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.

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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

[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 μg 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$_2$HPO$_4$; 1.0 g L^{-1} KH$_2$PO$_4$; 0.2 g L^{-1} MgSO$_4$·7H$_2$O; 10 g L^{-1} glycerol; 3.0 g L^{-1} yeast extract; pH 6.9) or PSYL medium (1.2 g L^{-1} K$_2$HPO$_4$; 0.8 g L^{-1} KH$_2$PO$_4$; 0.2 g L^{-1} MgSO$_4$·7H$_2$O; 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 μg 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

of the log–log plot of viscosity versus shear rate. The value of 'K' was determined by the intercept of the same graph.

2.6. Emulsifying activity

2.6.1. Preparation of emulsions

The emulsifying activity of cell suspensions and the both EPSs to stabilize emulsions with liquid paraffin oil and hexane were prepared as described previously by Freitas et al. [25] with a slight modification [15]. The emulsifying activity is defined as given below depending on the assay: $En = (he/ht) \times 100$, where n is time in hours, he is the emulsion layer height and ht is the mixture overall height. All samples were stored at 30 °C. All tests were performed in triplicate.

3. Results and discussion

In this research, the presence of sucrose (as carbon source) plus diesel oil in liquid medium PSYL was investigated. Consequently, to examine if the presence of diesel oil in growth medium affected the growth and viscosity in *R. tropici* SEMIA 4080 and mutant strain, the cultures were monitored by measuring the optical density at 600 nm (OD600) after 96 h incubation. For initial screening procedures using 0.1–10% diesel oil concentration, *R. tropici* SEMIA 4080 and mutant strain (MUTZC3) were able to grow on presence the diesel oil and 3% sucrose as evidenced by an observed increase in the turbidity of the cultures after 4 days' incubation (Fig. 1). The growth (OD_{600nm}) of wild-type *R. tropici* SEMIA 4080 and mutant MUTZC3 strains in liquid medium PSYL supplemented with diesel oil (0.1%, v/v) are 2.42 and 2.1 values, respectively. However, higher concentrations of diesel oil (10%, v/v) and sucrose (3%, v/v) have determined the growth' inhibition for the mutant strain MUTZC3 ($OD_{600nm} = 1.17$). These results revealed differences in the rates of growth for control (without diesel oil). In contrast, incubations with various concentrations of diesel oil little or no differences in the growth rates between the concentrations. Poor growth was observed for the medium containing only diesel oil ($OD_{600nm} = 1.2–1.4$).

Growth of the wild-type strain of *R. tropici* SEMIA 4080 and mutant strain (MUTZC3) using PSYL medium supplemented with and without diesel oil (0.1%, v/v) were also monitored by recording optical density (OD) at 600 nm until 144 h incubation, and the growth curve is shown in Fig. 2. It is remarkable that the wild-type strain of *R. tropici* SEMIA 4080 has been able to grow on diesel oil, as well as the mutant strain (MUTZC3). Growth of *Rhizobium* strains was found maximum in 0.1% diesel oil concentration during 120 h, that is, 4.36 for *R. tropici* (SEMIA 4080) and 3.1 for the mutant strain (MUTZC3). Similar work has been done using other substrates. According to the studies of Singh et al. [26], growth of *Rhizobium* strains was found maximum in 60% Dairy sludge concentration at 48 h, that is, 0.625 for *R. trifolii* (MTCC905), 0.804 for *R. trifolii* (MTCC906), and 0.793 for *Rhizobium meliloti* (MTCC100) and found minimum in rhizobial minimal media (RMM). Whyte et al. [27] reported that some surface-active compounds such as EPS, bioemulsifiers and biosurfactants are also closely related with the degradation of aliphatic hydrocarbons. Addition of diesel oil and surfactants into the culture medium may be an effective strategy to increase the yields of cells and EPS in fermentation processes since this strategy was proved to be successful in the bacterial fermentation, in which addition of surfactants could increase the yield of xanthan in cultures of *X. campestris* [28]. It is well known that *R. tropici* and others rhizobia isolates from *Phaseolus vulgaris* L. grow and produce measurable EPS using sucrose as a carbon source as tested in liquid medium [14] and our results showed that this species growth with the addition of diesel oil showed a strong stimulating effect on EPS production and physical-chemical characterics and culture medium. In the studied conditions (sucrose plus diesel oil), the cultures produced a flocculent, moderately turbid suspension with scantly viscid sediment and there has been observed biofilm formation.

