Chemical characterization of *Pseudomonas veronii* 2E soluble exopolymer as Cd(II) ligand for the biotreatment of electroplating wastes

Maria L. Ferreira [a, b], Adriana C. Casabuono [c], Sabrina T. Stacchiotti [c], Alicia S. Couto [c], Silvana A. Ramirez [a], Diana L. Vullo [a, b, *]

[a] Área Química, Instituto de Ciencias, Universidad Nacional de General Sarmiento, J.M. Gutierrez 1150, B1613GSX, Los Polvorines, Buenos Aires, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas, Av. Rivadavia 1917, C1033AAJ, Buenos Aires, Argentina
[c] Universidad de Buenos Aires, Facultad de Ciencias Exactas y Naturales, Departamento de Química Orgánica - Consejo Nacional de Investigaciones Científicas y Técnicas, Centro de Investigación en Hidratos de Carbono (CIHIDECAR), Buenos Aires, Argentina

ARTICLE INFO

ABSTRACT

The production, isolation, purification and characterization of soluble Extracellular Polymeric Substances (EPS) secreted by *Pseudomonas veronii* 2E were studied. Optimal production was obtained in medium M9-14 g l^{-1} glycerol, 25 °C. Spectrophotometric and potentiometric titration studies showed that EPS consisted on extracellular DNA, proteins and polysaccharides as the main components. DNase I, Proteinase K and further sodium deoxycholate treatments yielded similar proportions of lipopolysaccharides (LPSs) and exopolysaccharides (ExPs). ExP was subjected to total acid hydrolysis and then analyzed by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD), showing the presence of fucose, galactosamine, glucosamine, galactose, glucose, mannose and glucuronic acid. Further treatment with oxalic acid confirmed the presence of a pyruvilated sugar. The ability of the secreted products to interact with Cd(II) was studied by anodic stripping voltammetry (ASV). LPS fraction contained the highest ligand concentration (279 ± 10 μmoles Cd(II) binding sites g LPS^{-1}). This information is a valuable tool for the application of EPS in industrial wastewater treatment.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

Several industrial activities release amounts of metals such as cadmium, chromium, cobalt, copper, nickel and zinc to the environment. In particular, discharges from extractive metallurgy and metal finishing processes typically contain high concentrations of such dissolved metals. The most common methods for metal removal include chemical precipitation, solvent extraction, dialysis, electrolytic extraction, ion-exchange resins, membrane process, carbon and zeolite adsorption and ultrafiltration (Farooq et al., 2010). Some of these treatments are very expensive and non-applicable in remediation processes on site (*in situ*). Moreover, these methods are not effective when an extremely low concentration of metal in the treated effluent is required according to the local legislations. The increasing concern for metal contamination and the need for sustainable and environmental friendly methods have encouraged the search of new procedures for metal removal. From this point of view, biological treatment processes based on biosorption and bioaccumulation are presented as a more efficient and innovative technologies (Lloyd and Lovley, 2001; Valls and de Lorenzo, 2002; Vullo et al., 2008; Gadd, 2010).

The extracellular polymeric substances (EPS) are an example of the survival strategies developed by bacteria in metal polluted environments. EPS are microbial products associated to (bound) or secreted outside (soluble) the cell surface that form the framework of microbial mats. EPS are typically composed by polysaccharides, lipids, proteins and DNA in small quantities (Nielsen and Jahn, 1999; Wingerder et al., 1999). Polysaccharides are the major component of EPS matrix (Frølund et al., 1996), with a molecular weight from 1 to 5000 kDa (Freitas et al., 2011). Monomers like hexoses, pentoses, uronic acids and deoxysugars are commonly present in exopolysaccharides while phosphate, sulphate, acetate

and pyruvate are characteristic functional groups of the primary structure (Sutherland, 1997, 2001; Meisen et al., 2008). Ionisable functional groups on EPS matrix (carboxylate, phosphate, amino and hydroxyl groups) can strongly bind a wide range of metals. Thus, EPS may be considered as natural detoxifying compounds of polluted water (Loaëc et al., 1997; Guibaud et al., 2005, 2008; Comte et al., 2006c; Pal and Paul, 2008; Weia et al., 2011; More et al., 2014). Although the role of EPS present in sludge flocs has been widely reported in different articles (Xuan et al., 2010; Geyik and Çeçen, 2015; Bao et al., 2016), there is still very limited information on the interaction of metallic ions with soluble EPS extracted from bacterial cultures. Soluble EPS-metal interactions contribute to metal speciation in natural or engineered environments, representing an alternative strategy for metal retention in soluble systems.

The aim of this work is focused on the production of *Pseudomonas veronii* 2E soluble EPS, the chemical characterization and the evaluation of Cd-EPS interactions, considering EPS as a potential tool for metal loaded wastewater biotreatments.

2. Materials and methods

2.1. Microorganism

Pseudomonas veronii 2E is an autochthonous bacterium isolated from sediments associated to the Reconquista River basin (Buenos Aires Metropolitan Area). This strain was identified by 500 bp 16S r-RNA gene sequencing and is able to retain Cd(II), Zn(II) and Cu(II) and biotransform Cr(VI) from aqueous systems, as described in previous studies (Vullo et al., 2008; Garavaglia et al., 2010; Mendez et al., 2011).

2.2. Production of soluble EPS by P.veronii 2E

For the effect of carbon and nitrogen sources on soluble EPS production test, an enriched medium PY (g l^{-1}: 2.5 peptone, 1.25 yeast extract) and a minimal medium M9 (g l^{-1}: 7.3 K$_2$HPO$_4$, 3.0 KH$_2$PO$_4$, 6.6 NH$_4$Cl, 3.3 NaCl, supplemented with 0.1 yeast extract) were used. In both culture media, sodium citrate, glutamate and succinate with final concentration of 5 g l^{-1}, 14 g l^{-1} glycerol or 20 g l^{-1} glucose were used as carbon source. In PY media a final concentration of glucose in a range 0.5–100 g l^{-1} was assayed.

P. veronii 2E was grown in M9 at two temperatures (25 °C and 32 °C) with different carbon sources. First, *P. veronii* 2E was pre-cultured in a 50 ml medium at 32 °C or 25 °C and 120 rpm until late exponential phase of growth. A total 10 ml culture was transferred into another 100 ml M9 medium and incubated in the same conditions of the pre-culture for 48 h. A non-inoculated medium was used as negative control. In all cases, biomass was estimated by OD at 600 nm.

2.3. Soluble EPS extraction and purification

A scheme of the EPS extraction and purification process is shown in Fig. 1. Cells were centrifuged (7000 g) and the soluble EPS in culture supernatants were precipitated by adding 2.2 vol of cold ethanol 95% to keep overnight at −20 °C. The resulting precipitated EPS were recovered by centrifugation (7000 g, 20 min, 4 °C). The precipitation step was repeated three times for EPS purification. EPS content in the final fraction was determined by dry weight (50 °C up to constant weight). EPS content was expressed as g EPS (l OD)$^{-1}$. The culture medium that yielded the maximum EPS production was applied in the following studies.

Before chemical analysis, the soluble EPS were centrifuged and pellets were redissolved in the minimum volume of ultrapure water (18 MΩ cm, Millipore). Then the resultant solutions were dialyzed through a cellulose membrane (Sigma Aldrich, cut off 12,400 Da) against ultrapure water for 48 h to remove low molecular weight impurities. The fraction was called EPSpp (Fig. 1).

2.4. Polysaccharide fraction enrichment

To obtain the polysaccharide fraction of the soluble EPS, the solution was lyophilized and redissolved in 50 mM Tris buffer (Tris(hydroxymethyl)aminomethane, pH 7.5) supplemented with 10 mM MgCl$_2$. DNase was added to a final concentration of 7 μg ml^{-1} and incubated at 32 °C for 4 h. After this operation, Proteinase K was added (2.5 μg ml^{-1}) and the fraction was incubated overnight (32 °C). The mixture was then heated at 60 °C for 1 h and centrifuged at 7000 g for 20 min at 4 °C. The soluble fraction was precipitated with ethanol and dialyzed as described in the previous section. The polysaccharide-enriched EPS (EPSenz) was lyophilized and stored at −20 °C (see Fig. 1).

