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New emulsifying and cryoprotective exopolysaccharide from Antarctic *Pseudomonas* sp. ID1

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ABSTRACT

Pseudomonas sp. ID1 is a cold-adapted bacterium isolated from a marine sediment sample collected from South Shetland Islands (Antarctica) that is noted for the highly mucous appearance of its colonies. In this work, we have characterized an exopolysaccharide (EPS) produced by this strain, which is mainly composed of glucose, galactose and fucose, and has a molecular mass higher than 2×10^6 Da. We have also studied its potential biotechnological applications as an emulsifier and cryoprotectant agent. The EPS emulsifying activity against different food and cosmetic oils was much higher than commercial gums such as xanthan gum and arabic gum, and surfactants such as Span 20. It formed highly stable emulsions against the cosmetic oil cetiol V, exhibiting pseudoplastic flow behavior, low thixotrophy and yield stress. The EPS of *Pseudomonas* sp. ID1 conferred significant cryoprotection for the strain itself as well as for other bacteria, including *Escherichia coli*, suggesting a universal cryoprotectant role. The cryoprotective activity of the EPS showed a clear dose–response relation at −20 °C and −80 °C and was significantly higher than that observed for the membrane stabilizer fetal bovine serum (FBS). These properties make the EPS of *Pseudomonas* sp. ID1 a promising alternative to commercial polysaccharides as an emulsifier and cryoprotectant agent for food, pharmaceutical and cosmetic industries.

1. Introduction

Exopolysaccharides (EPSs) are high molecular weight polymers that constitute a substantial component (40–95%) of the extracellular polymeric substances surrounding most microbial cells in the marine environment (Mancuso Nichols, Guezennec, & Bowman, 2005a; Poli, Anzelmo, & Nicolaus, 2010). Most EPSs produced by marine bacteria are heteropolysaccharides containing three or four different monosaccharides that may be pentoses, hexoses, amino sugars or uronic acids, arranged in groups of ten or less to form repeating units. Organic or inorganic substituents, such as sulphate, phosphate, acetic acid, succinic acid and piruvic acid, may also be present (Decho, 1990; Mancuso Nichols et al., 2005a; Nicolaus, Kambourova, & Oner, 2010; Poli et al., 2010; Rehm, 2009). In nature, bacterial EPSs serve various biological functions, for example, as a reserve material of biodegradable compounds and trace metal nutrients, or as part of a protective structure against predators, desiccation, salinity, cytotoxic compounds and high or low temperatures (Nicolaus et al., 2010; Poli et al., 2010; Rehm, 2009).

Owing to the wide diversity in their composition and physical–chemical properties, EPSs have emerged as new, industrially important polymeric materials, which are gradually becoming economically competitive with natural gums produced by marine algae and plants (Freitas, Alves, & Reis, 2011; Nicolaus et al., 2010; Rehm, 2009). In addition, EPSs derived from natural resources have a competitive advantage, due to their sustainable production from renewable resources, their biodegradability and often their biocompatibility (Rehm, 2010). Bacterial EPSs have been extensively used in high-value applications, such as food, pharmaceutical, medical and cosmetic products or processes, wherein they act mainly as thickening, stabilizing, emulsifying, binding, and structure creation agents. Among other applications, EPSs can also be used as a source of oligosaccharides and sugar monomers, or applied in bioremediation processes due to their capacity for toxic compound sequestration (Freitas et al., 2011; Krembs, Eicken, Junge, & Deming, 2002; Poli et al., 2010; Rehm, 2010).

There is a growing interest in isolating new EPS-producing bacteria from extreme environments because they have adopted special metabolic pathways and protective mechanisms to survive adverse conditions. In addition, the microbial biodiversity of harsh environments is relatively unexplored, making them a potential source of diverse EPSs with novel and unusual characteristics and functional activities. Moreover, EPS-producing bacteria from harsh

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habitats represent a valuable resource not only for exploitation in innovative biotechnological processes but also as a models for investigating how biomolecules are stabilized when subjected to extreme environmental conditions (Freitas et al., 2011; Mancuso Nichols et al., 2005a; Nicolaus et al., 2010; Poli et al., 2010).

Pseudomonas sp. ID1 is a novel cold-adapted bacterium isolated from marine sediment collected from South Shetland Islands, Antarctica. This Antarctic strain, which is able to grow at temperatures ranging from 0 °C to 30 °C, caught our attention, since its mucoid appearance suggested EPS production. In the present work, we present the chemical characterization of an EPS produced by *Pseudomonas* sp. ID1. The functional properties of this EPS, including its emulsifying capacity as well as its cryoprotective activity, were also studied to evaluate its potential industrial applications.

2. Materials and methods

2.1. EPS production

The strain used in this work was isolated from marine sediment collected from South Shetland Islands, Antarctica and identified as *Pseudomonas* sp. ID1 *Pseudomonas* sp. ID1 was grown on minimal medium MM1 composed of the following ($g\,l^{-1}$): glucose, 20; bacto-peptone, 0.5; yeast extract, 0.1; citrate, 0.4; $NaNO_3$, 7; K_2HPO_4, 2; $NaNH_5PO_4{\cdot}4H_2O$, 0.7; $MgSO_4{\cdot}7H_2O$, 0.1; $FeSO_4{\cdot}7\ H_2O$, 0.018; trace elements, 1 ml. *Pseudomonas* sp. ID1 cultures were incubated at 11 °C on an orbital shaker at 150 rpm for 120 h.