3.1. EPS production

Assays were carried out to know the ability of wild-type strain of *R. tropici* SEMIA 4080 and the mutant strain (MUTZC3) to produce extracellular biopolymers with a great capacity for bioemulsification and adequate rheological properties, when there has been used an inoculum size at a final concentration of 10% (v/v) and PSYL medium supplemented with diesel oil (0.1% v/v) under aerobic conditions. The EPS production was evaluated after 96 h. The results from EPS production are shown in Table 1. The maximum amounts of rhizobia biomass ($0.77–1.44\,g\,L^{-1}$) and EPS ($5.26–5.52\,g\,L^{-1}$) were obtained at 96 h after 0.1% (v/v) diesel oil was added to the culture (Table 1). Under the conditions of this study, after 96 h it was observed a significant increase ($p < 0.05$) of 22.4% in the production of EPS, when compared with the control. This result showed that the optimal concentration of diesel oil with sucrose (as carbon source) could stimulate the production of EPS. Higher amounts of carbohydrate (79 and 85%) were found in EPS from the *R. tropici* SEMIA 4080 strain cultivated on PSYL medium supplemented without and with diesel oil, respectively, when compared with EPS from mutant strain cultivated on PSYL medium plus diesel oil, with only 60% of carbohydrate.

Kang and Park [29] evaluated the environmental factors that influence the biodegradation of diesel oil in *Acinetobacter* sp. strain DR1. These authors also noted that the induction of EPS via the addition of hexadecane, diesel oil, and NaCl. These bacterial EPS tend to facilitate the production of flocculation with hydrocarbons due to bridging with multivalent cations and hydrophobic interactions. In particular, this is an important and characteristic feature when contaminated areas are considered since it allows for in situ bioremediation through bio-barrier technology.

In previous studies with our testing conditions, using PSYL medium, the maximum yield of EPS was observed at 144 h, inoculated with different inoculum sizes (0.1%, v/v), after that a plateau was attained. Moreover, the cell lysis could lead to the enzymatic degradation of EPS towards the late growth stages. In this study, it was therefore, planned to study the fermentation pattern from 96 h onwards. The growth curves demonstrated that the maximum EPS production were achieved during the stationary phase of these strains (Fig. 2). According to Sutherland [30], a possible explanation for this uncoupling is that a competition exists between EPS and cell-wall polymer (e.g., lipopolysaccharides) biosynthesis and consequently, EPS production generally occurred only in the post-stationary growth phase of the microorganism. Generally, the influence of growth phase on EPS production is controversial [1].

pH is an important parameter for the growth of organisms, during the experiment it was found a lower acidification, in which the pH decreased from 6.9 ± 0.09 (0 h) to 6.35 ± 0.06 and 6.49 ± 0.03 at the end of the incubation period (96 h). The pH of the culture medium significantly influenced the EPS production. In general, the optimal medium pH for EPS production varies from 5 to 7.

3.2. Physicochemical characteristics of EPS

All EPS are highly soluble in aqueous solutions, whereas the low amount of protein and nucleic acids in the EPS indicated that in this study there were obtained an yield of high quality EPS (Table 1). However, it was possible to find some EPS with significant levels of proteins and nucleic acids, e.g. EPS from biofilms [31].
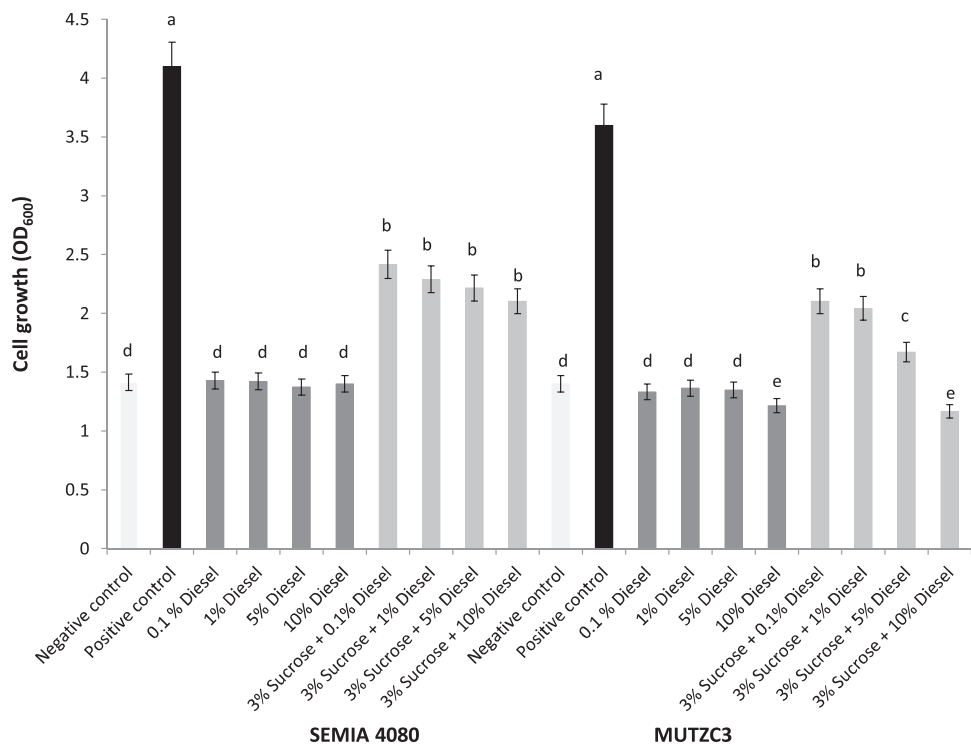
Fig. 1. Diesel oil concentration was varied from 0.1% to 10% for analysing the growth of *Rhizobium tropici* strains (SEMIA4080 and MUTZC3) in liquid medium PSYL. The values are the means ±SD of three different experiments performed on different days, determined by measuring the optical density after 96 h incubation (each experiment was conducted in triplicate). Growth under anaerobic conditions at 30 °C in shaker at 150 rpm. 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 oil. Different letters above the bars indicate a significant difference (p < 0.05).

