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Exopolysaccharide produced by *Gordonia polyisoprenivorans* CCT 7137 in GYM commercial medium and sugarcane molasses alternative medium: FT-IR study and emulsifying activity

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ABSTRACT

EPS Gp CCT 7137 produced by *Gordonia polyisoprenivorans* CCT 7137 in GYM commercial medium and sugarcane molasses alternative medium showed a carbohydrate structure composed mainly of α-anomers with presence of carboxylic and amide functional groups. The presence of carboxyl groups can be responsible for the low pH of EPS aqueous solutions, probably associated with the protein structure, acting as binding sites for cations. This characteristic is important in emulsification applications. When produced in a GYM commercial medium, EPS Gp CCT 7137 showed superior or similar emulsifying activity than commercial surfactant Triton X-100 and a better performance than EPS produced in a SM alternative medium. The results suggest the potential of EPS Gp CCT 7137 for removal of contaminating oil.

1. Introduction

Several microorganisms produce exopolysaccharides (EPS) which are found adhered to cell surface (capsule) or released into the adjacent medium in the form of amorphous slime. Microbial polysaccharides are ionic or non-ionic water-soluble polymers with regular, branched or unbranched repeating units connected by glycosidic linkages and the variety of chemical structures found in miocrobial EPS leads to a broad spectrum of biological, physical and chemical properties, allowing a great diversity of applications in the food, chemical and pharmaceutical industries, such as gelling, thickening, antitumor, flocculation agents and emulsifiers (Sutherland, 1998). Recently studied microbial EPS are: bacterial cellulose (β-D-glucan), produced by *Gluconacetobacter xylinus* (Barud et al., 2008; Legnani et al., 2008); curdlan ((1 → 3)-β-D-glucan) from *Agrobacterium* (McIntosh, Stone, & Stanisich, 2005); pullulan (α-D-glucan) from *Aureobasidium pullulans* (Singh, Gaganpreet, & Kennedy, 2008); gellan from *Sphingomonas paucimobilis* (Banik, Santhiagu, & Upadhyay, 2007; Sá-Correia et al., 2002); alginate from *Pseudomonas* and *Azotobacter* (Remminghorst & Rehm, 2006); xanthan from *Xanthomonas campestris* (Hamcerencu, Desbrieres, Popa, Khoukh, & Riess, 2007) and emulsan from *Acinetobacter calcoaceticus* RAG 1 (Singh, Van Hamme, & Ward, 2007).

Among EPS potential applications, biosurfactants have gained considerable interest due to their low toxicity, biodegradable nature and diversity (Banat, Makkar, & Cameotra, 2000) and have considerable potential in commercial applications within oil-processing operations, in bioremediation and manufacturing processes, mining, agriculture, microbial bioprocessing operations and medical applications (Singh et al., 2007). However, biosurfactants industrial application has been limited due to their cost of production relative to the cost of chemical surfactants (Banat et al., 2000; Singh et al., 2007).

EPS synthesis is influenced by environmental variables such as temperature, pH, oxygen, carbon source, which can be used to control its production for different purposes (López, Ramos, & Sanromán, 2003; Nampoothiri, Singhania, Sabarinath, & Pandey, 2003; Ricciardi et al., 2002). To reduce production costs and the use of clean technology, agro-industrial co-products such as corn-steep liquor and molasses, may be utilized as alternative EPS growth substrates. As the synthesis of EPS with surfactant activity is not always a consequence of microbial growth on hydrophobic substances (Calvo, Manzanera, Silva-Castro, Uad, & Gonzáles-López, 2009), soy molasses was proposed as carbon source supplement to generate a lower cost biosurfactant from *Acinetobacter venetianus* (Panilaitis, Castro, Solaiman, & Kaplan, 2007).