In order to separate and purify exopolysaccharides (Kachlany et al., 2001), the EPSenz was dissolved in 0.05 M Tris containing 0.1 M NaCl. Sodium deoxycholate (DOC) was added to a final concentration equivalent to 0.75 CMC (critical micellar concentration). The mixture was incubated for 15 min at 65 °C, and then cooled on ice. Acetic acid was added to a final concentration of 1% (v/v), and the mixture was centrifuged (20000 g, 5 min) to precipitate LPS. The supernatant containing the purified exopolysaccharide was dialyzed against a solution containing 4 mM Tris in 10% ethanol, then against ultrapurewater and lyophilized. This fraction was called ExP (Fig. 1).

The precipitate was repeatedly dialyzed (3500 cut off membrane) against a solution containing 4 mM Tris in 10% ethanol, then against ultrapure water and finally lyophilized (LPS).

2.5. Soluble EPS chemical characterization

Different assays were used to determine the composition of soluble EPS. Triplicate measurements were performed for quantification of neutral sugar, proteins, uronic acids and phosphorus of extracellular polymeric substances.

The procedures are summarized in Fig. 1.

2.5.1. Spectrophotometric methods
2.5.1.1. Protein content.
Protein content was determined by the Bradford protein assay (Sedmak and Grosserberg, 1977), with Bovine Serum Albumin 0.05% (w/v) solution as standard.

2.5.1.2. Carbohydrate content.
Content of neutral sugars was determined using the Anthrone/H$_2$SO$_4$ method (Frølund et al., 1996), with glucose solution 0.01% (w/v) as standard. Absorbance was measured at 578 nm.

2.5.1.3. Uronic acid content.
Sulfamate/m-hydroxydiphenyl method was used (Blumenkrantz and Asboe-Hansen, 1973; Filisetti-Cozzi and Carpita, 1991). A 700 μl sample was mixed with 40 μl of 4 M sulfamic acid (pH 1.6) and 2.5 ml of 75 mM of Na$_2$B$_4$O$_7$ in H$_2$SO$_4$(c). Samples were placed in water bath at 100 °C for 20 min and then cooled at room temperature. 80 μl of 0.15% (w/v) m-hydroxydiphenyl in 0.5% (w/v) NaOH were added. D-galacturonic acid was used as standard. Absorbance was measured at 525 nm.

2.5.1.4. Total phosphorus.
Total phosphorus was determined using Molibdenum Blue method (Bray and Kurtz, 1945). A 1 ml of sample was mixed with 1 ml of Molibdeum solution (1.66% (w/v) (NH$_4$)$_2$MoO$_4$ in 6% (v/v) H$_2$SO$_4$) and 0.6 ml of 1% (w/v) ascorbic acid. A 5 ppm KH$_2$PO$_4$ solution was used as standard. Absorbance was measured at 660 nm after 15 min.

100 mL de *P. veronii* 2E growing in M9 glycerol 14 g l⁻¹ , 48 h, 25 °C

1. 7000 *g* centrifugation, 4 °C, 20 min
2. 3X precipitation with 2.2 vol of ethanol
3. Purified with dialysis membrane (12400 Da), 20 °C, 48 h

Characterization

-Spectrophotometric methods   -Acid-Base titration

-CC (ASV)   -SDS-PAGE

EPSpp

1. Enrichment of sugar fraction by enzimatic treatment
2. Precipitation with 2.2 vol of ethanol
3. Purified with dyalisis membrane (12400 Da), 20 °C, 48 h

Characterization

- Spectrophotometric methods   -DOC-PAGE

-SDS-PAGE   -CC (ASV)

EPSenz

0.75 CMC DOC, 65 °C, 15 min

ExP

Hydrolysis (2M TFA, 120 °C, 2h)

0.5 M oxalic acid, 100 °C, 90 min

-DOC-PAGE
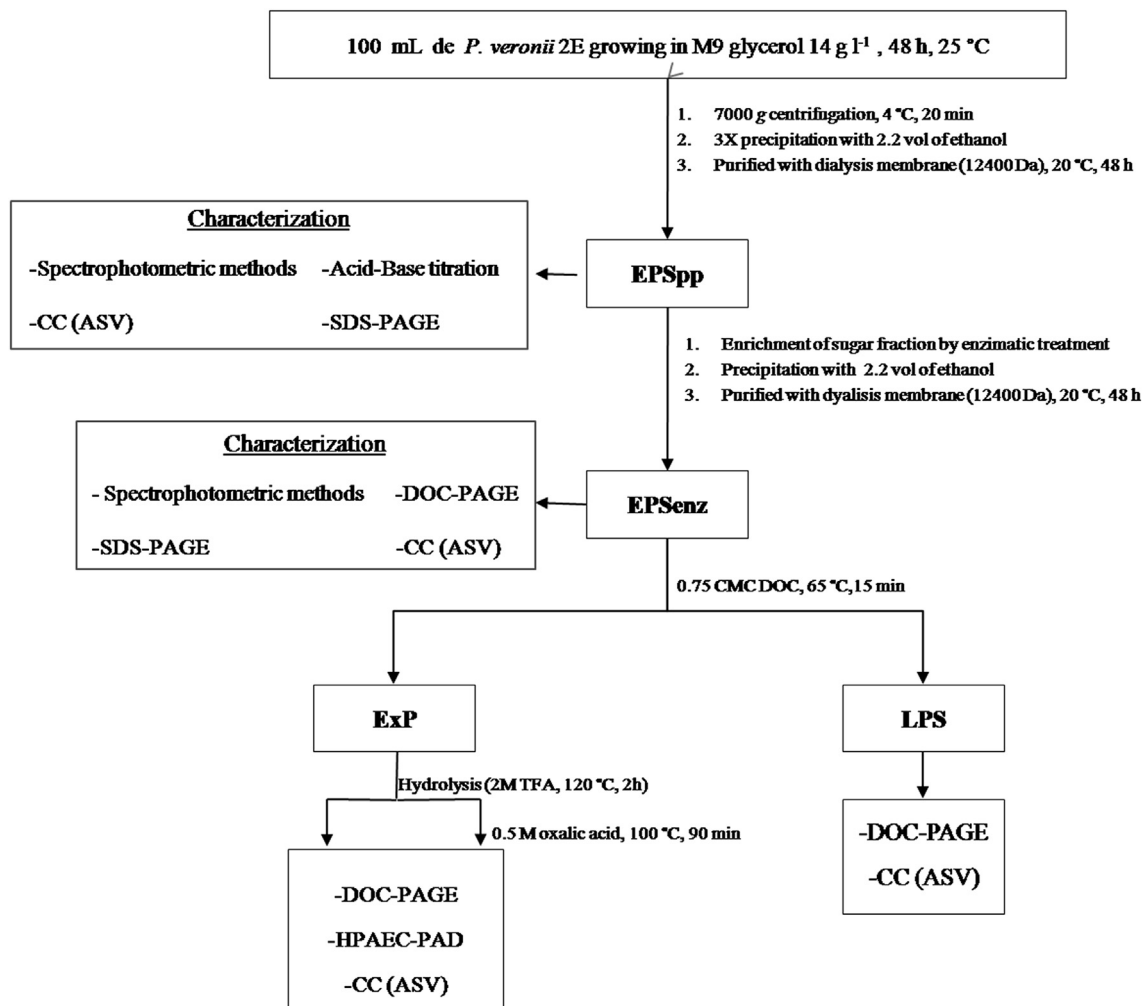
-HPAEC-PAD

-CC (ASV)

LPS

-DOC-PAGE

-CC (ASV)

Fig. 1. Extraction and characterization of soluble EPS produced by *P. veronii* 2E. EPSpp: partially purified EPS; EPSenz: EPSpp after enzymatic treatment; ExP: exopolysaccharide fraction and LPS: lipopolysaccharide fraction.

2.5.2. Acid-base properties

Two separate acid-base titrations were performed to determine the EPS pK_a values using 0.036 M HCl and 0.038 M NaOH. A volume of 25 ml of EPS solutions (0.6 mg EPS ml⁻¹) was titrated by adding in 50 μl increments of HCl or NaOH solutions. pH was measured with a 827 Metrohm pH lab at room temperature.

2.5.3. Analysis by SDS-PAGE and DOC-PAGE

Electrophoretic analysis was performed with a Bio-Rad Mini-Protean II cell. For protein analysis, a 12% SDS-PAGE analysis was carried out at 110 V. The gel was stained with Coomassie Brilliant Blue G250.