Table 1
Evaluation of the differences in the exopolysaccharide production and cell dry weight between wild-type (SEMIA 4080) and mutant (MUTZC3) strains of *R. tropici*.

Strains	Culture Media	pH[f]	EPS	CDW	EPS/ CDW	P	TS
		(g l^{-1})				(mg g^{-1})	(%)
SEMIA 4080							
	PSYL (3% Sucrose)	6.52a	4.51b	1.44a	3.12b	0.07b	79ab
	Modified PSYL (3% Sucrose, 0.1% Diesel)	6.61a	5.26a	0.77b	6.81a	0.12a	85a
MUTZC3							
	PSYL (3% Sucrose)	6.52a	4.39b	1.19a	3.68b	0.02b	79ab
	Modified PSYL (3% Sucrose, 0.1% Diesel)	6.61a	5.52a	0.87b	6.38a	0.04b	60b

* pH[f] = final pH (initial pH 6.9). CDW, dry cell weight; P, protein; TS, total sugars.
Mean values within the same column not sharing a common superscript differ significantly (P < 0.05).

3.3. Partial structures analysis

After washing several times with ethanol and dialysis, the isolated EPS freeze-dried from the *R. tropici* SEMIA 4080 and the mutant MUTZC3 strains cultivated on PSYL medium supplemented with and without diesel oil (0.1%, v/v) were analyzed by RP-HPLC, FTIR and solid-state ^{13}C NMR spectroscopies. Fig. 3 shows the FTIR spectra of EPS produced by SEMIA 4080 and MUTZC3 strains from 1800 to 800 cm^{-1}. As observed in the spectrum, the peak 1, at 1725 cm^{-1}, has been assigned to C=O ester group, normally acetate, and the peak 2, at 1631 cm^{-1}, to O—H and/or carboxylate groups [23]. Moreover, the peak 3 observed at 1050 cm^{-1} was assigned to C—O groups from the EPS [23,24]. The FTIR spectra from 1800 to 4000 cm^{-1} for all EPS samples (EPSWT, EPSWT-D, EPSC3, and EPSC3-D) show a strong and broad OH peak at 3250 cm^{-1} and the methyl and methylene signals from 2850 to 2980 cm^{-1}.

The major differences in the FTIR spectra of EPSWT (black) and EPSWT-D (grey) were at the signals at 1725 and 1631 cm^{-1}. As these signals are stronger in EPSWT-D that are related to acetate and carboxylate groups, this indicates that the bacteria add more ester side chain and acid groups to EPS when cultivated on modified PSYL medium, with diesel oil. On the other hand, the FTIR spectra of EPS from mutant strain MUTZC3 did not show any significant difference between the grown in PSYL media supplemented with and without diesel oil. Therefore, the diesel oil did not interfere in the EPS composition. The FTIR spectra of the EPSC3 (black) and EPSC3-D (grey) (Fig. 3B) were similar to the FTIR of the EPSWT-D (Fig. 3A).