For EPS recovery, cells were removed from culture by centrifugation (6000 rpm, 25 min, 4 °C). The cell-free supernatant was reserved and pellets were washed three times with a Ringer solution (Scharlau) and centrifuged (40,000 × *g*, 20 min, 4 °C) to remove the EPS adhered to the cell surface. Washing supernatants were pooled with the first culture supernatant and subjected to a tangential flow filtration process through 0.22 μm membranes (Millipore). The filtrate was submitted to a dialysis process with sterile distilled water 1:10 (v/v) through 10,000 Da membranes (Millipore) to remove salts, pigments and other components of the culture medium and to obtain a concentrated and purified EPS. Finally, the resulting product was freeze-dried.

2.2. EPS characterization

2.2.1. Colorimetric analysis

The total neutral carbohydrate content of the EPS was determined by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Uronic acid content was determined by the metha-hydroxydiphenyl method (Blumenkrantz & Asboe Hansen, 1973). Protein content was determined by the Bradford protein assay (BioRad).

2.2.2. Monosaccharide analysis

To analyse the neutral sugar composition of the EPS samples, they were hydrolyzed with 1% H_2SO_4 at 111 °C for 30 min. The hydrolysate was used to identify and quantify the constituent monosaccharides by high-perfomance liquid chromatography (HPLC) using Aminex HPLC Carbohydrate Analysis Columns HPX-87P (300 × 7.8 mm) and HPX-87C (300 × 7.8 mm) (BioRad). Milli-Q water at 85 °C was used as the eluent, and detection was carried out with a Waters 2414 Refractive Index Detector (Waters). Fifteen commercial sugars and amino sugars were used as patterns for monosaccharide identification.

2.2.3. Amino acid analysis

After hydrolysing EPS samples with 6 M HCl at 110 °C for 24 h, the hydrolysate was used to identify and quantify the constituent amino acids with a Biochrom 30 amino acid analyzer (Biochrom) with a cation-exchange chromatography column (200 × 4 mm) and post-column derivatization with ninhydrin, as described by Spackman, Stein, and Moore (1958). Lithium citrate buffers of increasing ionic strength and pH (Biochrom) were used as eluents at temperatures ranging from 34 °C to 80 °C. Amino acid detection was carried out with a UV detector at 440 nm and 570 nm, and 17 commercial amino acids were used as patterns for identification.

2.2.4. Elemental analysis

The elemental composition of the EPS was analyzed using a Thermo EA 1108 elemental organic analyzer (Thermo Scientific), working in the standard conditions recommended by the instrument supplier (helium flow at 120 ml/min, combustion furnace at 1000 °C, chromatographic column oven at 60 °C, oxygen loop 10 ml at 100 kPa).

2.2.5. Size exclusion chromatography

The molecular weight (M_r) of the EPS was determined by size-exclusion chromatography (SEC) in an HPLC Waters 2695 apparatus (Waters) equipped with an ultrahydrogel 500 column (7.8 × 300 mm; Waters) and a differential refractive index detector (Waters 2414). 0.1 M $NaNO_3$ was used as the eluent at room temperature. Dextran standards (Sigma-Aldrich) of M_r ranging from 8.8×10^3 to 2×10^6 Da were used to calibrate the column for M_r estimation.

2.2.6. Nuclear magnetic resonance analysis

Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained in D2O 99.96% (Euriso-top) at 25 °C using a Varian VNMRS500 spectrometer (500 MHz) (Varian Ltd.).

2.2.7. FT-IR spectroscopy

The infrared spectra of the EPS were acquired with a Thermo FT-IR Microscope IN10MX (Thermo Scientific), using a MCT detector cooled by liquid nitrogen. All the spectra were obtained in transmission mode, using a Thermo diamond compression cell (Thermo Scientific). The spectra were obtained in a 100 μm × 100 μm area, with a resolution of 4 cm^{-1} and 64 scans.

2.3. Emulsifying activity

2.3.1. Emulsifying activity against food, cosmetic oils and n-hexadecane

To assay EPS emulsifying activity, the protocol described by Gutiérrez, Shimmield, Haidon, Black, and Green (2008) was performed. Xanthan gum and arabic gum (Sigma-Aldrich) were used as positive controls. 5 ml of a solution of 0.02% (wt/v) of tested emulsifier (EPS, xanthan gum and arabic gum) were mixed with 0.8 ml of *n*-hexadecane (Sigma-Aldrich), olive oil, sunflower oil or corn oil using an Ultra-turrax T10 basic homogenizer (IKA) at speed 5 for 1 min. Emulsions were allowed to stand undisturbed at room temperature for 24 h. After that period, the emulsifying activity (A_{540}) was determined by measuring the turbidity of the lower aqueous layer using a UV-1800 spectrophotometer (Shimadzu) at 540 nm. Emulsifying activities were compared under neutral conditions (0.1 M PBS, pH 7). Samples of the corresponding food oils or n-hexadecane and phosphate-buffered saline solution (PBS) without the EPS were used as controls, and A_{540} values of these controls were subtracted from those of the EPS, xanthan gum and arabic gum to obtain their final emulsifying activities.

