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Characterization of new exopolysaccharide production by *Rhizobium tropici* during growth on hydrocarbon substrate

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ABSTRACT

Exopolysaccharide (EPS) are produced by a diverse of rhizobia species and has been demonstrated to be a bioemulsifier with potential applications in the degradation of hydrocarbons. In the present study, attempts were made to obtain the new exopolysaccharide production by *Rhizobium tropici* (SEMIA 4080 and MUTZC3) strains during growth on hydrocarbon substrate. Under the different cultivation conditions, the high molecular weight exopolysaccharides from *Rhizobium tropici* strains cultivated for 96 h mainly consisted of carbohydrates (79–85%) and a low percentage of protein. The EPSC3-D differed from the others, with only 60% of carbohydrate. However, all strains produced polymers with distinct rheology properties, such as viscosity of each EPS sample, suitable for different applications. In addition, RP-HPLC, FTIR and NMR studies revealed EPS produced by rhizobia strains were similar indicating minimal difference between EPS compositions.

1. Introduction

Polysaccharides produced by microorganisms can have diverse functional categories and can be subdivided into intracellular polysaccharides, structural polysaccharides and extracellular polysaccharides or exopolysaccharides (EPS) [1,2]. In response to the extreme environmental conditions, the bacteria produced these compounds [3]. Depending on the different physical chemical characterization, which is conferred by the individual monomer and non-carbohydrate composition, EPS can be applied in various industrial fields. For example, new bacterial EPSs reported over recent decades have emerged as biotechnology and bioremediation important biopolymers with high commercial value. This is due to, mainly to their use as new bioemulsifiers, that can efficiently emulsify vegetable oils and other aromatic hydrocarbons [4], as aqueous systems rheology modifiers (e.g., welan, xanthan, gellan) [5], biomaterials (e.g., bacterial cellulose) [6], sorption of exogenous organic compounds, and heavy metal sorption to bacterial cells [7], drug delivery agents [8], and others.

In the last decade, a large number of EPS-producing rhizobia have been described. The biopolymers derived from species of rhizobia are primarily composed of glucose and galactose with trace amounts of other neutral and acid monosaccharides, with particular charge and spatial arrangements [9]. These rhizobia strains are in the Rhizobiacae family in the alpha-proteobacteria and are in the *Rhizobium*, *Mesorhizobium*, *Ensifer*, or *Bradyrhizobium* genera [10–12]. Among EPS-producing rhizobia, *Rhizobium tropici* is famous for its potential biotechnology and bioremediation properties and has received considerable attention in recent years [4,13–15].

The manipulation of fermentation conditions is among the suggested tools for enhancing the chances of commercial scale production and thus the field applications of these biomolecules

Abbreviations: EPS, exopolysaccharide; CDW, cell dry weight; ATR-FTIR, attenuated total reflectance-fourier transform infrared spectrometer; RMN, nuclear magnetic resonance; VACP-MAS, variable amplitude cross polarization magic angle sample spinning sequence; HPSEC, high performance steric exclusion chromatography; RP-HPLC, reverse-phase high-performance liquid chromatography; UV–vis, ultraviolet-visible; PMP, 1-phenyl-3-methyl-5-pyrazolone; EPSWT, exopolysaccharide from the strain SEMIA4080 (*Rhizobium tropici*) cultivated in PSYL medium; EPSWT-D, exopolysaccharide from the strain SEMIA4080 (*Rhizobium tropici*) cultivated in modified PSYL medium with the addition of 0.1% diesel oil; EPSC3, exopolysaccharide from the mutant strain MUTZC3 cultivated in PSYL medium; EPSC3-D, exopolysaccharide from the mutant strain MUTZC3 cultivated in modified PSYL medium with the addition of 0.1% diesel oil E_{24} emulsification index.

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[16]. Hence, in our group many investigations have been conducted on the culture optimization conditions for EPS production by *Rhizobium* strains. The aim of this study was to analyze structures and determine some of the physical chemical properties of EPSs extracted from *R. tropici* SEMIA 4080 and the mutant strain (MUTZC3). Both were cultivated using the medium PSYL containing sucrose (3%; w/v), as carbon source, and supplemented with or without diesel oil, under aerobic conditions and then latter there has been an investigation on the bioemulsification potential of these EPS.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The wild-type strain of *R. tropici* SEMIA 4080 and mutant strain (MUTZC3) were used. YMA medium [17] supplemented with Congo Red (25 $\mu g\,mL^{-1}$) were used for routine rhizobia cultivation rhizobia. The cultures were incubated at 30 °C for 24 h.

For comparative growth curve analyses, EPS production, monosaccharide compositions, and the rheological properties of their EPS bacteria were cultivated either on PGYL medium ($1.4\,g\,L^{-1}$ K_2HPO_4; $1.0\,g\,L^{-1}$ KH_2PO_4; $0.2\,g\,L^{-1}$ $MgSO_4{\cdot}7H_2O$; $10\,g\,L^{-1}$ glycerol; $3.0\,g\,L^{-1}$ yeast extract; pH 6.9) or PSYL medium ($1.2\,g\,L^{-1}$ K_2HPO_4; $0.8\,g\,L^{-1}$ KH_2PO_4; $0.2\,g\,L^{-1}$ $MgSO_4{\cdot}7H_2O$; $30.0\,g\,L^{-1}$ sucrose; $1.0\,g\,L^{-1}$ yeast extract; pH 6.9). Since these procedures are being reported for first time using both media (PGYL and PSYL), their detailed composition and formula are under patent restriction (registration PI0304053-4). These strains were also cultured in modified PSYL medium supplemented with diesel oil.