In Fig. 4 it is shown the ^{13}C NMR spectra of EPSWT, EPSWT-D, EPSC3, and EPSC3-D obtained in PSYL medium supplemented with (black) or without diesel oil (grey). The CP-MAS spectra have shown broad peaks indicating that most of the EPS structure is very rigid and in an amorphous form. Crystalline structure such as the one observed in cellulose show shaper and more resolved lines [24]. The broad signals from 60 to 110 ppm, peaks 3 to 6 have been assigned to the six carbons of the EPS pyranoid ring. The signals between 60 and 70 ppm (peak 6) have been assigned to C6 carbons, from 70 to 80 ppm to C2, C3 and C5 carbons (peak 5), at 82.5 ppm (peak 4) to C4 and the signal at 105 ppm (peak 3) to C1, the carbon bonded to two oxygen atoms [23,24,32]. The broad peak at 175 ppm (peak 1)
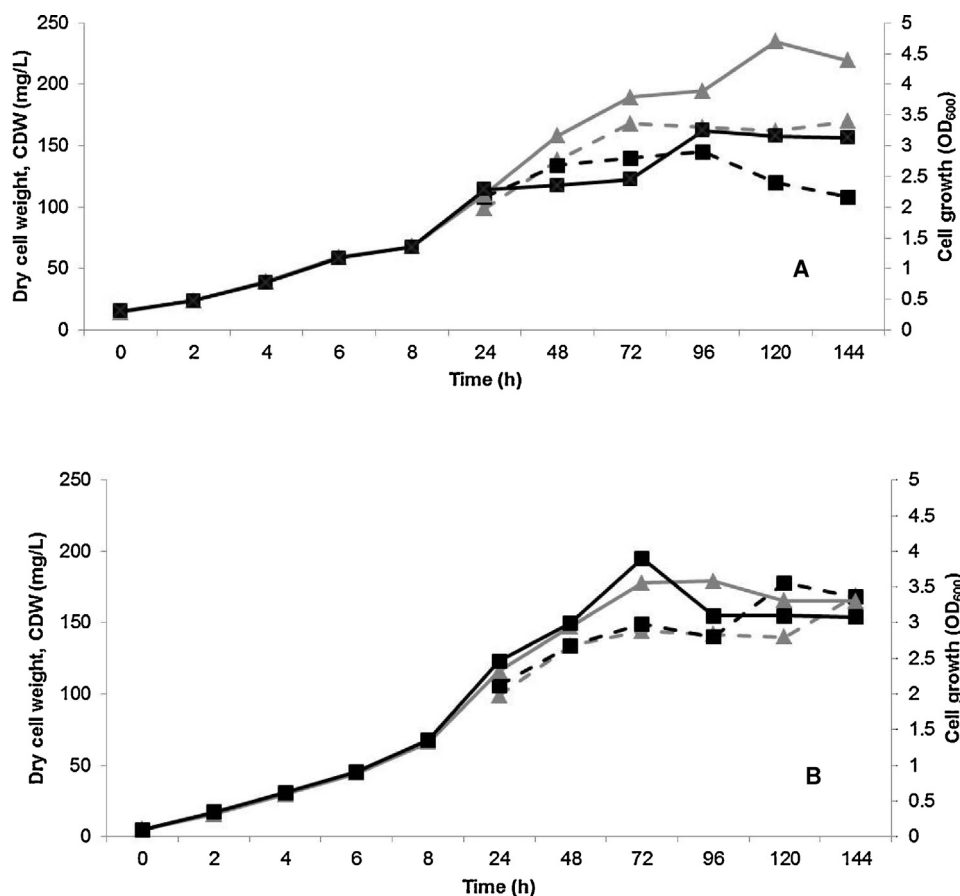
Fig. 2. Growth curves of *Rhizobium tropici* SEMIA 4080 (A) and mutant strain MUTZC3 (B) on the liquid medium PSYL supplemented with and without diesel oil (0.10%, v/v). *Rhizobium tropici* strains were incubated with (■) and without (▲) diesel oil (0.1%, v/v); dry cell weight, CDW (dashed line) and cell growth (solid line). The cell growth was monitored by measuring the optical density at 600 nm (OD600). Each point represents the average value obtained with triplicate flasks.

has been assigned to C=O ester and/or COOH of pyruvic and uronic acid, such as glucuronic acid and galacturonic acid.