Deoxycholic acid- PAGE (DOC-PAGE) electrophoresis were performed on 4% stacking and 12% resolving gels. Run conditions were 17 mA (stacking gel) and then 24 mA for the resolving gel. Also 4% stacking with 18% resolving gels were assayed at 100 V (Kachlany et al., 2001). The gels were visualized either with the silver staining method, according to Tsai and Frash (1982) with modifications (Krauss et al., 1988; Guard-Petter et al., 1995) or with Alcian Blue-silver staining (Reuhs et al., 1998).

2.5.4. Analysis of sugar components

The determination of the sugar residues was carried out by High pH Anion Exchange Chromatography (HPAEC-PAD). After total hydrolysis of the oligosaccharides with 2 M trifluoroacetic acid at 120 °C for 2 h, fractions were lyophilized and analyzed in a DX-3000 Dionex BioLC system with pulsed amperometric detection (HPAEC-PAD; Dionex Corp., Sunnyvale, CA). Separation of the monosaccharides was carried out on a CarboPac PA-20 column (150 × 0.3 mm) with a PA-20 precolumn (30 × 3 mm) and a 20 μl injection loop.

The following conditions were employed: for neutral sugar analysis, an isocratic elution with 16 mM NaOH at 0.4 ml min⁻¹ was used with 2-deoxyglucose as internal standard. For acidic sugars analysis, an isocratic elution with 50 mM NaOH and 100 mM sodium acetate at 0.4 ml min⁻¹ was used, and galacturonic acid was added as internal standard. The average from triplicate injections was calculated for each sample. Also, quantifications with external standard were performed. Peaks were identified by comparison with commercial standards, and a sample of *Brown algae* 516 MA sodium alginate was used to obtain D-mannuronic acid and D-guluronic acid standards.

An additional treatment was introduced to recognize pyruvilated sugars. After total hydrolysis of ExP, oxalic acid 0.5 M was added to the sample, heated at 100 °C for 90 min and further analyzed by HPAEC.

2.6. Soluble EPS as Cd(II) ligand

The ability of EPSpp to complex Cd(II) was studied in the pH

range of 5.5–9.2 using an electrochemically monitored titration where the non-bound metal was measured after the addition of increasing amounts of a standard Cd(II) solution (10 ppm or 100 ppm). An EPSpp mass of 0.65 mg was dissolved in 15 ml of the following buffer solutions, prepared with ultrapure water: 0.01 M MES (2-[N-morpholino]ethanesulfonic acid) for pH 5.5 and pH 6.2, 0.01 M HEPES(N-[2-hydroxyethyl]piperizine-N`-[2-ethanesulfonic acid] for pH 7.5 and 8.1, and 0.01 M Borax (sodium borate) for pH 9.2. A final concentration of 0.05 M KNO_3 was added as supporting electrolyte in all cases. These buffers were chosen because of the proved absence of Cd(II) complexing capacity (Ceretti et al., 2010). After each Cd(II) addition, the mixture was stirred during 5 min for equilibration (determined in preliminary experiments). All the titrations were monitored by Square Wave Anodic Stripping Voltamperometry (SW-ASV), applying −0.75 V during 2 min to achieve Cd(II) preconcentration. Each measurement was performed twice with good reproducibility.

Interaction of EPSenz, ExP and LPS with Cd(II) was studied at pH 7.5, and the conditions were identical to those described above for the EPSpp fraction (see Fig. 1).

The calculation of the number of binding sites and the conditional complexation constants was carried out using Ružić and Scatchard linearization (Scatchard, 1949; Ružić, 1996).

A 663VA polarographic stand and an Autolab PGStat10 potentiostat (Metrohm - Ecochemie) were used, both instruments controlled by the GPES software. A three electrode cell containing a mercury drop electrode was used to perform the titrations. All potentials were referred to an Ag/AgCl reference electrode (3 M KCl). Measurements were performed at $(25 \pm 1)\,^{\circ}C$ under N_2 atmosphere.

3. Results

3.1. Production of soluble EPS by P.veronii 2E

In PY medium, the increase in glucose concentration promoted biopolymer production up to 20 g l^{-1} glucose (Fig. 2a). Surprisingly, the highest glucose concentration evaluated (100 g l^{-1} glucose), did not allow cell growth and hence no EPS production was observed. Compared to PY, soluble EPS production (Fig. 2b) in the minimal medium M9 with NH$_4$Cl as nitrogen source is higher. These results show that inorganic nitrogen had a greater influence on EPS production in comparison with an organic nitrogen source as peptone. No matter the carbon source, EPS production was higher in M9 and maximal when using 5 g l^{-1} sodium citrate and 14 g l^{-1} glycerol.

To evaluate the effect of temperature on biopolymer production, bacterial growth in M9 medium containing different carbon sources was evaluated at 25 °C and 32 °C. Results in Fig. 3 confirmed that the production of biopolymers was clearly affected by temperature. At 25 °C the biopolymer production was enhanced in M9-14 g l^{-1} glycerol and M9-5 g l^{-1} sodium glutamate, while much smaller amounts of EPS were excreted with sodium succinate, sodium citrate or glucose. Optimal results were obtained using M9-14 g l^{-1} glycerol at 25 °C (2.99 ± 0.04 g EPS (l OD)$^{-1}$), for it was used in further studies.

3.2. Soluble EPS chemical characterization

The EPSpp excreted in M9-14 g l^{-1} glycerol was characterized and results of the spectrophotometric determinations are summarized in Fig. 4.

EPSpp contained carbohydrates 48% (m/m), protein 37% (m/m) and total phosphorus 14% (m/m). Uronic acids were also present in low concentration (ca. 1%). These results clearly indicate that EPS is a complex matrix of biopolymers with carbohydrates as main components.
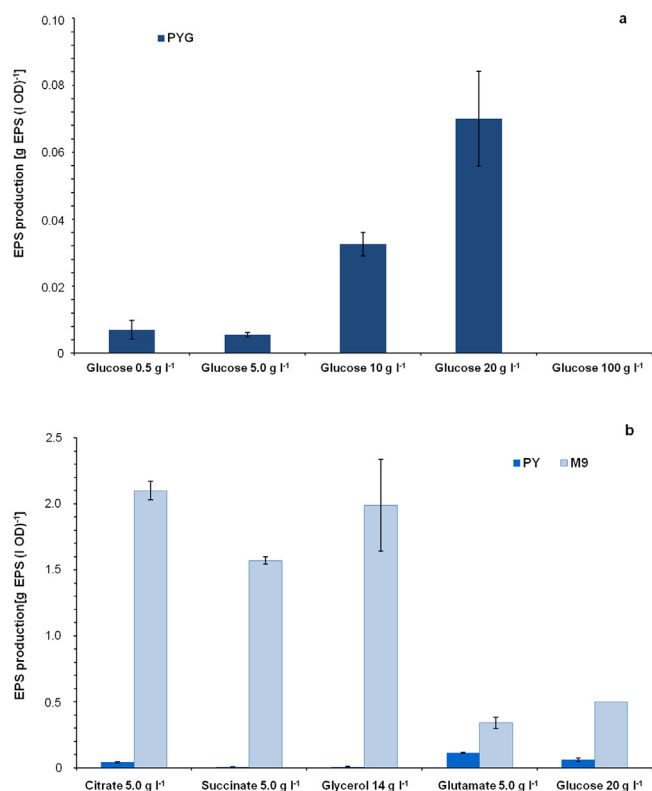

Fig. 2. Effect of carbon and nitrogen sources on soluble EPS production: a. Effect of glucose concentration in PY media. b. Effect of PY or M9 media with different carbon sources.
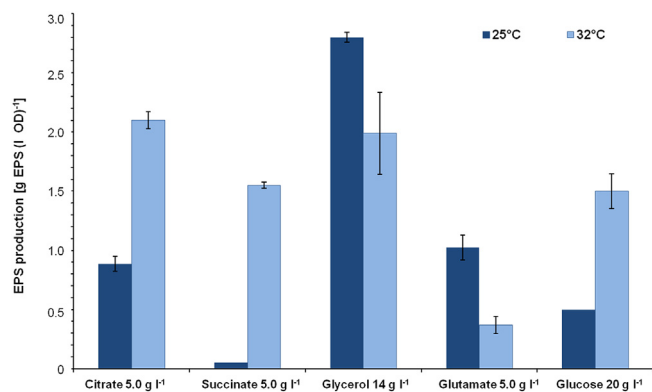

Fig. 3. Effect of temperature on soluble EPS production in M9.