EPS emulsifying activity was also tested against the cosmetic oil cetiol V (Fagron), and compared to the commercial emulsifier Span 20 (Croda). Emulsions with the EPS or Span 20 ranging from 1% to 12% and a mixture of water and cetiol V (1:2; v/v) were prepared using an Ultra-turrax T10 basic homogenizer (IKA) at speed 5 for 2 min.

2.3.2. Particle size analysis

Particle size analysis of emulsions with 2% of EPS, and a mixture of water and cetiol V (1:2; v/v) was performed by laser diffractometry (LD) using a Mastersizer Hydro 2000MU (Malvern instruments) yielding the volume distribution of the particles. For the LD analysis the diameters 10, 50 and 90% were used: for example, an LD90% value indicates that 90% (volume distribution) of the measured particles possess a diameter equal to or lower than the given value.

2.3.3. Rheological measurements

Rheological measurements of emulsions with 2% of EPS, and a mixture of water and cetiol V (1:2; v/v) were performed on a Haake RheoStress 1 rheometer (Thermo Scientific) equipped with a cone-and-plate geometry (plate diameter 35 mm, cone angle 2°). All measurements were carried out at 25 °C. Continuous shear investigations were performed to evaluate the shear stress (Pa) as a function of the shear rate (s^{-1}). This study was carried out with a shear rate of 0–100 s^{-1} for 3 min, 100 s^{-1} for 1 min and back to 0 s^{-1} for 3 min. The resulting shear stress and viscosity were measured.

2.3.4. Zeta potential measurements

The zeta potential (ZP) of emulsions with 2% of EPS and a mixture of water and cetiol (1:2; v/v) was determined using a Zetasizer Nano ZS (Malvern Instruments) at 25 °C. The ZP was calculated from the electrophoretic mobility using the Helmholtz–Smoluchoswski equation (Deshiikan & Papadopoulos, 1998). The processing was run by the software included in the system.

2.3.5. Emulsion stability

The stability of emulsions with 2% of EPS and a mixture of water and cetiol V (1:2; v/v) was assessed by measuring the variations in backscattering using a Turbiscan Lab Expert (Formulaction), based on multiangle laser light scattering. Due to the opacity of the samples, only backscattering (BS) profiles were used to evaluate the physicochemical stability of the emulsions. The measurements were performed at 25 °C with the freshly prepared emulsion (day 0) and one month later after storage at room temperature (day 30). EPS emulsion stability was also studied under several conditions of temperature, salinity and pH. For pH stability tests, 2% of EPS, and mixtures of water at pH ranging from 5 to 9 and cetiol V (1:2; v/v) were prepared and stored at room temperature. For temperature stability assays, mixtures with 2% of EPS, water adjusted to pH 7 and cetiol V (1:2; v/v) were prepared and stored at temperatures ranging from 4 °C to 42 °C. Finally, for salinity stability studies, emulsions with 2% of EPS and mixtures of water containing up to 1 M NaCl and cetiol V (1:2; v/v) were prepared and also stored at room temperature. The stability of the emulsions was evaluated over a period of 4 months by observing changes in their macroscopic characteristics.

2.4. Cryoprotective activity

2.4.1. Obtaining a non-EPS-producing mutant strain

A non-EPS-producing mutant strain was obtained by mutagenesis with ultraviolet light. To confirm that the mutant strain did not produce EPS, ruthenium tetraoxide staining was performed because it allows revealing polysaccharides around bacterial cells (Waller, Fox, Fox, Fox, & Price, 2004). First, cells were submitted to chemical fixation with 5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.3 and 4 °C for an overnight period. After being washed for 10 min with 0.1 M cacodylate buffer at pH 7.3 five times, samples were twice incubated with 0.25% ruthenium tetraoxide and 0.25% potassium ferrocianyde in 0.1 M cacodylate buffer at pH 6.8 for 1 h in darkness at 4 °C. Then, samples were washed five times for 15 min with Milli-Q water at 4 °C and were kept in 0.1 M cacodylate at pH 6.8 and 4 °C until cryofixation by High Pressure Freezing. For freeze substitution, a solution containing 1% osmium tetroxide, 0.5% uranyl acetate and 3% glutaraldehyde in methanol was used. The freeze substitution started at 72 h at −90 °C, followed by a warming up to 4 °C with a slope of 5 °C h^{-1}, at which point the temperature was maintained for 4 h. Once the process was finished, samples were kept at room temperature and in darkness at 4 °C. Afterwards, they were twice washed with methanol for 2 h and washed three times with acetone for 15 min. Finally, samples were infiltrated with Epon: methanol 1:3, 2:2 and 3:1 for 3 h in each step and embedded in Epon. Ultrathin sections were obtained using the UCT ultramicrotome (Leica Microsystems), and stained with 2% uranyl acetate and lead citrate. Samples were observed with a transmission electron microscope TEM Tecnai Spirit Twin (FEI) 120 kV LaB6.