However, the major difference between samples is observed in the sharp peaks of the EPSWT or EPSWT-D (Fig. 4A). These differences are more pronounced in the single pulse excitation (SPE-MAS) spectra. The sharp peak at 170 ppm (peak 2), has been assigned to C=O of ester or carboxylic group of the acetate or succinate, the peaks at 66 ppm (peak 7) to C6 succinate [33], the peak 7 at 43 ppm to C2 and C3 of succinate, and the peaks 8 and 9, at 26 and 21 ppm to methyl groups of pyruvate and acetate, respectively [23,34]. The succinate has been reported in the EPS of rhizobia [33]. The peak at 20 ppm has been assigned to the methyl group of rhamnose. The presence of succinate and acetate groups were confirmed in the ^{1}H NMR spectra of the hydrolyzed EPS, by the singlet at 2.67 and 2.25 ppm. However, the pyruvate signal at 1.4 ppm was not observed in the samples.

Fig. 4B shows that the CP-MAS and SPE MAS (inset) of spectra of EPSC3 and EPSC3-D were similar indicating minimal difference between the two EPS. Therefore, the FTIR and ^{13}C NMR results show that the major effect of diesel oil in the EPS is observed in EPSWT that is the increase of succinate, pyruvate and acetate contents.

Similarly, to most rhizobial EPS, the repeating sugar units are mainly composed of glucose and galactose (Table 2). As seen in Fig. 5, the EPS elution profile by HPSEC/RID analysis showed one peak, suggesting the presence of only one type of EPS. For all samples (EPSWT, EPSWT-D, EPSC3, and EPSC3-D), the molecular weight were estimated to be higher than 1.4×10^6 Da, according to a standard calibration curve obtained from definite molecular weight dextrans (Fig. 5). These results indicate that the high molec-

Table 2
Comparative monosaccharide composition of EPS (%) produced by the wild-type (SEMIA4080) and mutant (MUTZC3) strains of *Rhizobium tropici*[a].

SAMPLES[b]	Monomers Compositions (%)		Reference
	Glucose	Galactose	
EPSWT	55.48	32.47	[14]
EPSWT-D	71.13	25.54	In this study
EPSC3	53.53	40.52	[14]
EPSC3-D	74.37	21.92	In this study

[a] On the basis of peak area, the percentage of monosaccharides was calculated.
[b] 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.

ular weight exopolysaccharides from *Rhizobium tropici* strains, a water soluble heteropolysaccharides, which consists of six repeating units monosaccharides and non-sugar components like uronic acid, methyl and esters. Also, based on the above results from FTIR, RMN and RP-HPLC, it concluded that these EPS obtained in PSYL medium supplemented with or without diesel oil (0.1%, v/v), indicating similar monosaccharide and non-carbohydrate compositions.

3.4. Rheological properties of purified EPSs solution

To evaluate the rheological properties of rhizobial EPS we determined the viscosity of its 10% w/v aqueous solution over a range
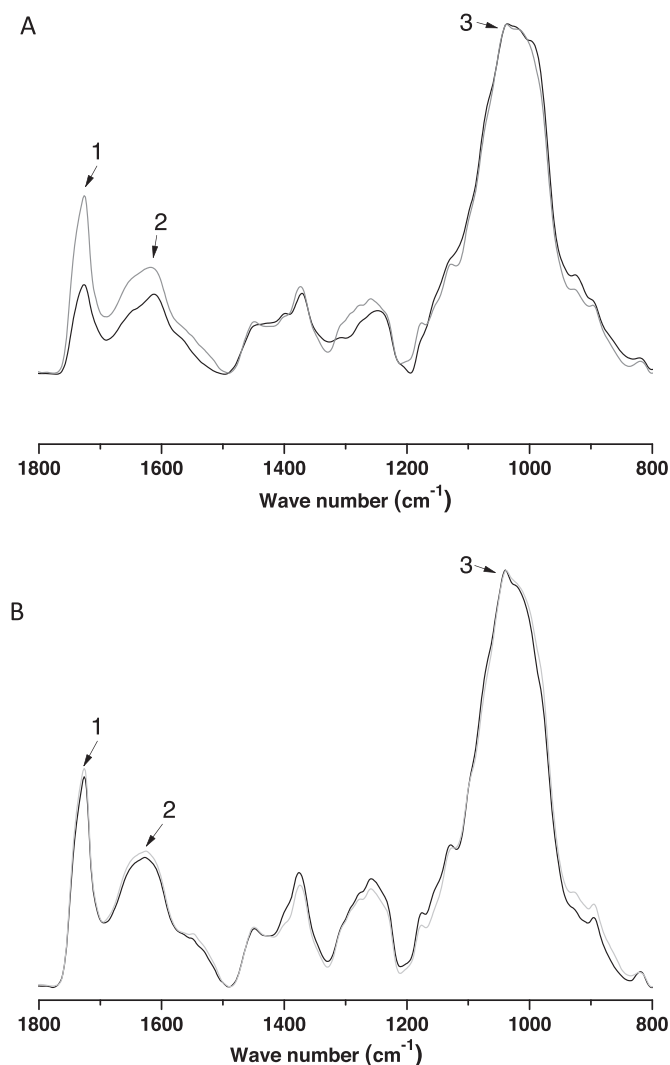
Fig. 3. FTIR spectra of the EPS from *Rhizobium tropici* SEMIA 4080 (A); mutant strain MUTZC3 (B). *R. tropici* strains were grown in PSYL medium supplemented with (black) and without diesel oil (grey) for 96 h.