2.2. Growth curves

Initial screening conducted under PSYL medium with different concentration of diesel oil (range 0.1–10%, v/v) were carried out to assess the broad range effect of the hydrocarbon and its tolerance by the analyzed strains. Cultures were grown in Erlenmeyer flasks in a rotary shaker at 30 °C and 150 rpm. After 96 h of incubation, the optical density was measured. Positive controls were prepared in the PSYL medium with bacteria, while negative controls were prepared in the same culture medium with bacteria inoculated but without sucrose and diesel fuel. One-way analysis of variance was used to determine the differences and Tukey's test was used to calculate statistical significance, and probability value $p < 0.05$ or $p < 0.01$ was considered to be significant in statistic.

Bacterial growth was determined by measuring optical density at 600 nm and based on the dry weight per volume of the culture. Aliquots of the cultivated strains were sampled at 0, 2, 4, 6, 8, 24, 48, 72, 96, 120 and 144 h. The cell growth was monitored by measuring the optical density at 600 nm (OD600). The OD readings were carried out by placing the sample (diluted) in a 1 mL cuvette, which was scanned using a Beckman, DU7400 (CA, USA) spectrophotometer.

After the initial 24 h of incubation, at each time course, the cell pellet was used for the gravimetric determination of the biomass concentration, after washing twice with deionized water (resuspension in water, centrifugation at 10,000 × *g*, 4 °C, 30 min), filtration through 0.20-μm membranes and filter drying at 50 °C until constant weight. The results were presented as relative growth in comparison to growth in diesel oil free medium. All experiments were performed in triplicate.

2.3. EPS production

For the evaluation of EPS production, pre-inoculum were prepared using the conditions described by Castellane et al. [4]. Aliquots of the corresponding cultures were transferred to 1000-mL Erlenmeyer flasks containing 500 mL of liquid PSYL medium supplemented with and without diesel oil at a final concentration of 10% (v/v) and incubated for 96 h at 140 rpm and 29 °C.

2.4. EPS extraction

For EPS extraction, the cell-free supernatant obtained by culture broth centrifugation (12,000 × *g*, 4 °C, 30 min; viscous samples were diluted with deionized water, for viscosity reduction, prior to the centrifugation) was mixed with cold 96% ethanol (3:1, v/v) [18]. The mixture was refrigerated at 4 °C for 24 h. After the refrigeration period, the samples were centrifuged once again (10,000 × *g*, 4 °C, 30 min) to separate the pellet from the solvent. The pellet was washed several times with ethanol followed by dialysis and then dried using a Hetovac VR-1 lyophilizer until a constant weight was observed. A precision balance was used to verify the amount of EPS obtained (grams of EPS per liter of culture); the results are presented as the means ± standard error.

2.5. Analysis of the EPS physical properties

2.5.1. Chemical composition

The solubility of biopolymers was tested by mixing amount of EPS (1%, w/v) in different solvents using 2 mL eppendorf tubes and vortexed for 60 s and observed for pellet dissolution. The concentration of total sugars and protein were determined by colorimetric assays, [19,20] using glucose (100, 75, 50, 37.5, 25 and 12.5 $\mu g\,mL^{-1}$) and bovine serum albumin (BSA) (2.0, 1.0, 0.5 and 0.25 $mg\,mL^{-1}$), respectively, as the standards for the calibration purposes.

2.5.2. Molecular weight (M_w)

Rhizobial EPS solutions (0.1% w/v) were filtered through a Millipore® membrane (0.22-μm pore size) and then injected (200 μL) in high performance size exclusion chromatography (HPSEC) coupled to a refractive index (RI) detector model RID 10A, and UV–vis detector (Shimadzu Co., Kyoto, KYT, Japan) as described by Orlandeli et al. [21]. A dextran standard curve with 1400, 1100, 670, 500, 410, 266 and 150 kDa was prepared to determine the Mw. Data analysis was performed using LC solution software (Shimadzu Corporation).

2.5.3. Chemical characterization

Hydrolysis of the EPS was performed with $2\,mol\,L^{-1}$ trifluoroacetic acid (200 μL) at 120 °C for 120 min. After 1-phenyl-3-methyl-5-pyrazolone-labeled monomers [14,22], the samples were analyzed by RP-HPLC.

FTIR spectra of the EPS samples were obtained on a Attenuated Total Reflectance-Fourier Transform Infrared Spectrometer (ATR-FTIR) (Bruker-VERTEX 70, Germany) using a KBr disc containing freeze-dried EPS samples as described by Osiro et al. [23]. Solid-state ^{13}C NMR spectra of EPSs were acquired using a 9.4 T Avance III, 400 Bruker spectrometer. The solid-state spectra were acquired using a Variable Amplitude Cross Polarization Magic Angle Sample Spinning sequence (VACP-MAS). The speed of rotation was 9 kHz, and the proton 90° pulse was 4 μs. The contact time was 1 ms [24].

2.5.4. Rheological properties in aqueous medium

Apparent dispersion viscosity (η) was determined at different shear rates, $\eta = K\gamma^{n-1}$ (where γ is the shear rate), using a controlled stress rheometer (Rheometrics Scientific). The rheological tests were conducted at 25 °C in triplicate. The ranges were determined using a shear rate control experiment in which the maximum shear rate value was $100\,s^{-1}$. The value of 'n' was obtained from the slope