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Production of exopolysaccharide from rhizobia with potential biotechnological and bioremediation applications

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ABSTRACT

The potential use of rhizobia under controlled fermentation conditions may result in the production of new extracellular polymeric substances (EPS) having novel and superior properties that will open up new areas of industrial applications and thus increase their demand. The production of EPS and the stability of emulsions formed with soybean oil, diesel oil and toluene using different concentrations of purified EPS derived from wild-type and mutant strains of *Rhizobium tropici* SEMIA 4077 was investigated. The EPS was defined as a heteropolysaccharide composed of six constituent monosaccharides that displayed higher intrinsic viscosity and pseudoplastic non-Newtonian fluid behavior in an aqueous solution. The ratio between the total EPS production and the cellular biomass was 76.70 for the 4077::Z04 mutant strain and only 8.10 for the wild-type strain. The EPS produced by the wild-type *R. tropici* SEMIA 4077 resulted in more stable emulsions with the tested toluene than xanthan gum, and the emulsification indexes with hydrocarbons and soybean oil were higher than 50%, indicating strong emulsion-stabilizing capacity. These results demonstrate that the EPS of *R. tropici* strains could be attractive for use in industrial and environmental applications, as it had higher intrinsic viscosity and good emulsification activity.

1. Introduction

Exopolysaccharides (EPS) are organic macromolecules that are formed by polymerization of similar or identical building blocks, which may be arranged as repeated units within the polymer [1]. A wide range of bacteria from various environmental habitats are known to produce complex and diverse EPS as capsular polysaccharides intensively associated with the cell surface or as slime polysaccharides, which are loosely associated with the cell [2]. EPS are being investigated for use in a wide range of commercial, medical, and industrial applications. However, the EPS have aroused great interest among biotechnologists because of their potential applications in the bionanotechnology sector, such as viscosifiers and syneresis-lowering agents, for their pseudoplastic rheological behavior and water-binding capacity [3]. Because of their sustainable production from renewable resources, generally nontoxic status, and biodegradability compared with oil-based nonrenewable polymers, the demand for EPS as newly emerging industrially important biopolymers has kept increasing [4].

Exopolysaccharides are also used as bioemulsifiers and increase the solubilities of hydrocarbons and the efficiency of hydrocarbon degradation [4,5], bioflocculating [4] and bioadsorption of heavy metals from wastewater and natural water [6] in the environmental sector. They are known to increase the viscosity of solutions and emulsify several hydrocarbon compounds [7] and are intriguing many researchers trying to harness their extraordinary properties and considerable potential applications in various fields [8]. Polysaccharide-based emulsifiers of microbial origin have drawn attention, as they offer several advantages over synthetic emulsifiers, including lower toxicity, higher degradability, better compatibility with the environment and selectivity [9]. In addition, they can remain effective even at extreme conditions of pH, temperature, and salinity. These properties increase their scope of applicability in a diverse range of biotechnological areas. However, considering the biodiversity of the microbial

Abbreviations: EPS, exopolysaccharide; CDW, cell dry weight; RP-HPLC, reverse-phase high-performance liquid chromatography; UV–vis, ultraviolet–visible; Glc, glucose; Gal, galactose; GalA, galacturonic acid; GlcA, glucuronic acid; Man, mannose; Rha, rhamnose; PMP, 1-phenyl-3-methyl-5-pyrazolone; EPSWT, exopolysaccharide from *Rhizobium tropici* SEMIA 4077; EPSMUT, exopolysaccharide from the 4077::Z04 mutant strain; E24, emulsification index.

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world and the number of papers published each year describing new microbial exopolysaccharides, it is astonishing to realize that only four microorganisms (i.e., *Xanthomonas*, *Leuconostoc*, *Sphingomonas*, and *Alcaligenes*, which produce xanthan, dextran, gellan, and curdlan) are the best known and most industrially used. However, there is a lack of studies on the applications of rhizobial EPS in bioemulsifiers.

Recently, Castellane et al. [10] examined different types of *Rhizobium* strains and reported three strains have been described that may prove to be an excellent model species for the development of biotechnology products because of their ability to produce large quantities of polysaccharides. *Rhizobium tropici* strain CIAT 899 (=USDA 9030; USDA 2744; =UMR 1899; =TAL 1797; =HAMBI 1163; =CM01; =SEMIA 4077; =ATCC 49672; BR322), another rhizobia symbionts of common bean (*Phaseolus vulgaris*) crop, was identified as member of the species *R. tropici*, is abundant in all Brazilian biomes [11]. The *R. tropici* CIAT 899 (=SEMIA 4077) and PRF 81 (=SEMIA 4080) are different strains that can produce a gel-like, extracellular polymeric substance or exopolysaccharides. However, EPS production by *R. tropici* populations is not crucial for the establishment of successful symbiosis with *P. vulgaris* L. but may contribute to competitiveness [12]. Large numbers of rhizobial EPS are potentially available, but relatively few have been commercially developed. To date, numerous types of exopolysaccharides have already been described [10,13–16]. Acidic EPS produced by all biovars of *R. leguminosarum* is a polymer of repeating units that consists of five glucoses, two glucuronic acids, and one galactose with acetyl, pyruvyl, and hydroxybutanoyl modifications [13]. The repeating units are assembled at undecaprenyl phosphate, the phosphorylated lipid carrier, and are then polymerized to high-molecular-weight EPS and transported to the cell surface [14]. Some exceptions to this structure have been reported [3]. One type of rhizobial EPS has been studied in more detail, namely succinoglycan, extracted from *Sinorhizobium meliloti* [1]. In addition, the galactoglucopolysaccharides obtained from *Rhizobium* spp., *Achromobacter* spp., *Agrobacterium radiobacter*, *Pseudomonas marginalis* and *Zooglea* spp. have been described.