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Owing to its potential as a biopolymer source of industrial interest, EPS-producing microorganisms have been isolated from a wide range of environments. Among them, *Gordonia polyisoprenivorans* CCT 7137 was isolated as an EPS-producing strain from contaminated groundwater (Fusconi & Godinho, 2002). The strain has been investigated concerning its ability to grow and produce EPS from sugarcane molasses, an agriculture-based co-product rich in fermentable sugars, as sole nutrient source (Fusconi, Godinho, & Bossolan, 2005; Fusconi, Godinho, & Bossolan, 2008).

The objectives of the present work were to isolate, purify the EPS produced by *G. polyisoprenivorans* CCT 7137 in GYM commercial medium and sugarcane molasses as an alternative medium, to characterize its main functional groups and to study its emulsifying activity on monoaromatic petroleum hydrocarbons.

2. Materials and methods

2.1. Bacterial strain

Gordonia polyisoprenivorans CCT7137 was isolated during sorting and selection of EPS-producing bacteria from groundwater contaminated with leachate from an old controlled landfill in the city of São Carlos (São Paulo, Brazil) (Fusconi & Godinho, 2002). The strain belongs to the genus *Gordonia*, class *Actinobacteria*, order *Actinomycetales*, suborder *Corynebacterineae*, family *Gordoniaceae,* it is an EPS-producing bacterial strain, Gram-positive, catalase-positive, oxidase-negative, non-motile, non-sporing and did not reduce nitrate (Fusconi et al., 2005).

2.2. Culture media

Solid media, used for plate and stock cultures, were nutrient agar (g L^{-1}: 5.0 peptone, 3.0 meat extract, 15.0 agar) and glucose-yeast-maltose (GYM) agar. The media used for liquid culture were GYM, as commercial medium (Kondo et al., 2000) (g L^{-1}: 4.0 glucose, 4.0 yeast extract, 10.0 maltose, 15.0 agar), and sugarcane molasses (SM) as alternative gowth medium. The raw molasses had to be clarified, to eliminate interference from solid residues (Mattos, Volpon, Previato, & Previato, 1997), before the final medium is made up. Molasses was diluted with an equal weight of distilled water (1:1) containing a solution of NaH_2PO_4 (final concentration of 1.5 g L^{-1}), autoclaved at 120 °C for 30 min and allowed to settle for 24 h. The liquid phase (clarified molasses) was then siphoned off and diluted in distilled water to obtain sugarcane molasses media 6% (v/v) with 2.13% of sugars. The concentration of sugar in raw molasses was 71.5% while in clarified molasses it was 38.5%, according to the refractive index measured with a Zema refractrometer (Carl Zeiss). The pH was adjusted to 7.0 ± 0.2 and the media were autoclaved at 121 °C for 15 min.

2.3. Production of EPS

Batch fermentation for EPS production was carried out by a modified method previously described by Fusconi et al. (2005): stock cultures of *G. polyisoprenivorans* CCT 7137, stored at 4 °C, were plated on nutrient agar and resuspended in 10 mL GYM broth. 7.5 mL of this suspension was added to 142.5 mL fresh GYM broth in 500 mL Erlenmeyer flasks to obtain the pre-culture. After 22–24 h growth at 30 °C, the exponential phase, 11 mL of the pre-culture was withdrawn and used to inoculate 209 mL of the medium being tested (GYM and SM) in 1000 mL Erlenmeyer flasks. The inoculum was standardized at $O.D._{600}$ 0.3 (10^7 cfu mL^{-1}). The liquid cultures grew aerobically at 30 °C, being stirred in a rotary shaker set at 150 rpm throughout the experiments. Samples for emulsification assays and for EPS extraction and purification were taken after 72 h of incubation, at the end of the stationary phase.