2.4.2. Cryoprotection assays with Pseudomonas sp. ID1

Wild type (wt) and mutant (mt) *Pseudomonas* sp. ID1 cells were grown on Tryptone Soya Agar (TSA; Oxoid) plates at 10 °C for 5 days to reach a confluent growth. Suspensions of wt and mt cells were prepared in a Ringer solution and adjusted to an optical density of 0.6 (540 nm). 1 ml aliquots were prepared and centrifuged at 12,000 rpm for 30 min. Supernatants were discarded, and cell pellets were frozen at −20 °C and −80 °C. After one week, samples were thawed, and pellets were resuspended in 1 ml of Ringer solution. Cell viability was determined by the dilution plating method, and the survival rate was expressed as a percentage of viable cells with respect to unfrozen cells.

2.4.3. Cryoprotection assays with E. coli

Escherichia coli ATCC 10536 was grown on Tryptone Soya Broth (TSB, Oxoid) to an optical density of 0.6 (540 nm). 0.1 ml aliquots were mixed with different EPS concentrations (0–10%) to a final volume of 1 ml. Samples were frozen at −20 °C and −80 °C for one week, thawed and the number of viable cells was determined by the dilution plating method. Survival rate was expressed as the percentage of viable cells with respect to unfrozen cells. Fetal bovine serum (FBS) was used as a positive control.

3. Results and discussion

3.1. EPS characterization

The carbohydrate content of the purified EPS was 33.81% ± 2.59, composed of glucose (17.04% ± 0.32), galactose (8.57% ± 1.15) and fucose (8.21% ± 1.12). The total uronic acid content of EPS was 2.40% ± 0.33%. The EPS protein content determined by the Bradford assay (BioRad) was 2.81% ± 0.13. Amino acid analysis of hydrolyzed samples identified the presence of four major amino acids: glutamic acid (0.54% ± 0.01), aspartic acid (0.33% ± 0.02), glycine (0.26% ± 0.05) and alanine (0.22% ± 0.03). All these percentages are related to the total weight of the purified EPS.

Chemical composition based on elemental analyses was determined as C (37.29% ± 1.07); H (6.17% ± 0.10); N (2.25% ± 0.21); and S (0.41% ± 0.16).

The molecular mass (M_r) of the EPS produced by *Pseudomonas* sp. ID1 was determined by size exclusion chromatography (SEC) and estimated to be higher than 2×10^6 Da.

The FT-IR spectrum of EPS is presented in Fig. 1. The broad and intense band around 3400 cm^{-1} represents O—H stretching of hydroxyls and bound water. C—H stretching peaks of CH_2 and CH_3 appear at 2930 cm^{-1} and 2985 cm^{-1}, respectively. The well-defined envelope found between 1200 and 900 cm^{-1} represents skeletal C—O and C—C vibration bands of carbohydrates. The band at 1720 cm^{-1} may be attributed to the C—O stretching of carbonyls in acyl groups. The C—O stretching peak of amide 1 at 1640 cm^{-1}

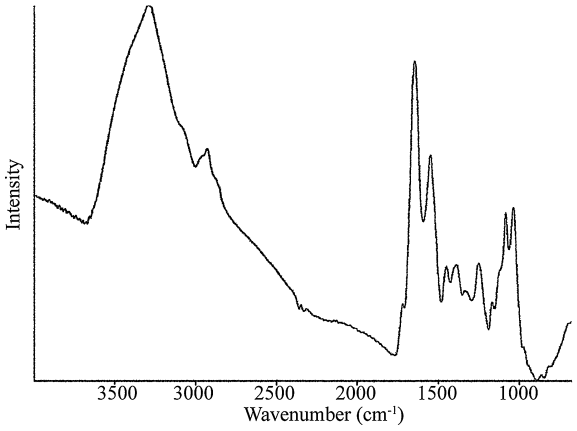

Fig. 1. Infrared spectrum of the EPS produced by *Pseudomonas* sp. ID1.

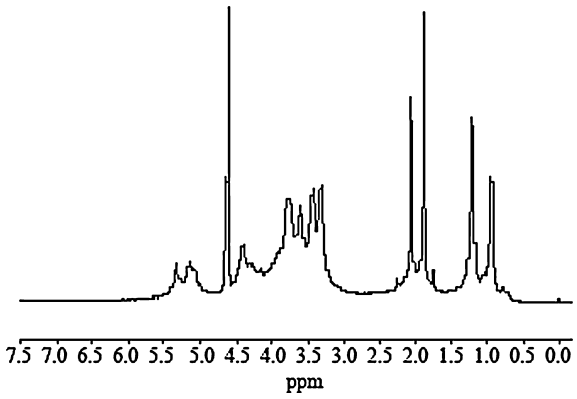

Fig. 2. ^{1}H NMR spectrum of the EPS produced by *Pseudomonas* sp. ID1.

and N—H stretching peak of amide 2 at 1540 cm^{-1} are characteristic of proteins. This protein detection is in agreement with the low protein content detected by the Bradford assay and is probably due to cellular lysis during the EPS obtention and purification processes. In any case, the high speed centrifugation process is validated as one of the best methods to avoid cell lysis in EPS recovery from bacterial cultures (Brown & Lester, 1980).