Four pKa values were determined from the analysis of the first and second derivate of the EPSpp titration curves (see supplementary material), all of them corresponding to proton binding sites compatible to weak acids: pKa$_1$ = 3.6, pKa$_2$ = 6.2, pKa$_3$ = 7.0 and pKa$_4$ = 8.1. Considering the chemical composition obtained from spectrophotometric results and the possible functional groups involved, pKa$_1$ can be assigned to carboxylic acid (Comte et al., 2006b; Guibaud et al., 2006) and phosphate diester groups (Martinez et al., 2002; Barkleit et al., 2008), pKa$_2$ and pKa$_3$ to phosphate monoester groups (Barkleit et al., 2008) and carboxylic acid groups (Guibaud et al., 2003, 2006); and pKa$_4$ to amino groups (Comte et al., 2006b; Guibaud et al., 2006; Braissat et al., 2007).

An enzymatic purification was performed on EPSpp to study the main component polysaccharides. The EPSpp was treated with
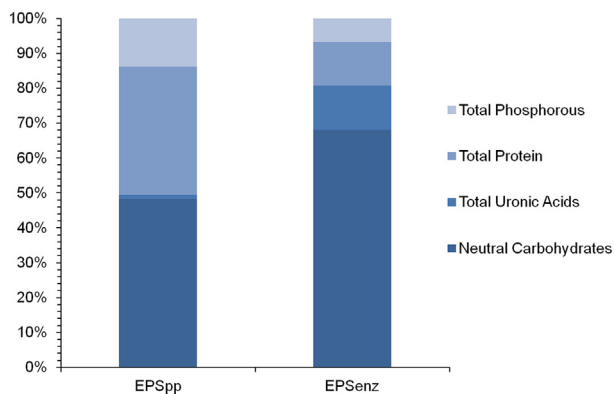
Fig. 4. Percentage composition of EPSpp and EPSenz.

DNase and Proteinase K to ensure the removal of DNA and proteins. Enrichment in the polysaccharide fraction accompanied by a decrease in protein and phosphorus content was observed as a result of enzymatic treatment (Fig. 4). The decrease of protein content was confirmed by SDS-PAGE (polyacrilamide gel electrophoreris) stained with Coomassie Brilliant Blue G250 (see supplementary material).

A deoxycholate polyacrylamide gel electrophoresis (DOC-PAGE) carried out with the EPSenz fraction was revealed with a combination of silver staining, to detect only LPS, and Alcian blue/silver staining to detect both LPS and ExP. This experiment confirmed the presence of LPS in EPSenz (see supplementary material). A ExP:LPS ratio ca. 1:1 was detected (55%–45%).

HPAEC columns were used for the separation of monosaccharides present in ExP after total acid hydrolysis. The results showed that most of the soluble EPS secreted by *P. veronii* 2E in M9 with glycerol as carbon source were composed of the neutral sugars fucose ($t_R = 3.84$), galactose ($t_R = 9.67$), glucose ($t_R = 10.41$) and mannose ($t_R = 11.71$). Aminosugars such as galactosamine ($t_R = 6.65$) and glucosamine ($t_R = 7.90$) were also detected (Fig. 5).

The analysis of uronic acids in ExP exposed the presence of two important peaks (Fig. 6). One of them was recognized as glucuronic acid ($t_R = 12.98$). To characterize the peak at $t_R = 6.38$, oxalic acid treatment after acid hydrolysis revealed a pyruvilated sugar, then confirmed as mannose by the detection of the released pyruvate and the mannose fraction increase (Cavallero et al., 2015).

3.3. Soluble EPS as Cd(II) ligand

EPSpp, EPSenz, ExP and LPS interactions with Cd(II) were characterized at pH 7.5 and the ASV- monitored titration curves of the different fractions are shown in Fig. 7.

Fig. 7a is an example of a typical titration curve. The first curved zone corresponds to the complexation equilibrium originated in the interaction between Cd(II) and EPSpp. The linear zone corresponds to the saturation of the binding sites. The EPSpp conditional binding constants and the number of binding sites were determined at pH 5.5, 6.2, 7.5, 8.1 and 9.2. Cadmium hydroxide precipitation was not observed, evidence of the complexation with the organic ligands.

The analysis of the titration curves by Ružić and Scatchard linearizations (Scatchard, 1949; Ružić, 1996) allowed the determination of the conditional constant K' and the concentration of binding sites for EPSpp. The linear behaviour observed in Fig. 8 corresponds to the formation of a 1 metal: 1 ligand complex at pH 7.5. The conditional binding constant K' and the concentration of binding sites Lt were obtained from the slopes of the Scatchard and Ružić plots, respectively (Scarponi et al., 1996).
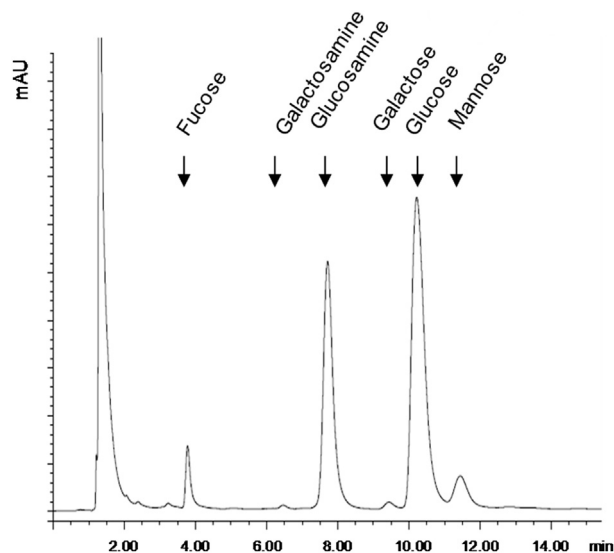

Fig. 5. HPAEC-PAD analysis for ExP. Monosaccharides present in EPS produced by *P. veronii* 2E in M9 medium with glycerol as carbon source.
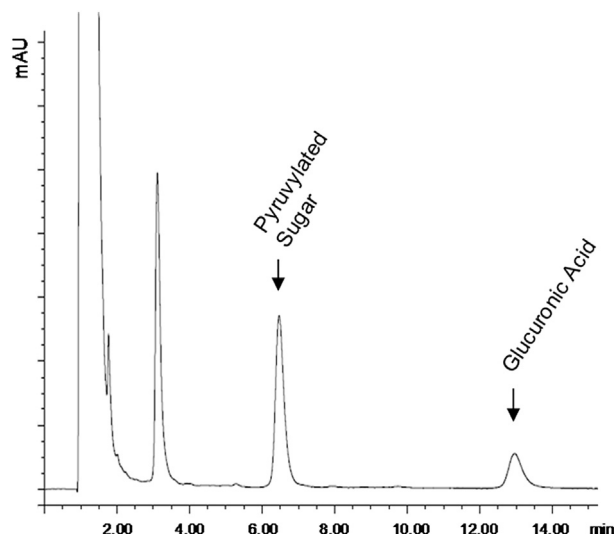

Fig. 6. HPAEC-PAD analysis for ExP. Acid sugars present in EPS produced by *P. veronii* 2E in M9 medium with glycerol as carbon source.

Table 1 summarizes the conditional binding constants (expressed as Log K') and the number of binding sites (expressed as Cd(II) μmoles (g EPSpp)$^{-1}$) for all the evaluated pH. Log K' was 5.46 ± 0.09 for pH 6.2–9.1 and ligand concentration was 60 ± 8 μmoles of Cd(II) binding sites (g EPSpp)$^{-1}$. At pH 5.5 no interaction between Cd(II) and EPSpp was detected. The strength of Cd-EPSpp interaction can be considered as moderate in the pH range evaluated. EPS contained ionisable functional groups which could be considered the potential binding sites for the interaction with metal ions.

The titration curve for EPSenz fraction is showed in Fig. 7b. In this case, ligand concentration was $[L]_t = 496 \pm 14$ μmoles of Cd(II) binding sites (g EPSenz)$^{-1}$. This result showed that, after the enzymatic treatment, there was an enrichment of cadmium binding sites. The conditional constant obtained was consistent with one family of moderate ligands (Log K' 5.7 ± 0.1), a value similar to EPSpp conditional constant.