2.4. EPS extraction and purification

EPS produced by *G. polyisoprenivorans* CCT 7137 was extracted and purified following the modified method of Faber, Van den Haak, Kamerling, and Vliegrnhart (2001), described by Dogsa, Kriechbaum, Stopar, and Laggner (2005). After incubation, proteins in the fermented broth were precipitated by adding trichloroacetic acid to a final concentration of 8% (w/v). The mixture was stirred for 45 min and the cell debris removed by centrifugation at 5000 rpm for 1 h at 4 °C (Allegra 21R Centrifuge-Beckman Coulter). The EPS in the supernatant was precipitated by addition of two volumes of cold ethanol (95%), and left overnight at 4 °C. EPS was recovered by centrifugation (6000 rpm, 20 min, 4 °C) and dialyzed (cellulose dialysis tube, molecular weight cut-off: 12,000–16,000) against distilled water for 48 h (water was changed four times each day) to eliminate residual sugars from the culture medium. After dialysis, EPS was stored in two volumes of ethanol (95%) at 4 °C, concentrated in a rotary evaporator and dried for 48–72 h at 35 °C.

2.5. Infrared spectra of EPS

The main functional groups of the purified EPS were assigned using Fourier-transformed infrared (FT-IR) spectroscopy. Pellets for infrared analysis were prepared by pressing a mixture of EPS with dry KBr (1:100 EPS:KBr w/w). FT-IR spectra were recorded covering the 4000– 400 cm^{-1} region with 20 accumulated scans and resolution of 4 cm^{-1}, using a Shimadzu Prestige – 21 FT-IR spectrometer.

2.6. Viscosity assays

The intrinsic viscosity, $[\eta]$, of EPS solutions was determined with a Cannon-Fenske capillary viscosimeter in 0.1 mol L^{-1} NaCl aqueous solution as solvent. The measurements were made at 25 °C. The Huggins equation was used for intrinsic viscosity determination. The concentration of the EPS solutions was in the range 5.0×10^{-5} and 1.0×10^{-3} g mL^{-1}.

2.7. Emulsification assays

Emulsification assays were carried out according to Iqbal, Khalid, and Malik (1995) using cell free GYM and 6% SM liquid media from *G. polyisoprenivorans* CCT 7137 grown during 72 h. After incubation, proteins in the fermented broth were precipitated by adding trichloroacetic acid to a final concentration of 8% (w/v). The mixture was stirred for 45 min and the cell debris removed by centrifugation at 5000 rpm for 1 h at 4 °C (Allegra 21R Centrifuge-Beckman Coulter). In each test tube (12-mm-diameter glass tubes), 2 mL of the culture supernatant was mixed with an equal volume of hydrocarbon compound (benzene, toluene and *o*-xylene). The tubes were vortexed for 2 min and left to stand for 24 h. The emulsifying activity was investigated after 24 h and the emulsification index (E_{24}) expressed as the percentage of total height occupied by the emulsion and was calculated as follows: E_{24} = (height of emulsion layer/total height) × 100. The higher the emulsification index, the higher the emulsification activity. Triton X-100 (Sigma) was used as chemical surfactant for comparison. A control was prepared using the same method but replacing the sample by non-innoculated GYM and SM liquid media. Each culture was done in triplicate and results were tested by analysis of variance (Anova), followed by the Tukey test at a 0.05 level of significance.