Fig. 2 shows the proton spectrum acquired from the EPS synthesized by *Pseudomonas* sp. ID1. The spectrum is in agreement with the carbohydrate composition of this biopolymer, with strong peaks from the predominating glycan component. Detailed analysis of the EPS spectrum was not possible due to the complexity of the sample, but the presence of sugars in the anomeric proton region (4.2–5.5 ppm) and in the ring proton region (3.2–4 ppm) can be observed. Intense signals at 1.9 ppm and 2.1 ppm are consistent with the presence of acetyl groups. The intense peak at 1.2 ppm in the EPS spectrum could correspond to methyl H6 protons of fucose.

The presence of glucose, galactose and fucose residues in microbial polysaccharides is rather common, although their amounts vary. Fucose-containing EPSs are seen as interesting products for pharmaceutical and cosmetic industries not only for their thickening, emulsifying and/or film-forming properties, but also as a source of fucose. The biological properties of this rare sugar, which is difficult to obtain, potentiate its use in pharmaceuticals, e.g. as an anticarcinogenic or anti-inflammatory agent, and cosmetic products as an anti-aging agent (Freitas et al., 2011; Péterszegi, Fodil-Bourahla, Robert, & Robert, 2003; Vanhooren & Vandamme, 1999).

In recent years, characterization of bacterial EPSs from polar environments has become an active research field, but few have been characterized from cold-adapted psychrotolerant marine bacteria. Most of the characterized EPSs are from the *Pseudoalteromonas* genus, as is the case of *Pseudoalteromonas* sp. SM20310, isolated from Arctic sea ice, which secretes an EPS composed mainly of mannose, and traces of glucose, galactose, rhamnose, *N*-acteylglucosamine, *N*-acetylgalactosamine and xylose (Liu et al., 2012). Other cold-adapted bacteria isolated from the Antarctica are *Pseudoalteromonas haloplanktis* TAC125, which synthesizes an EPS consisting of mannose and traces of glucose (Corsaro et al., 2004), and *Pseaudoalteromonas arctica* KOPRI 21653, with an EPS composed of galactose and glucose (Kim & Yim, 2007).

More complex EPSs have also been described from Antarctic environments, including *Pseudoalteromonas* sp. CAM025 and *Pseudoalteromonas* sp. CAM036. The EPS produced by the former is a sulfated heteropolysaccharide with high levels of uronic acids with acetyl groups, and its monosaccharide composition is estimated to be glucose, galactose, rhamnose and galacturonic acid. The latter strain also synthesizes a sulfated heteropolysaccharide with high levels of uronic acids with acetyl groups, but it is composed mainly of glucose, mannose, arabinose, galacturonic acid and N-acetyl-galactosamine (Mancuso Nichols, Garon, Bowman, Raguénès, & Guézennec, 2004; Mancuso Nichols et al., 2005b).The *Pseudomonas* genus is one of the most studied sources of EPSs, some of which are currently commercially exploited in food, cosmetic and pharmaceutical industries, including alginates and levans (Celik, Aslim, & Beyatli, 2008; Freitas et al., 2009; 2011; Hung, Santschi, & Gillow, 2005; Kachnaly et al., 2001; Osman & Fett, 1989; Read & Costerton, 1987; Rehm, 2009, 2010; Sudhamani, Tharanathan, & Prasad, 2004). However, to our knowledge, no EPS has been described from *Pseudomonas* bacteria isolated from the polar regions, as is the case of our strain *Pseudomonas* sp. ID1.

The EPS from *Pseudomonas* sp. ID1 is a new biopolymer with a composition that differs from that of other EPSs produced by other cold-adapted bacteria or other *Pseudomonas* strains reported so far.

3.2. Emulsifying activity

Pseudomonas sp. ID1 EPS emulsifying activity was assayed against several hydrophobic compounds, namely food oils, cosmetic oils and hydrocarbons. Fig. 3 shows the measured emulsifying activities of the EPS under neutral conditions against three different food oils and n-hexadecane. The EPS exhibited a high emulsifying capacity for olive, sunflower and corn oils, with emulsifying activities (A_{540}) of 1.28, 1.15 and 0.66, respectively. In all cases, the EPS showed a higher emulsifying activity than xanthan gum and arabic gum, with the exception of sunflower oil, for which the EPS showed the same emulsifying activity as xanthan gum. The EPS also demonstrated a higher emulsifying capacity against n-hexadecane