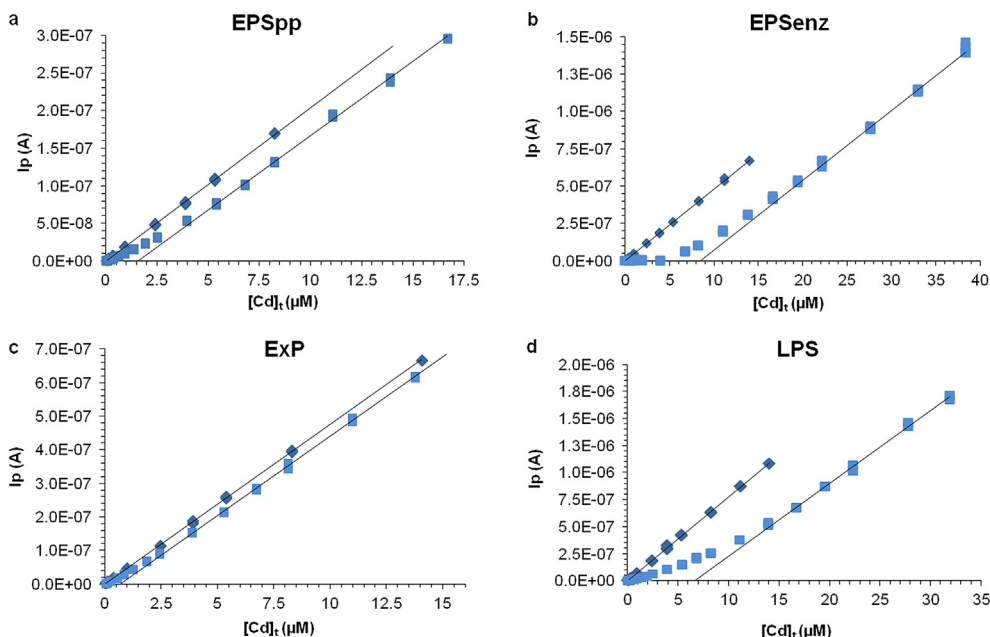Fig. 7c and d show titration curves obtained for the two fractions

Fig. 7. ASV monitored titration curves of EPSpp (a), EPSenz (b), ExP (c) and LPS (d). ($t_{deposition}$ 30 s, $E_{deposition}$ −0.75 V, $t_{equilibrium}$ 5 min, buffer HEPES pH 7.5). ($\blacklozenge$) buffer HEPES, ($\blacksquare$) titration curve with Cd(II). Cd(II) was used as titrant agent. Ip is peak current and $[Cd]_t$ is Cadmium total concentration.
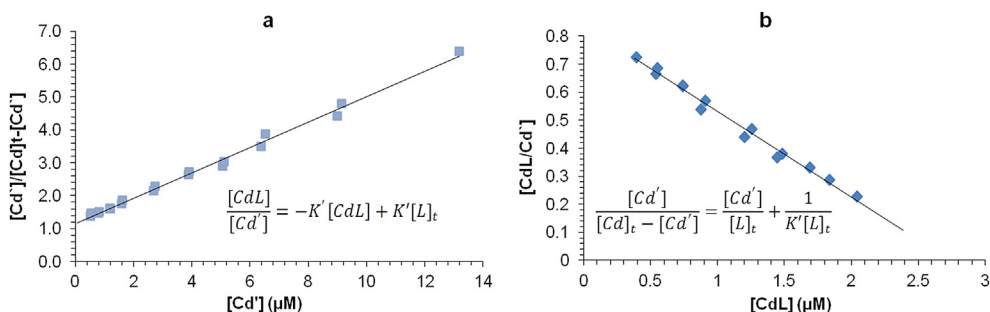

Fig. 8. Calculation of K'and Lt from the ASV monitored titration curve, pH 7.5 from Ružić (a) and Scatchard (b) linearizations. The corresponding equations are given. [Cd'] is free metal concentration; [CdL] is Cadmium complex concentration and $[Cd]_t$ is cadmium total metal concentration.

Table 1
Conditional binding constants (Log K') and number of binding sites ($[L]_t$) expressed as μmoles of Cd(II) binding sites (g EPSpp)$^{-1}$ at different pHs.

	pH 5.5	pH 6.2	pH 7.5	pH 8.1	pH 9.2
Log K'	[a]	5.48 ± 0.05	5.47 ± 0.01	5.48 ± 0.08	5.61 ± 0.01
[L]$_t$	[a]	61 ± 2	65 ± 3	66 ± 6	48 ± 3

[a] Non observable.

ExP and LPS. The conditional constants determined were in the same order as for EPSpp and EPSenz. However, the ligand concentration in LPS ($[L]_t = 279 \pm 10$ μmoles of Cd(II) binding sites (g LPS)$^{-1}$) was higher in this fraction than the value found in ExP ($[L]_t = 16 \pm 1$ μmoles Cd(II) binding sites (g ExP)$^{-1}$), showing these sites mainly belonged to LPS.

4. Discussion

The production of EPS was affected by factors such as temperature, carbon and nitrogen sources. In previous research, it was shown that PY medium was optimal for the development of biofilms by *P.veronii* 2E (Daniel et al., 2016). For that reason, as a preliminary approach, the PY medium with peptone as nitrogen source was tested for soluble EPS production, resulting that an increase in glucose concentration caused an increase in biopolymer excretion. However, *P. veronii* 2E produced larger quantities of soluble EPS in M9 minimal medium.

The optimal conditions for production used glycerol as carbon source and room temperature (2.99 ± 0.04 g EPS (l OD)$^{-1}$). These conditions are relevant when considering a future application in biotechnological processes (Oliveira et al., 2007; Silva et al., 2009; Freitas et al., 2011; More et al., 2014). Minimizing thermostating by working at room temperature (25 °C) is desirable for the production of exopolymers due to lower energy requirements, resulting in cost reduction during a final bioreactor design. The use of glycerol, a by-product of biodiesel industry, could also contribute to a sustainable EPS production process.

In order to study the EPS composition, a simple extraction method using ethanol has been applied. Precipitation followed by centrifugation led to the greatest EPS recovery in short time with low costs. This simple method avoided the introduction of substances to the biopolymer, as reported by different authors (Klock et al., 2007; Chen et al., 2009; More et al., 2014). Also, when using ethanol in polysaccharide separation, other compounds such as proteins, extracellular DNA and LPS are extracted (D'Abzac et al.,

2010a). Thus ethanol precipitation was combined with a cut off 12,400 Da-dialysis to remove low molecular weight compounds. For this reason characterization of EPS was only focused on the high molecular weight polymer fraction.

Other requisite for a good EPS extraction is to avoid cell lysis. EPS can be easily contaminated with intracellular components if cell lysis occurs due to an inadequate speed during centrifugation (Fang and Jia, 1996). Several authors proposed that a high protein:polysaccharide ratio confirms the present of intracellular contaminants in EPS extracts (Liu and Fang, 2003; McSwain et al., 2005; Comte et al., 2006a; D'Abzac et al., 2010a). In this work, protein:polysaccharide ratio was clearly below 0.8 and hence ensured that the EPS excreted by *P. veronii* 2E were not contaminated by intracellular materials.

Analysis of *P. veronii* 2E soluble EPS showed that the major components are polysaccharides (48% dry mass). Proteins present in the EPSpp (37% total mass) were possibly related to exoenzymes or structural proteins entrapped in the biopolymer as described by Liu and Fang (2003), and Flemming and Wingender (2010). Phosphorus content could be related to extracellular DNA or lipopolysaccharides (LPSs) associated to the biopolymer (Wingerder et al., 1999; Comte et al., 2006b) since the determination of total phosphorus did not provide information about the P-compounds in EPS. The LPS contained oligosaccharides moieties that could interfere with the polysaccharide ExP structural analysis. Thus the EPSenz sample was treated with DOC to separate the LPS fraction. The insoluble fraction obtained after the DOC-treatment contained the extracellular purified precipitate (Epp) and the supernatant fraction extracellular polysaccharide (ExP). The ExP fraction corresponded to the purified polysaccharide fraction and Epp was mainly related to LPS. The lack of LPS in the ExP obtained after the DOC-treatment was confirmed by DOC-PAGE, suggesting that total phosphorus content was mainly related to the LPS contribution. In addition, the LPS fraction in the *P. veronii* 2E soluble EPS obtained in this work, should not be disregarded as potential Cd(II) ligand as it represented a 45% of the EPS dry weight. Kachlany et al. (2001) reported that the soluble EPS produced by *Pseudomonas putida* G7 were also formed by LPS, but in this case the LPS fraction was not considered for metal interaction studies despite the non negligible potential binding capacity. This work demonstrated that EPS produced by *P.veronii* 2E corresponded to a heteropolysaccharide structure and besides their monosaccharide compositions that were mainly similar to those reported for other *Pseudomonas* (Celik et al., 2008; Chen et al., 2009; Freitas et al., 2009; Kachlany et al., 2001), some different monosaccharides or their derivatives were found. The study of the monosaccharides present in ExP by HPAEC-PAD showed that the ExP was composed by a variety of sugars (fucose, galactose, glucose, mannose, galactosamine, glucosamine, glucuronic acid and pyruvilated mannose). This differential composition of soluble EPS was not reported before for *Pseudomonas* genus. Further experiments on a deeper structural analysis by NMR should not be disregarded for an integral description of the backbone sequences.