3. Results and discussion

3.1. FT-IR spectroscopy

Fig. 1a and b shows the FT-IR spectra of EPS produced by *G. polyisoprenivorans* CCT 7137 in GYM and SM media. From this point, EPS produced by *G. polyisoprenivorans* CCT 7137 is referred to as EPS Gp CCT 7137. EPS Gp CCT 7137 produced in both media showed a carbohydrate structure characterized by a broad band at 3415 cm^{-1}, assigned to O–H, stretching and a set of intense bands between 1160 and 1000 cm^{-1}, particularly the bands at 1142, 1082 and 1042 cm^{-1} (attributed to C–O–C antisymmetric bridge stretching), 1082 (related to primary C–OH group at the C_6 position (Shingel, 2002) and 1042 cm^{-1} (associated to C–O stretching) (Fig. 1b) (Beech, Hanjagsit, Kalaji, Neal, & Zinkevich, 1999; Bramhachari & Dubey, 2006; Shingel, 2002; Yim, Kim, Ahn, & Lee, 2007). Considering the carbohydrate structure, a small band at 830 cm^{-1}can be seen in the spectra. This band is related to anomers in polysaccharides since the region between 950 and 700 cm^{-1} is strongly dependent of the anomeric carbon (Xiao, Sun, & Sun, 2001). In the region between 2000 and 750 $cm^{-1,}$ (in detail in Fig. 1b), the presence of the band at 830 cm^{-1} combined with the lack of observation of the bands at 895–905 cm^{-1} indicates that the EPS Gp CCT 7137 samples, produced in GYM and SM media, are composed mainly of α-anomers. Other important absorption bands which can be seen in FT-IR spectra of EPS samples are the one at 1741 cm^{-1}, assigned to C=O stretching of acetyl ester bonds, two bands at 2924 and 2854 cm^{-1}, assigned to C–H asymmetric stretch and C–H symmetric stretch of CH_2 and CH_3 groups, respectively, and the band at 1655 cm^{-1} attributed to C=O stretching of carboxyl group and/or protein related bands of amide I. Another band attributed to amide II of the protein structure can be seen at 1551 cm^{-1} (Beech et al., 1999). The low intense band at 1260 cm^{-1}, assigned to C–O, confirms the presence of *O*-acetyl ester bonds.

The presence of these functional groups in the EPS samples is important for technological applications of biopolymers as bioemulsifiers and bioflocculants (Yim et al., 2007). Some hydrocolloids have demonstrated interfacial properties. Yapo, Wathelet, and Paquot (2007) and Lutz, Aserin, Wicker, and Garti (2009) showed that pectin can be used as emulsifier and/or emulsion stabilizer and that several factors such as the degree of ester and carboxylic groups and weight average molar mass can be associated to the emulsifier ability of EPS. The quantitative analysis of FT-IR related to bands associated to acetyl and carboxylic groups provides information about esterification degree and/or presence of acid groups in the EPS. The relationship between the absorbance intensity of 1741 cm^{-1} divided for sum of those corresponding to 1655 and 1741 cm^{-1} band (Eq. (1)) should be proportional to esterification degree (R) of the sample. This ratio has been used by Manrique and Lajolo (2002) for determination of esterification degree of pectin an important hydrocolloid used in food industries. Considering the use of this technique for EPS produced by *G. polyisoprenivorans* CCT 7137, the samples showed a ratio of 0.45 and 0.38 for EPS from SM and GYM media, respectively.

$$R = \frac{A_{1741\ cm^{-1}}}{A_{1655\ cm^{-1}} + A_{1741\ cm^{-1}}} \quad (1)$$

The presence of carboxyl groups can be responsible for the low pH of EPS aqueous solutions (4.0–4.5).

Both FT-IR spectra of EPS Gp CCT 7137, produced in GYM and SM media showed the same patterns indicating that the EPS produced have similar chemical structures.

3.2. Viscosity assays

The values of intrinsic viscosity of EPS, produced from GYM and SM media, obtained from Huggins plots are shown in Fig. 2a and b. The intrinsic viscosity of EPS from GYM medium (726.9 mL g^{-1}) is higher than the value observed for EPS produced in SM medium (609.9 mL g^{-1}). Despite the similar chemical structure of the EPS Gp CCT 7137 produced in different culture media, as supported by FT-IR measurements, the higher viscosity value of EPS produced in GYM medium compared to EPS produced in SM medium indicates that the differences in nutritional culture medium affect the production of EPS polymers. These results are probably due to differences in molecular weight of the biopolymers produced. This supposition is confirmed by the recent study of Laws, Chadha,