Fig. 4. ^{13}C CP-MAS NMR spectra of the EPS samples of SEMIA4080 (A) and mutant strain MUTZC3 (B). *R. tropici* strains were grown in PSYL medium supplemented with (black) and without diesel oil (grey). The insets are the single pulse excitation (SPE-MAS) spectra of the same samples.

of different shear rates. Aqueous solutions of rhizobial EPS showed high viscosity values and pseudoplastic behavior, when the viscosity decreased concomitantly with an increase in shear stress (Fig. 6).

According to previous research, the viscosity values of the solution of EPSWT-D and EPSC3-D were higher than those of the EPSWT and EPSC3 [14]. By the definition the consistency, stability and viscosity are the important rheological parameters that govern the quality of biopolymers.

3.5. Emulsifying activity

The wild-type strain of *R. tropici* SEMIA 4080 and the mutant strain (MUTZC3) were found to produce an extracellular and cells with specific activities, could be an emulsifying agent (bioemulsifiers). This fact was noticeable for paraffin liquid oil and hexane, using PSYL medium supplemented with and without diesel oil for culture growth and EPS production. For both of the culture media (with cells) in which *Rhizobium tropici* strains were grown with diesel oil, the higher emulsifying activity was observed with hexane and paraffin liquid oil, as shown by its emulsification index (E_{24}) higher than 50%, SEMIA 4080 with values of 84.09 and 74.34% and mutant strain (MUTZC3) with values 89.59 and 60.84% for hexane
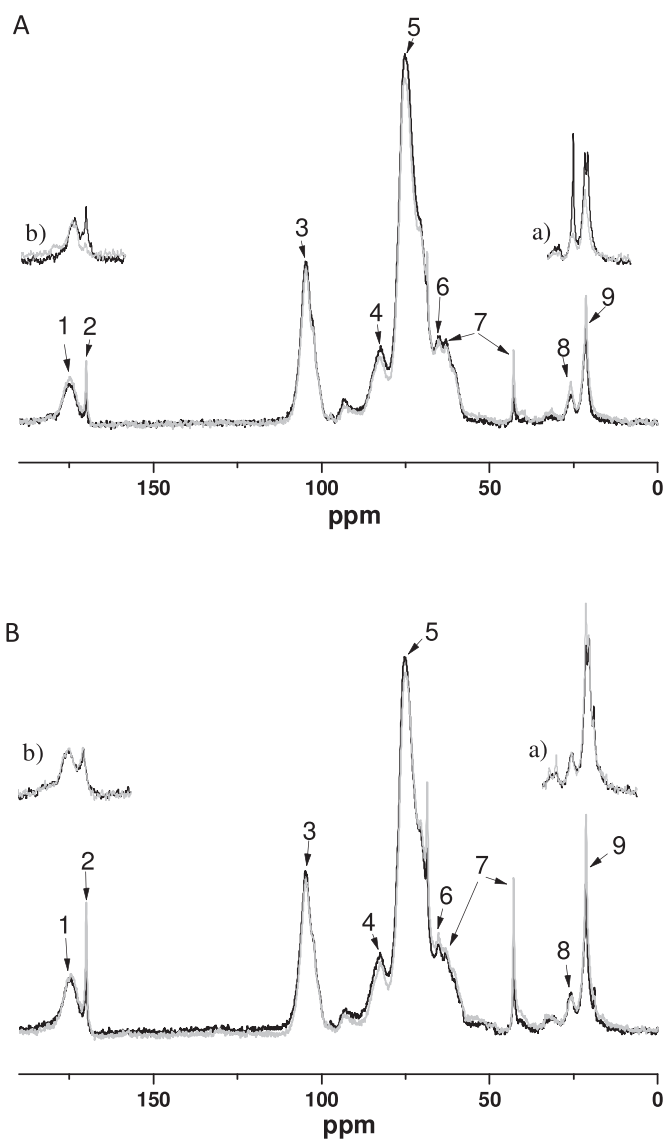
and paraffin liquid oil, respectively (Table 3). One possible explanation, the capsule (hydrophilic) could be released together with the bound protein (hydrophobic) into the medium, forming a complex with high emulsifying activity [35]. However, results here reported that the bioemulsifier produced by the cell-free medium from this bacterial cell cultures and in the presence hexane and paraffin liquid oil low values of 50% and, these bioemulsifiers exhibit considerable substrate specificity, p.e., Emulsan does not emulsify pure aliphatic, aromatic or cyclic hydrocarbons; however, mixtures of those compounds can be efficiently emulsified [36].