Recent research and reviews have demonstrated potential practical applications of great value for EPS produced by rhizobia in agriculture because of it adhesive properties [17] and its ability to form gels that promote the adhesion of soil particles, forming stable aggregates that contribute to significantly improved vegetative growth [18]. The biopolymer drastically increases root structure, resulting in higher node densities, which results in significantly increased biomass. Some authors have observed that the presence of the EPS released by the microorganisms present in biological soil crusts is capable of creating a number of micropores that facilitate water movement in silt soils [18]. The aggregation of soil particles in large and compact structures, due to the presence of EPS, has been demonstrated to be a key factor for reducing evapotranspiration [19]. Although the role of bacterial EPS in soil aggregation is recognized, little information is available in the literature about the specific action of known bacteria and possible methods of management [3].

In the context that *Rhizobium* species are likely to be involved in a broad range of functions in diverse ecosystems and the commercial and ecological importance of the rhizobial EPS, the present investigation addresses the isolation, purification and physicochemical characterization of extracellular polysaccharides from the wild-type and mutant strains of *R. tropici* SEMIA 4077. Moreover, the stability of emulsions formed with soybean oil, diesel oil and toluene using different concentrations of purified EPS derived from different strains was also demonstrated to elucidate the potential of EPS as soil-stabilizing agents or as bio-emulsifiers for use in the degradation of hydrocarbons.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The wild-type strain of *R. tropici* SEMIA 4077 and mutant strain (4077::Z04) were used in the present study. For routine rhizobia growth, YMA medium (0.4 g L^{-1} yeast extract, 10 g L^{-1} mannitol, 0.5 g L^{-1} K_2HPO_4, 0.2 g L^{-1} $MgSO_4$, and 0.1 g L^{-1} NaCl, 9 g L^{-1} agar, pH 7.0) [20] was used. For routine mutant growth, to verify the purity of each mutant culture the YMA medium was supplemented with Congo Red (25 μg mL^{-1}), and the antibiotic kanamycin. The cultures were incubated at 30 °C for 24 h.

For comparative analyses of the EPS production obtained with the wild-type, and mutant strains of *R. tropici*, the monosaccharide compositions, and the rheological properties of their EPS, pre-inocula and batch experiments were performed using PGYA, PGYL, PSYA, and PSYL media. The detailed contents of these cultivation media are not available because the formulas are under patent restriction (registration PI0304053-4).

2.2. EPS detection and production

For phenotypic comparisons, experiments were performed as previously described by Castellane et al. [10], using Petri dishes containing solid PSYA medium, with and without a fluorescent brightener 28 (calcofluor white; Sigma–Aldrich) with an emission wavelength of 430 nm at a final concentration of 200 μg mL^{-1}. This fluorescent pigment is specific to polysaccharides that contain β-1→4 or β-1→3 linkages [21]. After 48 h of growth in the dark, at 30 °C, on PSYA solid medium, colonies were photographed using an upright Leica MZ75 stereomicroscope equipped with appropriate filters and a Leica DPD 250 camera.

For the evaluation of EPS production, pre-inocula were initially prepared from cultures cultivated on solid PGYA medium containing glycerol (10 g L^{-1}), as a carbon source. After 24 h, each inoculating strain was cultivated in 125-mL flasks (20 mL of medium in each) containing PGYL liquid medium on a rotary shaker at 140 rpm for 30 h, at which time a suspension with an optical density at 600 nm (OD_{600}) of 1.0 was obtained. The temperature was maintained at 30 °C. Aliquots of the corresponding cultures were transferred to 1000-mL Erlenmeyer flasks containing 500 mL of half-liquid PSYL at a final concentration of 10% (v/v) and incubated for 144 h at 140 rpm and 29 °C.

2.3. Cell biomass determination

The cell pellet was used for the gravimetric determination of the biomass concentration, after washing twice with deionised water (resuspension in water, centrifugation at 10,000 × *g*, 4 °C, 20 min), filtration through 0.20-μm membranes and filter drying at 60 °C until constant weight [22]. The growth was measured based on the dry weight (CDW) per volume of the culture.

2.4. EPS extraction

Cold 96% ethanol was added to the supernatant obtained from the centrifugation at a 3:1 (v/v) ethanol:supernatant ratio to precipitate the EPS [23]. 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 precipitate from the solvent. The precipitate was washed several times with ethanol, and the ethanol was evaporated. The solvent precipitation also achieved a partial purification of the polymer by eliminating the soluble components of the culture media [15].

The precipitated product was dried using a Hetovac VR-1 lyophilizer until a constant weight was observed, and a

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