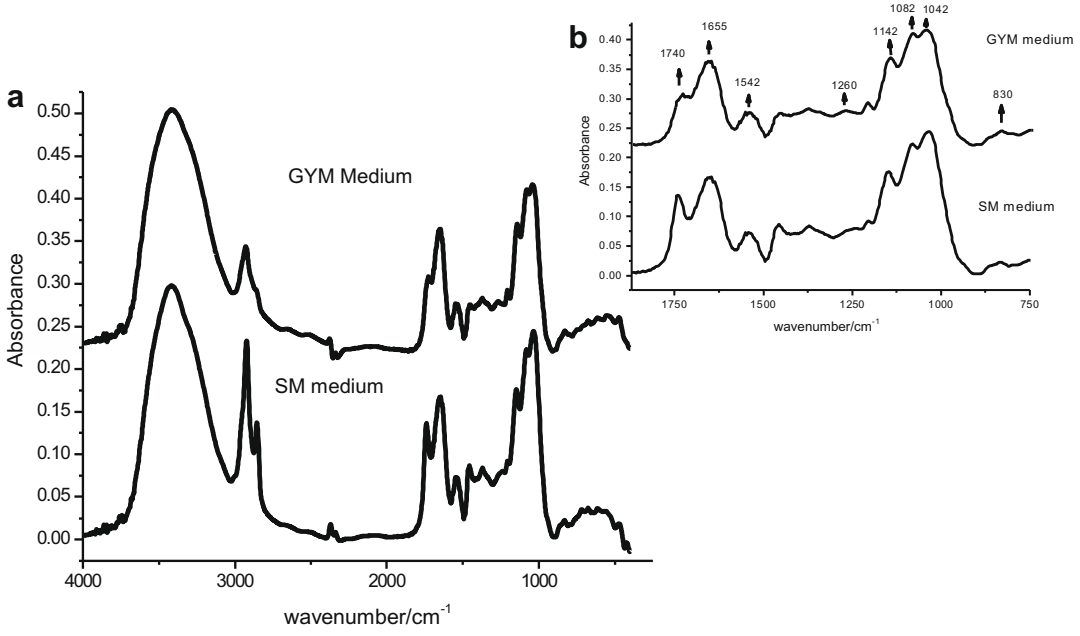

Fig. 1. (a) FT-IR spectra of EPS Gp CCT7137 produced from GYM and SM media. (b) Details of the 2000 and 750 cm^{-1} region.

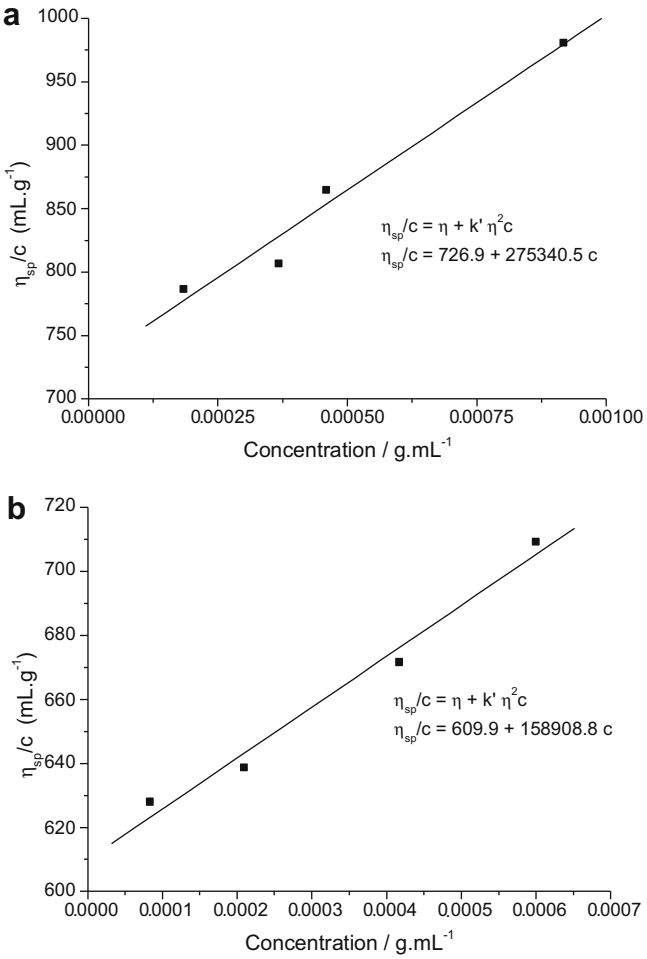

Fig. 2. Intrinsic viscosity of EPS Gp CCT 7137 produced in: (a) GYM and (b) in SM media with 0.1 mol L^{-1} NaCl solution.