Both strains possessed lower emulsion-stabilizing capacity for hexane and paraffin liquid oil, an except as shown by its emulsification index (E_{24}) of SEMIA 4080 with 55.85% in the presence paraffin liquid oil. However, the addition of diesel oil to the culture medium containing sucrose resulted in emulsification values higher than those obtained without hydrocarbons.

In order to study the possible effects of the EPS concentration on the emulsion stability, emulsions were prepared with two different concentrations of EPS and hexane and paraffin liquid oil, and the emulsification indexes (E24) were also determined (Table 3).
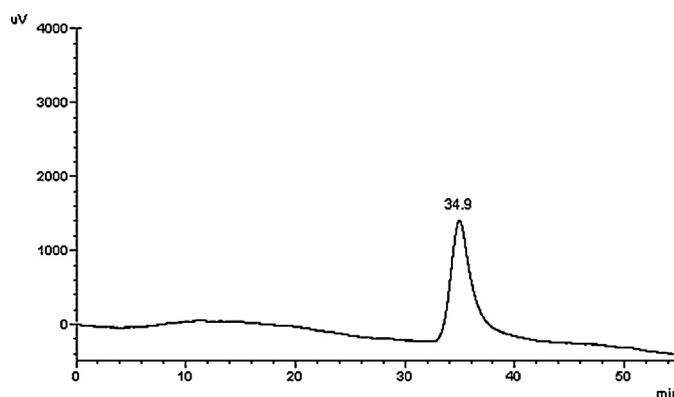
Fig. 5. Illustration of elution profile of EPS from *Rhizobium tropici* SEMIA4080 analyzed by HPSEC/RID coupled to a UV–vis detector.
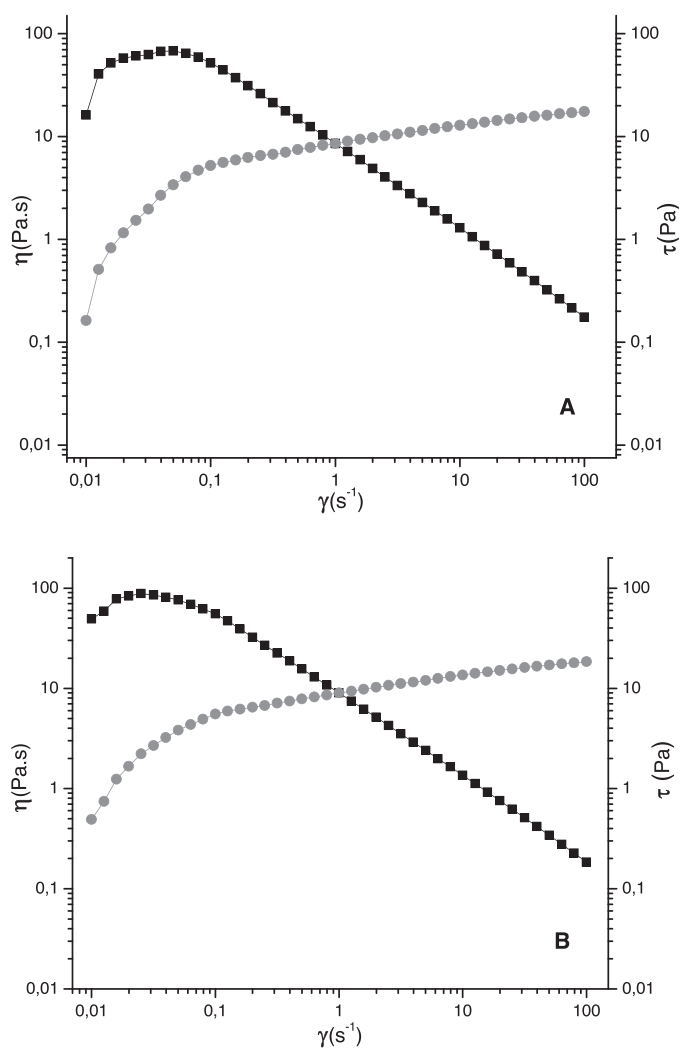

