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Solution properties of an exopolysaccharide from a *Pseudomonas* strain obtained using glycerol as sole carbon source

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ABSTRACT

We report the solution properties of a new exopolysaccharide (EPS) obtained from a *Pseudomonas* strain fed with glycerol as the sole source of carbon. This high molecular mass (3×10^6 g mol^{-1}) biopolymer is essentially made of galactose monomers with pyruvate and succinate groups imparting a polyelectrolyte character. The Smidsrod parameter *B* computed from the ionic strength dependence of the intrinsic viscosity indicates that the EPS backbone is rather flexible. In salt free aqueous solutions, the zero shear viscosity scaling with concentration follows a typical polyelectrolyte behavior in bad solvent, whereas at high ionic strength the rheological response is reminiscent from neutral polymers. Light scattering data indicate that the EPS adopts a globular conformation as a result of hydrophobic interactions. EPS solutions are stable within 4 days as particle sizing does not indicate EPS aggregation. Both globular conformation and stability against precipitation from solution are attributed to the low charge density of the polyelectrolyte.

1. Introduction

Microbial polysaccharides, such as xanthan, gellan, pullulan and bacterial alginate, may represent alternatives to polysaccharides obtained from plants (Guar gum, Arabic gum or pectins), algae (alginate, carrageenan or agar) and crustacean (chitin) (Stephen, 1995). In fact, microbial production is a much more controlled process, originating a product with tuned chemistry and properties and with a constant availability over time. This constitutes an advantage over the natural biopolymers isolated from plants and algae, whose availability and physical–chemical properties are dependent on external factors, such as climate conditions and the season of the year (Sutherland, 2001).

The most used carbon sources for EPS production have been sugars, namely glucose and sucrose, applied for instance in the production of xanthan gum (García-Ochoa, Santos, Casas, & Gómez, 2000) and bacterial alginate (Peña, Trujillo-Roldán, & Galindo, 2000). However, the high cost of these carbon sources has a direct impact on production costs, which limits the market potential of these biopolymers. In order to decrease the production costs, it is important to look for less expensive carbon sources, like industrial wastes or industrial by-products (Kumar, Mody, & Jha, 2007). Sugar molasses and potato starch wastes are examples of low cost carbon sources already used for the production of microbial polysaccharides such as exopolysaccharide based on cellulose (Paterson-Beedle, Kennedy, Melo, Lloyd, & Medeiros, 2000) and pullulan (Barnett, Smith, Scanlon, & Israilides, 1999).

More recently, glycerol, a by-product of many industrial processes, mainly from biodiesel production, has been generated in large quantities far beyond current consumption in traditional applications. Interesting applications for glycerol are still lacking. We reported recently the production of a new microbial polysaccharide by a *Pseudomonas* strain using glycerol as the sole carbon source (Freitas et al., 2009; Reis et al., 2008). The biopolymer is a high molecular weight extracellular heteropolysaccharide composed of neutral sugars (galactose, mannose, glucose and rhamnose) and acyl groups (pyruvil, succynil and acetyl). This exopolysaccharide (EPS) is amorphous, as inferred by thermal analysis and solid-state NMR. It possesses flocculating and emulsifying properties, along with film-forming capacity, making it a good alternative to other natural and microbial polysaccharides.

A systematic study of the EPS aqueous solutions properties is of major importance in order to screen potential application of this product to industrial activities such as water treatment, food,

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pharmaceutical, cosmetic, mining, paper and oil recovery. Preliminary rheological studies showed that the crude EPS has viscosity enhancing properties similar to Guar gum. Here, we explore in greater detail the solution properties of a purified EPS sample, in order to rationalize the good viscoelastic properties. EPS in salt free solutions as well as in NaCl solutions with various ionic strengths are studied at concentrations ranging from the dilute regime to the concentrated regime, using light scattering techniques and rheometry.

2. Materials and methods

2.1. Polysaccharide production and recovery

The bioprocess used to obtain the exopolysaccharide has been reported in detail elsewhere (Freitas et al., 2009). The culture was grown on Medium E* (Brandl, Gross, Lenz, & Fuller, 1988), supplemented with 25 g/l glycerol (Fluka, 86%) as carbon source and 3.3 g/l $(NH_4)_2HPO_4$ as nitrogen source. EPS production was carried out using *Pseudomonas oleovorans* NRRL B-14682 and was performed in a 10 l bioreactor (BioStat B-plus, Sartorius) operated in fed-batch mode, with controlled pH (6.75–6.85) and temperature (30 °C), and at a constant air flow rate of 0.125 vvm (volume of air per volume of reactor per minute). Glycerol and ammonium concentration were determined as described by Freitas et al. (2009).

Culture broth samples were diluted with deionised water for viscosity reduction and centrifuged at 48,384*g* for 1 h. The cell-free supernatant was subjected to protein thermal denaturation at 80 °C during 4 h, followed by their separation by centrifugation (48,384*g*, 1 h). The polymer was then precipitated from the cell-free supernatant by the addition of cold ethanol 96 vol% (3:1) and separated by centrifugation (27,216*g*, 15 min). The pellet was washed with ethanol 96 vol%, redissolved in deionised water, reprecipitated two times and freeze dried.