Chacon-Romero, and Marshall (2008) showed that EPS produced by *Lactobacillus acidophilus* 5e2 is independent on the carbon feed but its molecular weight distribution is dependent on the substrate.

EPS Gp CCT 7137 produced aqueous solution presenting a high viscosity value even at low concentration. This behavior can be attributed to the presence of acetyl ester (methoxylated material) and carboxylic acid groups. This is an important requirement for the use of EPS in many applications (foods, cosmetics, biomedical, water treatment, oil recovery applications) (Sutherland, 1990, 1998).

3.3. Emulsification assays

EPS GpCCT 7137 excreted in culture supernatant, during cultivation of *G. polyisoprenivorans* CCT 7137 in GYM and SM media, formed emulsions with benzene, toluene and *o*-xylene positioned on top of the aqueous phase and those observed in the control tubes (uninnoculated media) are in an intermediate position (Figs. 3 and 4). According to a classification of emulsion containing Winsor solutions, who identified four general types of phase equilibrium, emulsions formed in culture supernatant are classified as Winsor II, and emulsions formed in control tubes are classified as Winsor III. This observation suggests that the presence of EPS Gp CCT 7137 has influenced the quality of the emulsions. In Winsor II, the surfactant is localized mainly in the oil phase and water-in-oil emulsions, less dense than water, are formed. The surfactant-rich oil phase coexists with the surfactant-poor aqueous phase. In Winsor III, a three-phase system is formed where a surfactant-rich middle-phase coexists with both excess of water and oil surfactant-poor phases (Behjatmanesh-Ardakani & Nikfetrat, 2007).

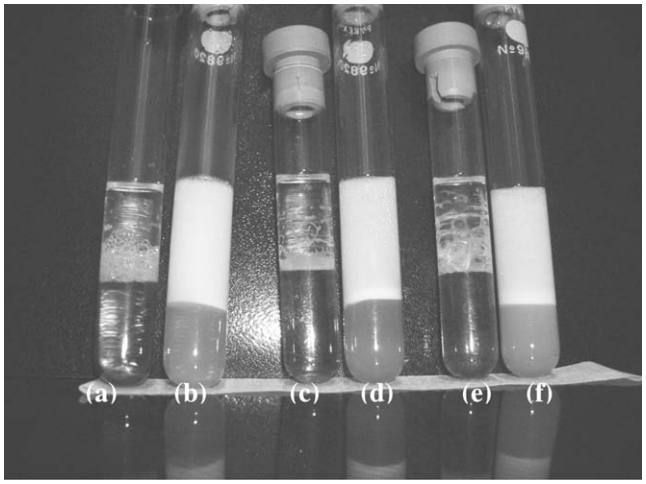

Fig. 3. Emulsification assay of EPS Gp CCT 7137 in SM supernatant free of cells (b, d and f) and control SM medium (a, c and e). Hydrocarbons: benzene (a and b); toluene (c and d) and *o*-xylene (e and f). Photos were taken after 48 h.