The potentiometric titrations performed on soluble EPSpp showed four pKas. pKa values were in agreement with the functional groups detected by spectrophotometry and proved the presence of carboxylate and phosphate groups. The first buffer zone, pH close to 4, can be assigned to carboxylic acid groups present in polysaccharides and uronic acids or phosphate monoester groups from LPS or extracellular DNA (Yee and Fein, 2001; Martinez et al., 2002; Comte et al., 2006b; Guibaud et al., 2006; Barkleit et al., 2008; D'Abzac et al., 2010b). pKas 2 and 3 were assigned to phosphate diester groups and carboxylic acid of extracellular DNA and LPS (Guibaud et al., 2003, 2006; Barkleit et al., 2008). pKa4 is characteristic of amine groups of proteins

(Guibaud et al., 2005; Comte et al., 2006b; Braissat et al., 2007). These functional groups are clearly involved in acid-base equilibrium, and can be either neutral or anionic depending on pH. Considering this, pH is expected to affect protonation of polyfunctional macromolecules such as EPS, conferring negative charge and, as consequence, the ability to interact with metals.

Cd(II)-EPSpp interaction was evaluated as a function of pH. pH changes affect both the metal and ligand. In fact pH is considered crucial due to the cation-proton competition for the binding sites (Guibaud et al., 2008). Results in Table 1 show no significant pH effect neither on the concentration of Cd(II) binding sites nor in the strength of the interaction as given by the conditional stability constant K'. In fact, one family of binding sites of moderate strength was detected, and the values of conditional formation constant were similar to those reported for other EPS from activated sludge (Guibaud et al., 2003; Comte et al., 2006b, 2008, 2006c). pH did not significantly affect Cd(II) binding. Independence of pH in Cd(II)-EPS interaction is beneficial to an electroplating waste biotreatment since pH would not be a variable needed to be strictly controlled. To explain these results, it should be kept in mind that EPS are polymer molecules continuously interacting with each other and with other molecules present in their surroundings, so conformational changes that may occur within the polymer should also be taken into account. According to Yokoi et al. (1986) and Morlay et al. (1998) in polyacid polymers, the interaction cation-binding sites cannot be explained as a pure ion exchange process: the availability of the functional groups related to the polymer spatial conformation is also a determinant factor. If such would be the case, Cd(II) binding sites in EPS polymer molecules may not be completely available as a result of the three dimensional structure adopted. At pH 5.5 Cd(II)-EPSpp interaction was not observed. Similar results were found by Comte et al. (2008) working with EPS extracted from activated sludge, and no Cd(II)-EPS interaction was observed at pH 4. K' obtained from the ExP and LPS fractions had similar values. In previous studies (Mendez et al., 2011), a metal concentration-dependent Zeta Potential (ζ) increase confirmed bacterial surface components (EPS and LPS)-Cd(II)/Zn(II) interactions. Future ζ measurements, FTIR and fluorescence spectroscopy (EEM, excitation-emission matrix) will help understanding which soluble EPS functional groups are specifically involved in Cd(II) interaction (Yin et al., 2011; Pan et al., 2012).

The more notorious difference remained in the concentration of Cd(II)-binding sites, which is mainly related to LPS structure. The content of phosphate groups in LPS may represent the fraction that would be mainly involved in Cd(II) binding. In fact, it has been stated that the phosphoryl groups of the core and lipid A region of the LPS are the most important sites involved in metal binding for *P. aeruginosa* (Langley and Beveridge, 1999). However, the cost of isolation of LPS from soluble EPS and the purification for a sustainable development of a bioreactor for the elimination of Cd(II) should be not disregarded. The presence of functional groups with potential ability to interact with metals is a requisite to explore any material to be applied in a metal-treatment process. Concentration of cadmium binding sites is connected to the amount of metal that EPS may retain, and the value of the conditional stability constant is a measure of the strength of the metal-EPS interaction. These two parameters are relevant and should be taken into account before considering any test in a bioreactor.

The properties of the EPS currently present in sludge flocs, including hydrophobicity, adhesion, flocculation, settling and dewatering and consequently their relevant role in anaerobic bioreactors such as an upflow anaerobic sludge blanket (UASB) reactor were well studied (Xuan et al., 2010; Li et al., 2013; More et al., 2014; Bao et al., 2016). The use of soluble EPS in the development of bioreactors for the removal of metal ions has not been fully

investigated yet. However, future experiments are being planned regarding the production of *P. veronii* exopolymer in full scale and EPS immobilization for the implementation of a biotreatment of electroplating wastes for cadmium removal. This research is the essential first step that will allow the potential application of soluble EPS as Cd(II) sorbent.

5. Conclusions

In literature the study of soluble EPS is limited. In this work, the extraction and characterization of soluble EPS produced by *P. veronii* 2E was studied. The major EPS production was observed in minimal medium with glycerol as the only carbon source at 25 °C. This result is of interest because glycerol is the main residue in biodiesel production and it could be used in the future as an economical carbon source for the production of biopolymers. In addition, EPS extraction is simple and completed in a short time. On the other hand, the biochemical characterization showed that the polysaccharides not only represented the main compounds in the EPS, but also contained similar concentration of ExP and LPS. Despite Log K' values corresponding to EPSpp, EPSenz, ExP and LPS -Cd(II) interactions showed no differences, the LPS evidenced the highest retention capacity for Cd(II) complexation. Thus, LPS functional groups would play a key role in the Cd(II) biosorption phenomena. Further research in the composition of the LPS present in EPS produced by *P. veronii* 2E will help in a deep understanding of the main functional groups and to which extent they are involved in Cd(II) binding.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibiod.2016.10.013.

References

Barkleit, A., Moll, H., Bernhard, G., 2008. Interaction of uranium(VI) with lipopolysaccharide. Dalton Trans. 2879–2886. http://dx.doi.org/10.1039/B715669C.

Bao, Z., Chen, X., Zhao, J., Lin, F., Li, J., Zhang, Y., 2016. Exploring the relationship between the EPS property and the toxicity of sludge for treating 4-chlorophenol synthetic wastewater in a sequencing batch reactor. Int. Biodeterior. Biodegrad. 110, 24–31. http://dx.doi.org/10.1016/j.ibiod.2016.02.003.

Blumenkrantz, N., Asboe-Hansen, G., 1973. New method for quantitative determination of uronic acids. Anal. Biochem. 54, 484–489.

Braissat, O., Decho, A., Dupraz, C., Glunk, C., Przekop, K., Visscher, P., 2007. Exopolymeric substances of sulfate-reducing bacteria: interactions with calcium at alkaline pH and implication for formation of carbonate minerals. Geobiology 5, 1–11. http://dx.doi.org/10.1111/j.1472-4669.2007.00117.x.

Bray, R.H., Kurtz, L.T., 1945. Determination of total, organic and available forms of phosphorus in soils. Soil Sci. 59, 39–46.

Cavallero, G., Casabuono, A.C., Ferreira, M.L., Vullo, D.L. Couto, A.S., 2015. Caracterización del exopolisacárido de *Pseudomonas veronii* 2E. Proceedings XX Simposio Nacional de Química Orgánica Sociedad Argentina de Investigación en Química Orgánica, p 86.

Celik, G.Y., Aslim, B., Beyatli, Y., 2008. Characterisation and production of exopolysaccharide (EPS) from *Pseudomonas aeruginosa* G1 and *Pseudomonas putida* G12 strains. Carbohydr. Polym. 73, 178–182. http://dx.doi.org/10.1016/j.carbpol.2007.11.021.