Fig. 6. Flow curves of solutions of the exopolysaccharides from: (A) the wild-type strain of *Rhizobium tropici* (SEMIA4080) and (B) the mutant (MUTZC3) strain at a $10 \, g \, L^{-1}$ concentration. These flow curves were measured at 25 °C. The ■ and ● symbols represent viscosity (η, Pa s^{-1}) and tension (τ, Pa), respectively, at $10 \, g \, L^{-1}$.

The high emulsification indexes observed reflect the stability of the emulsions thus formed with hexane and paraffin liquid oil. As shown in Table 3, significant differences in the emulsification indexes were observed for the EPS when the emulsion was prepared with EPS concentrations ranging from 1 to 5 mg/mL. Similar results have been obtained with the EPS of other bacterial species.

The hydrocarbons tested, namely, hexane and paraffin liquid oil, though having lower emulsification indexes (5.7 and 60%), formed sometimes unstable emulsions that breaked within several days after their preparation. Chemical characterization of rhizobial EPS revealed that its ability to associate with and emulsify hydrocarbons may be conferred by its presence uronic acids and deoxysugars

Table 3
Stability of emulsifying activity of culture medium, a substantially cell-free medium and exopolysaccharide (EPS) from a bacterial cell culture in which wild-type SEMIA 4080 or mutant (MUTZC3) strains were grown.

Samples	Hydrocarbon/oil	E24%		
		Culture medium	Cell-free medium	EPS (5%, wt/v)
SEMIA 4080 (3% Sucrose)	Liquid paraffin oil	**54.42**	23.19	**58.06**
	Hexane	49.63	31.96	43.51
SEMIA 4080 (3% Sucrose, 0.1% Diesel)	Liquid paraffin oil	**74.34**	36.93	38.89
	Hexane	**83.09**	**56.89**	34.67
MUTZC3 (3% Sucrose)	Liquid paraffin oil	26.45	49.8	41.85
	Hexane	**51.61**	34.74	**59.83**
MUTZC3 (3% Sucrose, 0.1% Diesel)	Liquid paraffin oil	**60.93**	38.62	**60.42**
	Hexane	**89.59**	41.39	42.58

[a]Results are expressed as percentages of the total height occupied by the emulsion; values are means of at least three determinations.
Values in bold: Emulsifier Index (E24) with values above 50%.

such as fucose and rhamnose content, moieties that can render EPS quite lipophilic and mediate the adsorption of polysaccharides to oil droplets [37]. The hydrophobic groups enhanced the interaction between EPS and hydrophobic compounds, and high emulsifying activity was thus presented [38].

Therefore, and since most published data reported E24 > 50% for potential biopolymers, bioemulsifiers and biosurfactant producing strains of *R. tropici* in the present work we have identified seven promising microbial candidates for used in numerous industrial areas. However, biosurfactants represent excellent ecological alternatives to their synthetic emulsifiers because they are less toxicity, potentially high activities and stabilities at extreme temperatures and pH. The EPS produced by *R. tropici* may be included among those bioemulsifiers.

4. Conclusions

Based on the above results, the exopolysaccharide production by *Rhizobium tropici* SEMIA 4080 and mutant (MUTZC3) strains were improved by increasing the initial inoculum and addition of diesel oil in PSYL medium, but cell growth was impaired. Moreover, under the different cultivation conditions, SEMIA 4080 and MUTZC3 strains produced polymers with distinct properties, suitable for different applications. The addition of diesel oil (0.1%, v/v) in culture medium resulted in emulsification values higher than those obtained without hydrocarbons, under aerobic conditions. EPS production during on growth media could act as biostimulant for bioremediation of oil-contaminated sites and be an effective and environmentally compatible green alternative used for improving the efficiency of oil remediation. The ability of these bioemulsifier to form stable emulsions with hexane and paraffin liquid oil maked it an interesting biotechnological product for oil industry, such as for bioremediation of oil contaminated sites. Hence, the strategy of supplementation of stimulatory agents in this study can be applied in other bacteria fermentation processes for enhancing production of extracellular metabolites and exopolysaccharide can be a useful tool to obtain an EPS with desired functionalities and with new characteristics. Further genetic studies on the factors associated with this enhancement will provide insight into the interactions occurring between aliphatic hydrocarbons and bacterial cells.

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