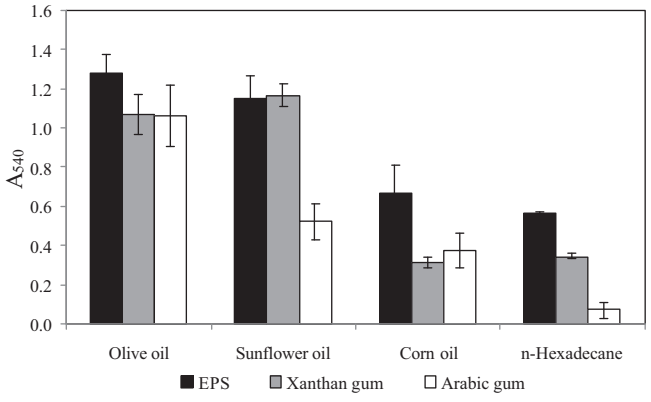

Fig. 3. Emulsifying activities (A_{540}) of the EPS, xanthan gum and arabic gum against olive, sunflower, and corn oils and *n*-hexadecane after 24 h at room temperature.

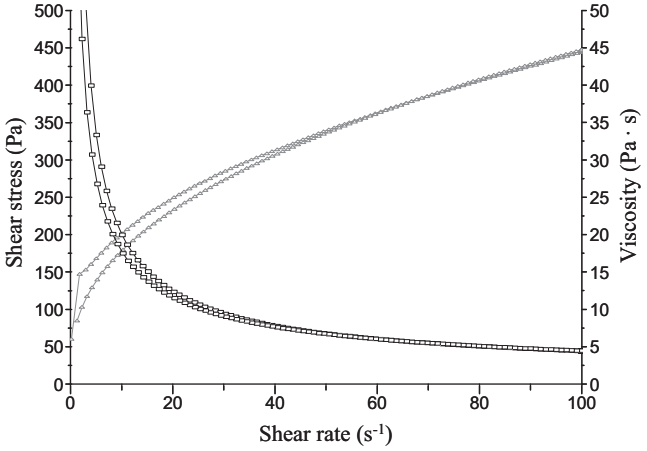

Fig. 4. Rheogram of emulsions with 2% of EPS from *Pseudomonas* sp. ID1 and a mixture of water and cetiol v (1:2; v/v). "-": Shear stress, "□": Viscosity.

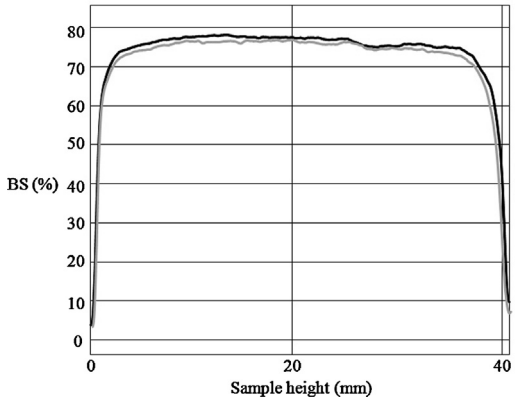

Fig. 5. Backscattering (BS) profiles of the emulsion with 2% of EPS and a mixture of water and cetiol V (1:2; v/v) as a function of sample height (mm), analyzed at days 0 and 30 of storage at room temperature. Thick line: Day 0. Soft line: Day 30.

than the positive controls (A_{540} of 0.56 compared with 0.35 and 0.07 for xanthan gum and arabic gum, respectively).

The EPS emulsifying activity was also tested against the cosmetic oil cetiol V and compared with Span 20. Stable emulsions with a creamy consistency were formed with 2% of EPS, whereas 6% of Span 20 was needed to achieve the same emulsification grade.

Particle size analysis of emulsions with 2% of EPS and a mixture of water and cetiol V (1:2; v/v) performed by laser diffractometry revealed LD10% values $\leq 4.57\,\mu m \pm 0.001$, LD50% $\leq 10.66\,\mu m \pm 0.006$ and LD90% $\leq 20.36\,\mu m \pm 0.03$, respectively, indicating that emulsion particles were in the micron range, with the major peak around 12 μm.

In rheological studies, the flow curve of emulsion with 2% of EPS exhibited pseudoplastic flow behavior, and low thixotrophy and yield stress (Fig. 4). Nevertheless, the flow curves of water and cetiol V without the EPS showed Newtonian behavior (data not shown), indicating that the pseudoplasticity was due to the EPS presence in the emulsion. The pseudoplastic behavior of the EPS from *Pseudomonas* sp. ID1 make it an attractive material for industrial and medical applications as a thickening, stabilizing, binding or structure creation agent, similar to other bacterial EPSs currently commercialized (Freitas et al., 2011; Rehm, 2010). In addition, the apparent viscosity of the emulsion was estimated to be 4.46 ± 0.007 Pa s at $100\,s^{-1}$.

The zeta potential (ZP) represents the electrical charge at the emulsion particle surface, also being an important parameter that allows the prediction of emulsion physical stability. In theory, when ZP is relatively high (>|25|), the forces of repulsion exceed those of attraction, which tends to stabilize particle suspensions. On the other hand, when ZP is low (<|25|), the forces of attraction are stronger and the particles come together, leading to flocculation (Lieberman, Rieger, & Banker, 1989). The ZP value of emulsions with 2% of EPS and a mixture of water and cetiol V (1:2; v/v) was 0.11 ± 0.4 mV, which indicated that the emulsion particles had a weak surface charge.