Ceretti, H.M., Vullo, D.L., Zalts, A., Ramírez, S., 2010. Effect of bacterial growth in the complexing capacity of a culture medium supplemented with cadmium(II). World J. Microbiol. Biotechnol. 26, 847–853. http://dx.doi.org/10.1007/s11274-009-0242-9.

Chen, X., Santschi, P.H., Schwehr, K.A., Hung, C.C., 2009. Optimized isolation procedure for obtaining strongly actinide binding exopolymeric substances (EPS) from two bacteria (*Sagittula stellata* and *Pseudomonas fluorescens* Biovar II). Bioresour. Technol. 100, 6010–6021. http://dx.doi.org/10.1016/j.biortech.2009.06.008.

Comte, S., Guibaud, G., Baudu, M., 2006a. Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and EPS complexation properties. Part I. Comparison of the efficiency of eight EPS extraction methods. Enzyme Microb. Technol. 38, 237–245. http://dx.doi.org/10.1016/j.enzmictec.2005.06.016.

Comte, S., Guibaud, G., Baudu, M., 2008. Biosorption properties of extracellular polymeric substances (EPS) towards Cd, Cu and Pb for different pH values. J. Hazard. Mater. 151, 185–193. http://dx.doi.org/10.1016/j.jhazmat.2007.05.070.

Comte, S., Guibaud, G., Baudu, M., 2006b. Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and complexation properties of Pb and Cd with EPS Part II. Consequences of EPS extraction methods on Pb^{2+} and Cd^{2+} complexation. Enzyme Microb. Technol. 38, 246–252. http://dx.doi.org/10.1016/j.enzmictec.2005.06.023.

Comte, S., Guibaud, G., Baudu, M., 2006c. Biosorption properties of extracellular polymeric subtances (EPS) resulting from activated sludge according to their type: soluble or bound. Process Biochem. 41, 815–823. http://dx.doi.org/10.1016/j.procbio.2005.10.014.

D'Abzac, P., Bordas, F., van Hullebusch, E., Lens, P., Guibaud, G., 2010a. Extraction of extracellular polymeric substances (EPS) from anaerobic granular sludges: comparison of chemical and physical extraction protocols. Appl. Microbiol. Biotechnol. 85, 1589–1599. http://dx.doi.org/10.1007/s00253-009-2288-x.

D'Abzac, P., Bordas, F., van Hullebusch, E., Lens, P., Guibaud, G., 2010b. Effects of extraction procedures on metal binding properties of extracellular polymeric substances (EPS) from anaerobic granular sludges. Colloids Surfaces B Biointerfaces 80, 161–168. http://dx.doi.org/10.1016/j.colsurfb.2010.05.043.

Daniel, M.A., Barrionuevo, M., Doyle, S., Vullo, D.L., 2016. Kinetics of *Pseudomonas veronii* 2E biofilm development under under different nutritional conditions for a proper bioreactor design. Biochem. Eng. J. 105, 150–158. http://dx.doi.org/10.1016/j.bej.2015.09.001.

Fang, H.H.P., Jia, X.S., 1996. Extraction of extracellular polymeric from anaerobic sludges. Biotechnol. Tech. 10, 803–808. http://dx.doi.org/10.1007/BF00154662.

Farooq, U., Kozinski, J., Khan, M., Athar, M., 2010. Biosorption of heavy metal ions using wheat based biosorbents-a review of the recent literature. Bioresour. Technol. 101, 5043–5053. http://dx.doi.org/10.1016/j.biortech.2010.02.030.

Filisetti-Cozzi, T., Carpita, N., 1991. Measurement of uronic acids without interference from neutral sugars. Anal. Biochem. 197, 157–162. http://dx.doi.org/10.1016/0003-2697(91)90372-Z.

Flemming, H.C., Wingender, J., 2010. The biofilm matrix. Nat. Rev. Microbiol. 8, 623–633. http://dx.doi.org/10.1038/nrmicro2415.

Freitas, F., Alves, D., Pais, J., Costa, N., Olivera, C., Mafra, L., Reis, M., 2009. Characterization of an extracellular polysaccharide produced by *Pseudomonas* strain grown on glycerol. Bioresour. Technol. 100, 859–865. http://dx.doi.org/10.1016/j.biortech.2008.07.002.

Freitas, F., Alves, V.D., Reis, M.A., 2011. Advances in bacterial exopolysaccharides: from production to biotechnological applications. Trends Biotechnol. 29, 388–398. http://dx.doi.org/10.1016/j.tibtech.2011.03.008.

Frølund, B., Palmgren, R., Keiding, K., Nielsen, P., 1996. Extraction of extracellular polymer from activated sludge using cation exchange resin. Water Res. 30, 1749–1758.

Gadd, G., 2010. Metals, minerals and microbes: geomicrobiology and bioremediation. Microbiology 156, 609–643. http://dx.doi.org/10.1099/mic.0.037143-0.

Garavaglia, L., Cerdeira, S., Vullo, D.L., 2010. Chromium (VI) biotransformation by beta- and gamma-*Proteobacteria* from natural polluted environments: a combined biological and chemical treatment for industrial wastes. J. Hazard. Mater. 15, 104–110. http://dx.doi.org/10.1016/j.jhazmat.2009.09.134.

Geyik, A.G., Çeçen, F., 2015. Variations in extracellular polymeric substances (EPS) during adaptation of activated sludges to new feeding conditions. Int. Biodeterior. Biodegrad. 105, 137–145. http://dx.doi.org/10.1016/j.ibiod.2015.08.021.

Guard-Petter, J., Lakshmi, B., Carlson, R., Ingram, K., 1995. On-farm monitoring of mouse-invasive *Salmonella enterica* serovar *enteritidis* and a model for its association with the production of contaminated eggs. Appl. Environ. Microbiol. 61, 2845–2851.

Guibaud, G., Bordas, F., Saaid, A., D'Abzac, P., van Hullenbusch, E., 2008. Effect of pH on cadmium and lead binding by extracellular polymeric substances (EPS) extracted from environmental bacterial strains. Colloids Surfaces 63, 48–54. http://dx.doi.org/10.1016/j.colsurfb.2007.11.002.

Guibaud, G., Comte, S., Bordas, F., Dupuy, S., Baudu, M., 2005. Comparison of the complexation potential of extracellular polymeric substances (EPS), extracted from activated sludges and produced by pure bacteria strains, for cadmium, lead and nickel. Chemosphere 59, 629–638. http://dx.doi.org/10.1016/j.chemosphere.2004.10.028.

Guibaud, G., Tixer, N., Bouju, A., Baudu, M., 2003. Relation between extracellular polymers' composition and its ability to complex Cd, Cu and Pb. Chemosphere 52, 1701–1710. http://dx.doi.org/10.1016/S0045-6535(03)00355-2.

Guibaud, G., van Hullebusch, E., Bordas, F., 2006. Lead and cadmium biosorption by extracellular polymeric substances (EPS) extracted from activated sludges: pH-sorption edge and mathematical equilibrium modelling. Chemosphere 64, 1955–1962. http://dx.doi.org/10.1016/j.chemosphere.2006.01.012.

Kachlany, S.C., Levery, S., Kim, J., Reuhs, B., Lion, L., Ghiorse, W., 2001. Structure and carbohydrate analysis of the exopolysaccharide capsule of *Pseudomonas putida*

G7. Environ. Microbiol. 3, 774–784. http://dx.doi.org/10.1046/j.1462-2920.2001.00248.x.

Klock, J.H., Wieland, A., Seifert, R., 2007. Extracellular polymeric substances (EPS) from cyanobacterial mats: characterisation and isolation method optimisation. Mar. Biol. 152, 1077–1085. http://dx.doi.org/10.1007/s00227-007-0754-5.

Krauss, J., Weckesser, J., Mayer, H., 1988. Structural analysis of the nontoxic lipid A of *Rhodobacter capsulatus* 37b4. Eur. J. Biochem. 38, 157–163. http://dx.doi.org/10.1111/j.1432-1033.1989.tb14677.x.

Langley, S., Beveridge, T., 1999. Effect of O-side-chain-lipopolysaccharide chemistry on metal binding. Appl. Environ. Microbiol. 65, 489–498.

Li, Z., Tian, Y., Ding, Y., Wang, H., Chen, L., 2013. Contribution of extracellular polymeric substances (EPS) and their subfractions to the sludge aggregation in membrane bioreactor coupled with worm reactor. Bioresour. Technol. 144, 328–336. http://dx.doi.org/10.1016/j.biortech.2013.06.127.

