made up of about 23.4 and 24.5% of carbon in terms of dry weight (Table 1). The organic matter content in the EPS thus appears to be relatively low, e.g. about 60% by weight (assuming that organic matter contains, on average, 40% of organic carbon), but is within the range reported in the literature for EPS and environmental colloids. The remainder of the mass of the EPS samples is likely hydration water, as these substances are strongly hygroscopic, a fact that had been discussed before by Buffle (1990) and Leppard (1995, 1997) for EPS and humic substances. The concentration of total carbohydrate-carbon (measured by the TPTZ method) in both particulate and dissolved EPS was 5.4 and 7.6% by dry weight. Uronic acids (URA), with COO^- functional groups, are the major acidic polysaccharides in both dissolved and particulate EPS, with 45 and 73% of total carbohydrate-carbon (or 3.4 and 3.9% of dry weight), respectively, resulting in a COO^-:6-C sugar ratio of 2.2 and 1.4, respectively. Protein concentration in both dissolved and particulate EPS accounted for 2.2 and 2.0% of dry weight (Table 1), resulting in a carbohydrate-C:protein-C ratio of 2.5 and 3.8, respectively. Other functional groups, SO_4^{-2} and PO_4^{-3}, were also found in the purified EPS, but at lower levels. The molar ratios for dissolved and particulate EPS, in terms of total carbon:PO_4:SO_4, were 2953:1.7:1 and 7722:1.4:1, respectively. In other words, the ratios of 6-C sugars:PO_4:SO_4 were 492:1.7:1 and 1287:1.4:1 for dissolved and particulate EPS, respectively.

3.2. Molecular characterization of EPS

The compositions of EPS in both particulate and dissolved fractions consisted of rhamnose, fucose, arabinose, ribose, xylose, mannose, galactose, and glucose (Fig. 2). The total content of neutral sugars (the summation of rhamnose, fucose, ribose, arabinose, xylose, mannose, galactose, and glucose, as carbon) in particulate and dissolved EPS was 1.9 and 2.8% in terms of dry weight, respectively. Galactose, arabinose and mannose are the three major neutral sugars in EPS (Fig. 3). The sum of neutral monosaccharides was a factor of 2.5 lower than the total carbohydrate concentration in both particulate and dissolved EPS, indicating that other sugar compounds

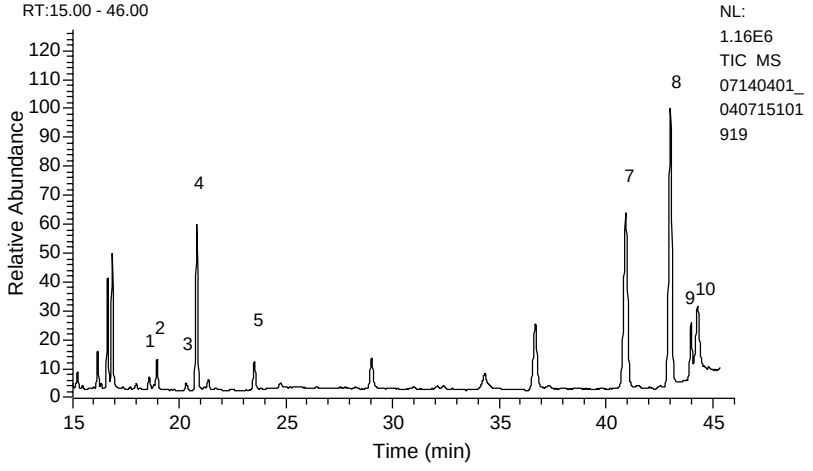

Fig. 2. Gas chromatograms of extracellular polysaccharides produced by *Pseudomonas fluorescence Biovar II*. (1) Rhamnose, (2) fucose, (3) arabinose, (4) D-ribose, (5) xylose, (6) allose, (7) mannose, (8) galactose, (9) glucose, (10) inositol.