The long-term stability of the emulsion with 2% of EPS and a mixture of water and cetiol V (1:2; v/v) was assessed by multi-angle laser light scattering, using Turbiscan Lab Expert, which yielded the percentage of backscattered (%BS) light along the vial containing the sample as a function of time. In the present study, the emulsion stability was evaluated from %BS at days 0 and 30 in samples stored at room temperature. As can be observed in Fig. 5, emulsion profiles at days 0 and 30 were extremely similar, and no coalescence, flocculation, creaming, or sedimentation phenomena were observed, indicating a long-term stability of the EPS emulsion.

The results from Turbiscan, ZP and rheological measurements demonstrated that the EPS emulsion stability is not due to the electrostatic charges of the particles, which is reflected by the low ZP value. This is in agreement with the low content of uronic acids in our EPS, since these normally contribute significantly to the global negative charge of bacterial exopolymers. It is likely that the stability of *Pseudomonas* sp. ID1 EPS emulsions is due to steric stabilization, a common process among polysaccharides, which stabilize emulsions by forming an extended network in the continuous phase. This consequently becomes highly viscous and droplet movements and encounters are reduced (Bouyer, Mekhloufi, Rosilio, Grossiord, & Agnely, 2012; Dickinson, 2009).

Additionally, EPS emulsion stability was tested under several conditions of pH, temperature and salinity. Emulsions remained stable for more than four months at a pH ranging from 5 to 8, temperatures of 4 °C to 42 °C and NaCl concentrations up to 1 M, making the *Pseudomonas* sp. ID1 EPS a good candidate for use as an emulsifying agent to form long-term emulsions for pharmaceutical, cosmetic and food products.

Although several exopolymeric substances with emulsifying activity have been described from marine bacteria (Gutiérrez, Mulloy, Bavington, Black, & Green, 2007a; Gutiérrez, Mulloy, Black, & Green, 2007b; Gutiérrez, Leo, Walker, & Green, 2009; Iyer, Mody & Jha, 2006), to our knowledge no EPSs from Antarctic bacteria with this property have been reported. The EPS from *Pseudomonas* sp. ID1 was found to be a more efficient emulsifier against several food oils and the hydrocarbon n-hexadecane than other bacterial EPSs or plant gums currently used in industry, such as xanthan gum and arabic gum. Similarly, the EPS demonstrated higher emulsification activity against the cosmetic oil cetiol V than the commercial emulsifier used as a positive control, Span 20. Moreover, the *Pseudomonas* sp. ID1 EPS was observed to form long-term, stable emulsions with pseudoplastic behavior. These results highlight the potential applications of the *Pseudomonas* sp. ID1 EPS in cosmetic and food industries as an emulsifying agent, with the additional attractions of a sustainable production, non-toxicity and biodegradability.

3.3. Cryoprotective activity

To study if the EPS from *Pseudomonas* sp. ID1 is able to preserve its cell structure and maintain its viability after being submitted to freezing temperatures, a non EPS-producing mutant strain (mt) was obtained by mutagenesis with ultraviolet light. We used electronic microscopy combined with ruthenium tetraoxide staining, which enables polysaccharides around bacterial cells to be visualized, to confirm that mt cells did not produce EPS (Fig. 6.) In wild-type

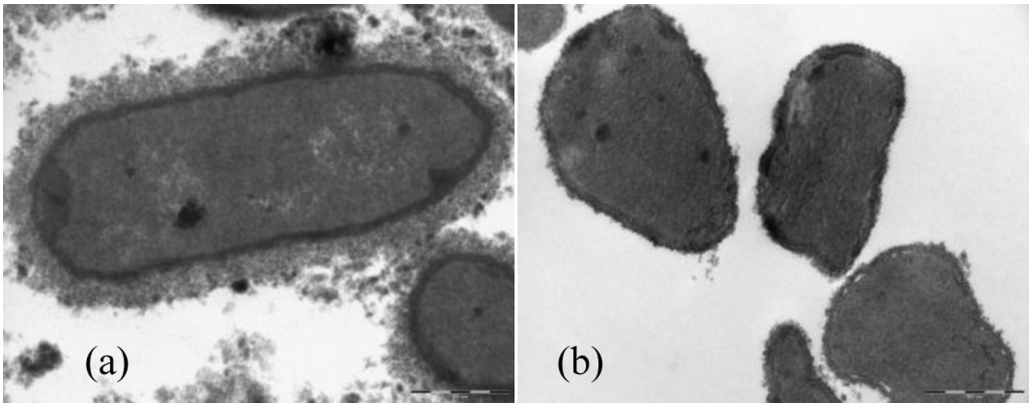

Fig. 6. Transmission electron microscopy images of wild-type cells of *Pseudomonas* sp. ID1 *Pseudomonas* sp. ID1 (a) and non-EPS-producing mutant cells (b).

Table 1
Survival rates of wild-type and mutant strains after freezing cells at −20 °C and −80 °C. The survival rate is expressed as the percentage of viable cells with respect to unfrozen cells ($n = 8$).