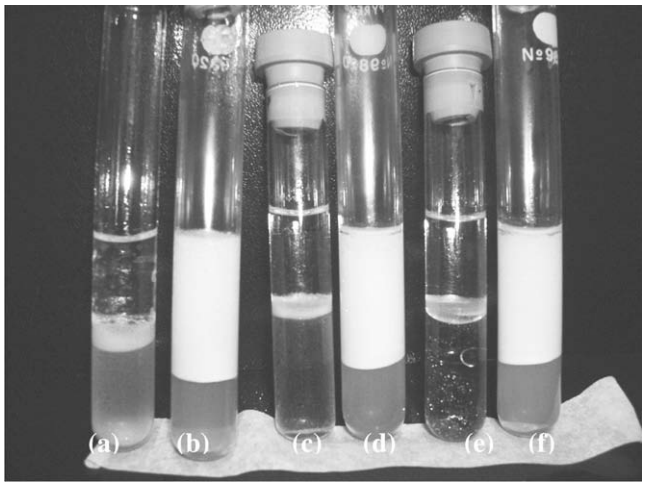

Fig. 4. Emulsification assays of EPS Gp CCT 7137 in GYM supernatant free of cells (b, d and f) and control GYM medium (a, c and e). Hydrocarbons: benzene (a and b); toluene (c and d) and *o*-xylene (e and f). Photos were taken after 48 h.

The emulsification index (E_{24}) of EPS Gp CCT 7137 excreted in culture supernatant on benzene, toluene and *o*-xylene are presented in Fig. 5. The emulsification index in GYM culture supernatant on benzene (61.67%) was higher than those observed for SM medium (34.91%) and Triton X-100 (47.16%). On toluene, the emulsification index in GYM culture supernatant (60.61%) was higher than the one obtained in SM medium (27.88%) and similar to that found in Triton X-100 (51.27%). On *o*-xylene, the emulsification index observed in GYM (62.88%) was higher than the one obtained in SM medium (35.61%) and similar to that obtained in Triton X-100 (59.69%). These results show that EPS Gp CCT 7137 exhibits different emulsifying activities according to the production media and the hydrocarbon investigated. When produced in a GYM commercial medium, EPS Gp CCT 7137 showed superior emulsifying activity to EPS produced in SM alternative medium with all the tested hydrocarbons and superior than Triton X-100 on benzene.