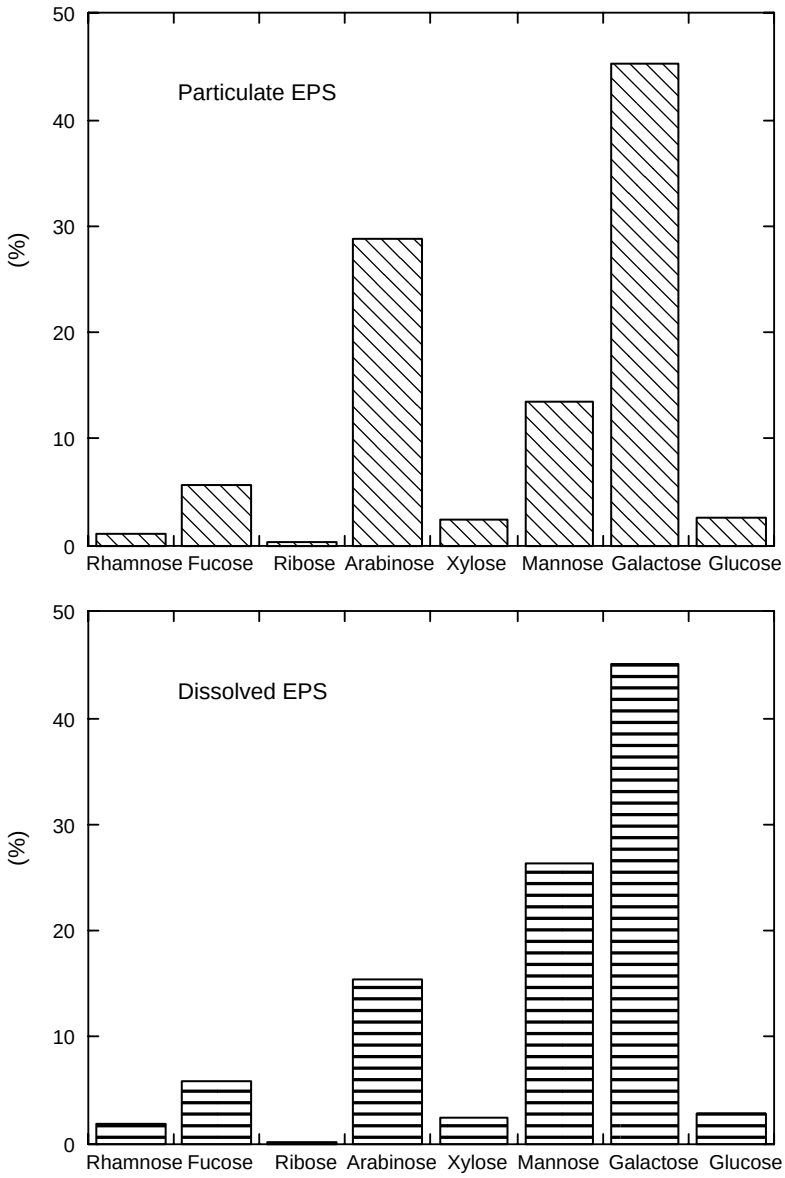

Fig. 3. Compositions of neutral monosaccharides in both particulate and dissolved EPS, as a percentage of each monosaccharide, normalized to the sum of eight monosaccharides.

existed that were not characterized at the molecular level, e.g. acidic sugars (see Discussion).

4. Discussion

Extracellular polysaccharides produced by *Pseudomonas* species have been widely reported in the literature (De Castro, Lanzetta, Molinaro, Parilli, & Piscopo, 2001; Jansson & Lindberg, 1983; Kachlany et al., 2001; Sudhamani, Tharanathan, & Prasad, 2004), but this is the first report on the characterization of EPS from *Ps. fluorescens* Biovar II. The EPS generated by *Ps. fluorescens* Biovar II appear to be very complex in both particulate and dissolved fractions, whereby EPS was characterized as carbohydrates (TCHO), neutral monosaccharides (N-CHO), proteins and polyanionic macromolecules, such as uronic acids, and as sulphated and phosphated moieties (Table 1).

The neutral monosaccharides isolated from *Ps. fluorescens* Biovar II were different from other *Pseudomonas* species, but the main sugar components (galactose, mannose, rhamnose and glucose) were similar to those reported by Kachlany et al. (2001) and Sudhamani et al. (2004). Interestingly, the major components (TCHO, N-CHO, and protein) in the dissolved EPS were higher than those in the particulate EPS, but the minor anions, sulphate

and phosphate, normalized to organic carbon in particulate EPS, were about twice higher than those in dissolved EPS. This might be a reflection of the fact that dissolved EPS are more easily released from the cell surface than the particulate EPS that adhere more closely to the bacterial cell wall. However, the concentration of URA in the particulate EPS was only slightly higher than that in the dissolved EPS. All in all, differences in composition of dissolved and particulate EPS were relatively minor. This suggests that significant differences in the treatment of these two EPS fractions (in terms of proteinase addition) lead to a similar product.

The concentration of total carbohydrates in both dissolved (26–31% of C) and particulate (22–30% of C) EPS was very similar to the sum of eight neutral monosaccharides (8–11% of C) and total uronic acids (14–19% of C). The good agreement in our mass balance might indicate that the isolated EPS are more uniform and less complex than natural organic matter (NOM), where a larger portion of the carbohydrate-type structures has not yet been characterized at the molecular level (Hung, Warnken, & Santschi, 2005).

Kenne & Lindberg (1983) reported that uronic acids, e.g. glucuronic and galacturonic acids, are common components of bacterial extracellular polysaccharides. Other important polysaccharides, lipopolysaccharides with phosphate groups, have also been isolated from other *Pseudomonas* species (De Castro et al., 2001; Kachlany et al., 2001). Therefore, the results reported here demonstrate that *Ps. Fluorescens Biovar II* likely produces similar common polysaccharides and anionic groups as other *Pseudomonas* species.

Extracellular polysaccharides play an important role as heavy metal biosorbents, whose effectiveness heavily depend on their chemical composition, molecular architecture, and metal binding capacity. Adsorption of heavy metals on the EPS is mainly based on the binding of ionic species to cell surface-associated functional groups of extracellular polysaccharides and proteins (Mullen et al., 1989). The EPS produced by *Ps. fluorescens* Biovar II, containing a high percentage of polyanionic compounds, uronate, sulphate and phosphate, and proteins, may thus be an excellent biosorbent for heavy metals or radionuclides.

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