	Wild-type strain	Mutant strain
−20 °C	75.19 ± 8.06	40.27 ± 5.97
−80 °C	93.53 ± 12.18	49.94 ± 7.43

(wt) *Pseudomonas* sp. a stained polymeric material around the cells was clearly visualized (Fig. 6a), while in mt cells this layer was not observed at all (Fig. 6b).

After freezing at temperatures of −20 °C and −80 °C, viability rates of mt cells were analysed by the dilution plating method and compared to wt cells. The wt strain exhibited significantly higher survival rates than the mt strain in all tested temperatures (Table 1), demonstrating that EPS production is an adaptation mechanism for survival in cold marine environments such as the Antarctica.

To determine if the EPS from *Pseudomonas* sp. ID1 had a cryoprotective role for other microorganisms, TSB cultures of *E. coli* ATCC 10536 were mixed with different EPS concentrations ranging from 0 to 10%, and frozen at −20 °C and −80 °C. *E. coli* viability was then analysed and compared with the viability of *E. coli* cells mixed with the same concentrations of fetal bovine serum (FBS), used as control for its capacity to stabilize membranes. As shown in Fig. 7, the EPS also conferred cryoprotection to *E. coli* cells, with a clear dose–response relation in all assayed temperatures and a maximal survival rate (35.68% at −20 °C, Fig. 7a and 64.13% at −80 °C, Fig. 7b) with an EPS concentration of 10%. This dose–response relation was not observed with FBS, and survival rates obtained at any concentration of FBS were lower than 0.04%.

Several studies have demonstrated that EPS production in bacteria at low temperatures is a cold temperature adaptation mechanism. Mancuso Nichols, Bowman, & Guezennec, (2005c) reported that *Pseudoalteromonas* sp. CAM025 produced 30 times more EPS at −2 °C and 10 °C compared to 20 °C. Marx and coworkers similarly observed that EPS production by the marine psychrophilic bacterium *Colwellia psychrerythraea* strain 34H was about 10-fold higher at −8 °C than at −4 °C (Marx, Carpenter, & Deming, 2009). Qin, Zhu, Chen, Wang, and Zhang (2007) also reported that the yield of EPS secreted by *Pseudoalteromonas* sp. SM9913 increased as the temperature decreased.

Other authors have reported that the EPS cryoprotective activity not only benefits the cold-adapted bacterial producer, but also non-producing cells, such as *E. coli*, suggesting a universal cryoprotectant role for these biopolymers (Kim & Yim, 2007; Liu et al., 2012). These observations are in accordance with the results obtained in our study of the *Pseudomonas* sp. ID1 EPS, which not only conferred cryoprotection for the strain itself, but also for *E. coli* cells, suggesting it can thus be applied as an agent for cell cryopreservation, alone or in combination with other cryoprotectants currently in use.

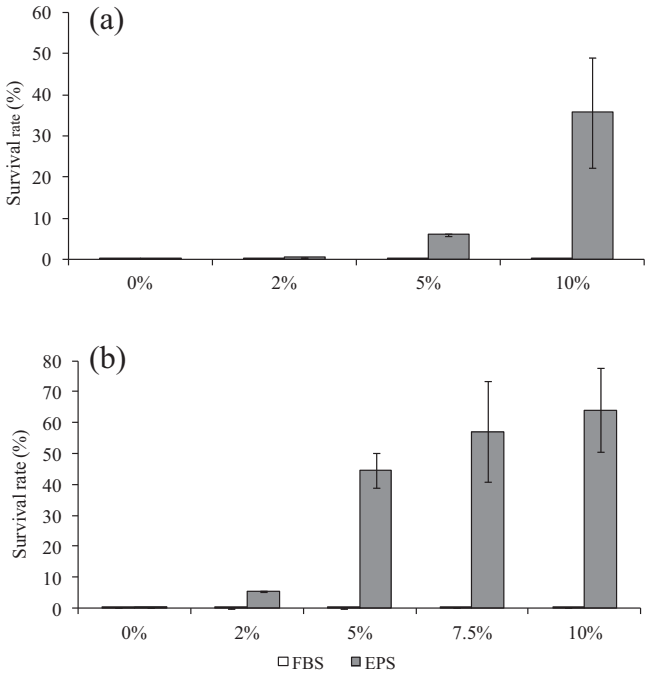

Fig. 7. Survival rates of *E. coli* ATCC 10536 cultures frozen at −20 °C (a) and −80 °C (b) with EPS or FBS. Survival rates are expressed as the percentage of viable cells with respect to unfrozen cells ($n = 3$).

4. Conclusions

In this work, an EPS produced by a novel Antarctic species, *Pseudomonas* sp. ID1, was characterized. The biopolymer is a high molecular weight heteropolysaccharide composed mainly of fucose, galactose and glucose. The ^{1}H NMR and FT-IR spectra were in accordance with the composition analysis. The main properties of the EPS, namely its ability to form a long-term stable emulsion against different food and cosmetic oils and its universal cryoprotective activity, make it a promising alternative to commercial polysaccharides currently in use.

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