Liu, H., Fang, H.H.P., 2003. Influences of extracellular polymeric substances (EPS) on flocculation, settling, and dewatering of activated sludge. Crit. Rev. Environ. Sci. Technol. 33, 237–273. http://dx.doi.org/10.1080/10643380390814479.

Lloyd, J.R., Lovley, D.R., 2001. Microbial detoxification of metals and radionuclides. Curr. Opin. Biotechnol. 12, 248–253. http://dx.doi.org/10.1016/S0958-1669(00)00207-X.

Loaëc, M., Olier, R., Guezennec, J., 1997. Uptake of lead, cadmium and zinc by a novel bacterial exopolysaccharide. Water Res. 31, 1171–1179.

McSwain, B.S., Irvine, R.L., Hausner, M., Wilderer, P.A., 2005. Composition and distribution of extracellular polymeric substances in aerobic flocs and granular sludge. Appl. Environ. Microbiol. 71, 1051–1057. http://dx.doi.org/10.1128/AEM.71.2.1051-1057.2005.

Martinez, R., Smith, D., Kulczycki, E., Ferris, F., 2002. Determination of intrinsic bacterial surface acidity constants using a Donnan Shell model and a continuous pKa distribution method. Methods Surf. Charact. 253, 130–139. http://dx.doi.org/10.1006/jcis.2002.8541.

Meisen, S., Wingender, J., Telgheder, U., 2008. Analysis of microbial extracellular polysaccharides in biofilms by HPLC. Part I: development of the analytical method using two complementary stationary phases. Anal. Bioanal. Chem. 391, 993–1002. http://dx.doi.org/10.1007/s00216-008-2068-y.

Mendez, N., Ramírez, S., Ceretti, H., Zalts, A., Candal, R., Vullo, D.L., 2011. *Pseudomonas veronii* 2E surface interactions with Zn(II) and Cd(II). Glob. J. Environ. Sci. Technol. 1, 3.

More, T., Yadav, J.Y., Tyagi, R., Surampalli, R., 2014. Extracellular polymeric substances of bacterial and their potential environmental applications. Environ. Manag. 144, 1–25. http://dx.doi.org/10.1016/j.jenvman.2014.05.010.

Morlay, C., Cromer, M., Mouginot, Y., Vittori, O., 1998. Potentiometric study of Cu(II) and Ni(II) complexation with two high molecular weight poly(acrylic acids). Talanta 45, 1177–1188. http://dx.doi.org/10.1016/S0039-9140(97)00229-4.

Nielsen, P., Jahn, A., 1999. Extraction of EPS. In: Wingender, J., Neu, T., Flemming, H. (Eds.), Microbial Polymeric Substances: Characterization, Structure and Function (1st ed.). Springer-Verlag, pp. 49–72. http://dx.doi.org/10.1007/978-3-642-60147-7.

Oliveira, M.R., Ferreira da Silva, R.S., Buzato, J.B., Calabone Celligoi, M.A., 2007. Study of levan production by *Zymomonas mobilis* using regional low-cost carbohydrate sources. Biochem. Eng. J. 37, 177–183. http://dx.doi.org/10.1016/j.bej.2007.04.009.

Pal, A., Paul, A.K., 2008. Microbial extracellular polymeric substances: central elements in heavy metal bioremediation. Indian J. Microbiol. 48, 49–64. http://dx.doi.org/10.1007/s12088-008-0006-5.

Pan, X., Liu, J., Song, W., Zhang, D., 2012. Biosorption of Cu(II) to extracellular polymeric substances (EPS) from *Synechocystis* sp.: a fluorescence quenching study. Front. Environ. Sci. Eng. 6, 493–497. http://dx.doi.org/10.1007/s11783-012-0416-9.

Reuhs, B., Geller, D., Kim, J., Fox, J., Kumar Kolli, V., Pueppk, S., 1998. *Sinorhizobium fredii* and *Sinorhizobium meliloti* produce structurally conserved lipopolysaccharides and strain-specific K antigens. Appl. Environ. Microbiol. 64, 4930–4938.

Ružić, I., 1996. Trace metal complexation at herogeneous binding sites in aquatic systems. Mar. Chem. 53, 1–15. http://dx.doi.org/10.1016/0304-4203(96)00008-4.

Scarponi, G., Capodaglio, G., Barbante, C., Cescon, P., 1996. The anodic stripping voltammetric titration procedure for study of trace metal complexation in seawater. In: Caroli, S. (Ed.), Element Speciation in Bioinorganic Chemistry. Wiley, New York, pp. 363–418.

Scatchard, G., 1949. The attractions of proteins for small molecules and ions. Ann. N. Y. Acad. Sci. 51, 660–672. http://dx.doi.org/10.1111/j.1749-6632.1949.tb27297.x.

Sedmak, J., Grosserberg, S., 1977. A rapid, sensitive, and versatile assay for protein using Coomassie Brilliant Blue G250. Anal. Biochem. 79, 544–552.

Silva, M.F., Fornari, R.C.G., Mazutti, M.A., Oliveira, D., Ferreira Padilha, F., Cichoski, A.J., Cansian, R.L., Di luccio, M., Treichel, H., 2009. Production and characterization of xanthan gum by *Xanthomonas campestris* using cheese whey as sole carbon source. J. Food Eng. 90, 119–123. http://dx.doi.org/10.1016/j.jfoodeng.2008.06.010.

Sutherland, I.W., 1997. Microbial exopolysaccharides-structural subtleties and their consequences. Pure Appl. Chem. 69, 1911–1917.

Sutherland, I.W., 2001. Microbial polyssacharides from Gram-negative bacteria. Int. Dairy J. 11, 663–674. http://dx.doi.org/10.1016/S0958-6946(01)00112-1.

Tsai, C.M., Frasch, C.E., 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. 119, 115–119. http://dx.doi.org/10.1016/0003-2697(82)90673-X.

Valls, M., de Lorenzo, V., 2002. Exploiting the genetic and biochemical capacities of bacteria for the remediation of heavy metal pollution. FEMS Microbiol. Rev. 26, 327–338. http://dx.doi.org/10.1111/j.1574-6976.2002.tb00618.x.

Vullo, D.L., Ceretti, H.M., Daniel, M.A., Ramírez, S.A., Zalts, A., 2008. Cadmium, zinc and copper biosorption mediated by *Pseudomonas veronii* 2E. Bioresour. Technol. 99, 5574–5581. http://dx.doi.org/10.1016/j.biortech.2007.10.060.

Weia, X., Fang, L., Cai, P., Huang, Q., Chen, H., Liang, W., Rong, X., 2011. Influence of extracellular polymeric substances (EPS) on Cd adsorption by bacteria. Environ. Pollut. 159, 1369–1374. http://dx.doi.org/10.1016/j.envpol.2011.01.006.

Wingerder, J., Neu, T.R., Flemming, H.C., 1999. What are bacterial extracellular polymeric substances? In: Wingender, J., Neu, T., Flemming, H. (Eds.), Microbial Polymeric Substances: Characterization, Structure and Function (1st ed.). Springer-Verlag, pp. 1–19. http://dx.doi.org/10.1007/978-3-642-60147-7.

Xuan, W., Bin, Z., Zhiqianq, S., Zhiqianq, Q., Zhaoli, C., Min, J., Junwen, L., Jinqfenq, W., 2010. The EPS characteristics of sludge in an aerobic granule membrane bioreactor. Bioresour. Technol. 101, 8046–8050. http://dx.doi.org/10.1016/j.biortech.2010.05.074.

Yee, N., Fein, J., 2001. Cd adsorption onto bacterial surfaces: a universal adsorption edge? Geochimica Cosmochimica Acta 65, 2037–2042. http://dx.doi.org/10.1016/S0016-7037(01)00587-7.

Yin, Y., Hu, Y., Xiong, F., 2011. Sorption of Cu(II) and Cd(II) by extracellular polymeric substances (EPS) from *Aspergillus fumigatus*. Int. Biodeterior. Biodegrad. 65, 1012–1018. http://dx.doi.org/10.1016/j.ibiod.2011.08.001.

Yokoi, H., Kawata, S., Iwaizumi, M., 1986. Interaction modes between heavy metal ions and water-soluble polymers. 2. Spectroscopic and magnetic reexamination of the aqueous solutions of cupric ions and poly(acrylic acid). J. Am. Chem. Soc. 108, 3361–3365.