2.2. Chemical characterization

The polymer samples were analyzed in terms of sugar composition, acyl groups, inorganic and protein content. For the analysis of the sugar composition, 2–3 mg of the extracted EPS were dissolved in 5 ml deionized water and hydrolyzed with trifluoroacetic acid (TFA) (0.1 ml TFA 99%), at 120 °C, for 2 h. The hydrolysate was used for the identification and quantification of the constituent monosaccharides by liquid chromatography (HPLC) using a CarboPac PA10 column (Dionex), equipped with an amperometric detector. The analysis was performed at 30 °C, with sodium hydroxide (NaOH 4 mM) as eluent, at a flow rate of 0.9 ml min^{-1}. The hydrolysate was also used for the identification and quantification of acyl groups present in the EPS. The analysis was performed by HPLC with an Aminex HPX-87H column (BioRad), coupled to an ultraviolet (UV) detector, using sulphuric acid (H_2SO_4 0.01 N) as eluent, at a flow rate of 0.6 ml min^{-1} and a temperature of 50 °C. The detection was performed at 210 nm.

For the determination of the EPS protein content, 5.5 ml samples of 4.5 g/l aqueous solutions were mixed to 1 ml 20% NaOH, placed at 100 °C for 5 min and cooled on ice. Each sample was mixed with 170 μl of $CuSO_4 \cdot 5H_2O$ (25% v/v) and centrifuged at 3500*g* for 5 min. The optical density was measured at 560 nm (Spectrophotometer Helios Alpha, Thermo Spectronic, UK). Albumin (Merck) solutions (0.5–3.0 g/l) were used as protein standards.

The total inorganic content of the extracted EPS was determined by subjecting the EPS to pyrolysis at a temperature of 550 °C for 48 h. The EPS was further analyzed by Inductively Coupled Plasma – Atomic Emission Spectroscopy, to quantify its content in sodium, calcium, potassium, magnesium and iron.

2.3. Biopolymer solutions

The EPS, isolated as described in Section 2.1 after 7 days operation of the bioreactor (see below), was added to hot NaCl solutions or deionised water and stirred at 80 °C for 1 h. Solutions were then further stirred overnight at room temperature, to ensure complete dissolution of the EPS, and then characterized without delay. EPS concentration ranged from 0.0005 to 0.04 wt% for light scattering experiments, and from 0.01 to 1.6 wt% for rheological analysis. Ionic strength of solutions ranged from roughly 10^{-4} for deionised water (Dou & Colby, 2006) to 0.5 M.

2.4. Rheological measurements

EPS solutions prepared as described above were directly loaded in the pre-heated (80 °C) cone and plate geometry (diameter 6 cm, angle 0.2 rad) of a stress rheometer (ARG2, TA Instruments Inc., New Castle, DE, USA) and the shearing geometry was covered with paraffin oil in order to prevent water loss. Such pre-heating step was performed in order to erase any thermal and mechanical history induced by the preparation of EPS solutions, and which might affect the rheological response of solutions at 25 °C. Solutions were then cooled (−5 °C/min) down to 25 °C and time was left for samples to equilibrate as demonstrated by the record of a time independent dynamic loss modulus G'' measured at 1 Hz with a 0.1 oscillatory shear strain amplitude. The mechanical spectrum of the solution was then recorded at 25 °C by performing a frequency sweep obtained with oscillatory strain amplitudes ranging from 0.01 to 0.15. The oscillatory torque response recorded on-line showed a sinusoidal wave form for all reported frequencies, thus ensuring a linear relationship between the applied sinusoidal strain and the measured stress. The solutions flow curves were then obtained from steady stress sweep tests (shear rate measured over the last 10 s of a step shear stress with 60 s duration, and steady state defined within a 2% tolerance for shear rate variation between two consecutive step shear stresses) performed between 0.1 and 100 Pa. Inspection of samples, right after performing the steady stress sweep tests, indicated that no flow instability (inertial, elastic or edge fracture leading to emulsion of the paraffin oil) took place within the range of applied stresses. As a result, a smooth shear thinning behavior was observed for all flow curves (see Figs. 3 and 5), which confirms that no secondary flow developed during the flow tests. For dilute EPS solutions showing viscosities below the sensitivity of the stress rheometer (such sensitivity limit was reached for concentration approaching 0.1 g/dl), a Cannon–Fenske capillary viscometer (COMECTA S.A., Barcelona, Spain) immersed in a temperature bath at 25 °C was used.

2.5. Light scattering

EPS solutions were passed through 0.45 μm polysulfone filters prior to analysis. Multi Angle Laser Light Scattering (MALLS) data were obtained by injecting the filtered EPS solutions at a flow rate of 19 ml/h with a syringe pump (Vial Medical, Program II) in the K5 flow cell of a MALS detector (Dawn, Wyatt Technology Corp., Santa Barbara, CA) irradiated by a Uniphase Argon laser (488 nm; 10 mW) and placed in series with the refractive index (RI) detector (Optilab DSP, Wyatt Technology Corp., Santa Barbara, CA). The MALLS and RI data were recorded with ASTRA software (Version 4.73.04, Wyatt Technology Corp., Santa Barbara, CA). Dynamic Light Scattering (DLS) was also performed on the same solutions, using an optical assembly equipped with a 20 mW HeNe laser (Photocor Instruments, Inc., College Park, MD). The time variations of light scattering intensity were analysed at 25 °C at an angle of 90° using the BI9000 correlator (Brookhaven Instruments Corp., Holtsville, NY). The mean light scattering intensities were analysed