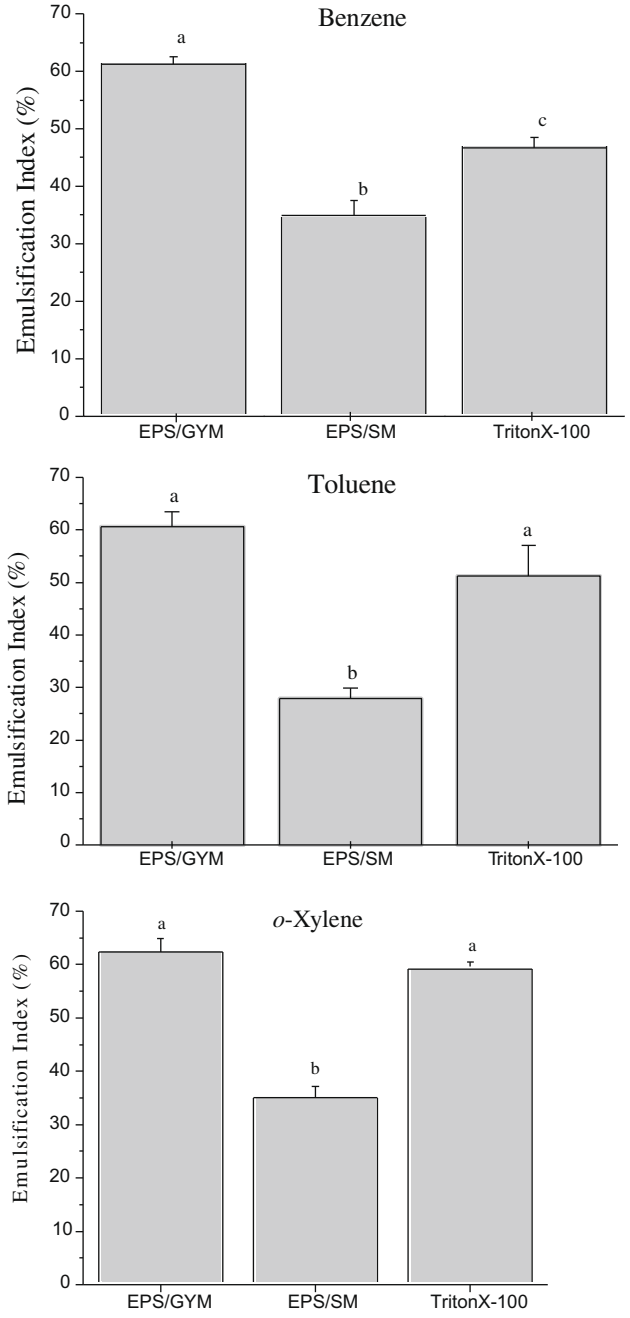

Fig. 5. Emulsification index (%) of EPS Gp CCT 7137, produced in GYM and SM media, and Triton X-100. Hydrocarbons: benzene (a), toluene (b) and *o*-xylene (c). Means followed by distinct letters differ from each other by the Tukey test at a 0.05 level of significance.

Moreover, EPS Gp CCT 7137 produced in GYM medium showed similar emulsifying activity than commercial surfactant Triton X-100 on toluene and *o*-xylene. When produced in a SM alternative medium, EPS Gp CCT 7137 showed the lower emulsifying activity than all the tested surfactants.

These results show that *G. polyisoprenivorans* CCT 7137 produces a surface-active agent referred to as bioemulsifier. The balance of esters and carboxylic groups and the distribution of these groups on polymer structure can be responsible for the emulsification properties. According to Lutz et al. (2009) the modified pectin reduces the surface tension and interfacial tension probably due to the preferred surface orientation of the carboxylic groups at the water/air or water/oil interfaces. Considering these aspects, the analysis of FT-IR indicates that EPS produced in GYM medium has higher degree of carboxylic groups than EPS from SM medium. The presence and probably the organization of these groups seem to be related to better performance of EPS produced in GYM medium in emulsification tests. Bisurfactants are classified according to their chemical composition and microbial origin (Desai & Banat, 1997) and can be divided in low-molecular-weight biosurfactants, generally glycolipids or lipopeptides, and high-molecular-weight exocellular polymers composed of polysaccharides, proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers (Ron & Rosenberg, 2001). EPS Gp CCT 7137 fall in the second category of biosurfactants.

Although EPS Gp CCT 7137 has not been produced with hydrocarbon supplementation, it was able to emulsify benzene, toluene and *o*-xylene. These solvents, together with ethyl benzene, are important monoaromatic petroleum hydrocarbons, indicated as BTEX, a main groundwater and health-risk contaminant group (Farhadian, Vachelard, Duchez, & Larroche, 2008). The presence of BTEX in solid waste disposed in the landfill were *G. polyisoprenivorans* CCT 7137 was originally isolated (Fusconi & Godinho, 2002), seem to suggest the potential of the strain in emulsifying these compounds. In addition, the genus *Gordonia* has ample potential for biotechnological use in the breakdown of environmental contaminants such as substituted or not substituted hydrocarbons (Arenskötter, Bröker, & Steinbüchel, 2004).

Gordonia polyisoprenivorans was originally described as a rubber-degrading bacterium (Linos, Steinbüchel, Spröer, & Kroppenstedt, 1999) and has been the subject of intense research concerning its ability to degrade rubber (Berekaa, Linos, Reichelt, Keller, & Steinbüchel, 2000; Arenskötter et al., 2001; Broker, Ditz, Arenskötter, & Steibüchel, 2008). However, the present study is the first to investigate the properties of EPS produced by a *G. polyisoprenivorans* strain aiming at biotechnological purposes.

4. Conclusions

Gordonia polyisoprenivorans CCT 7137 produced a water-soluble acidic EPS (EPS Gp CCT 7137), both in GYM commercial medium and in SM alternative medium with emulsifying activity for monoaromatic petroleum hydrocarbons. Both FT-IR spectra of EPS Gp CCT 7137 produced in GYM and SM media showed the same patterns indicating that the EPS produced have similar chemical structure. EPS Gp CCT 7137 showed a carbohydrate structure composed mainly of α-anomers with presence of carboxylic and amide functional groups, probably related to its emulsifying activity. The results suggest its potential for oil bioremediation studies. Further investigations such as structure analysis, are in progress.

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