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ORIGINAL PAPER

# Production, Characterization and Bioemulsifying Activity of an Exopolysaccharide Produced by *Sphingomonas* sp. Isolated from Freshwater

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**Abstract** This study aimed to evaluate the emulsion stability of solutions containing exopolysaccharide and culture medium of a *Sphingomonas* sp. strain with various hydrophobic compounds. The exopolysaccharide characterized belongs to a sphingan group, however, not being a gellan gum as produced by certain *Sphingomonas* strains. In general, the emulsifying indexes found in this study were above 70% for gasoline, hexane, kerosene and used frying oil. Nonetheless, the best results were achieved in kerosene solutions, which showed an index of 80% after 24 h, remaining stable for more than 168 h in combinations with various EPS concentrations. Interestingly, diesel oil best results were singly achieved in solution pH of 11, showing an index of around 65%. Furthermore, hexane obtained an index of 100% after 24 h when culture medium was used. Thus, these findings highlight the use of EPS as a potential bioemulsifier agent to enhance hydrocarbon degradation and emulsification effects in environmental biotechnology.

**Keywords** Bioemulsifier · Biopolymer · Diesel · Kerosene

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## Introduction

Bioemulsifier molecules have recently received increasing attention because of their advantages over synthetic counterparts, which include low toxicity, high biodegradability, better environmental compatibility, increased foaming and selectivity, high specific activity at extreme temperatures, pH, and salinity [1].

*Sphingomonas* is bacterial group widely spread in nature and commonly found in soil and aquatic environments. These microorganisms are able to survive and grow at low temperature, low nutrient concentration and toxic environments, being characterized, among other things, by a high capacity to degrade environmentally hazardous compounds [2, 3]. Several studies have reported production of extracellular polymers by different strains of *Sphingomonas* [3, 4]. Among them, the 'sphingans', which are structurally related exopolysaccharides (EPS) secreted by members of the genus *Sphingomonas*; of which, the 'gellan' is a multifunctional gelling agent highly produced by non-pathogenic strains, and currently, is one of the most important emulsifiers commercially produced, as well as xanthan gum that is produced by *Xanthomonas campestris* [5, 6].

Recently, there has been renewed interest in polysaccharides, particularly microbial-produced ones, since they have high relevance in industrial and environmental applications. These water-soluble carbohydrate polymers have a wide range of functional properties. They are able to modify aqueous solution properties by thickening, being also used as chelating, emulsifier, or stabilizer agents [7, 8]. In recent years, many microbial polymers have been used as bioemulsifiers due to their ability to stabilize emulsions between water and hydrophobic compounds [9].

In 1995, Ashtaputre and Shah [10] studied the emulsifying activity of an exopolysaccharide produced by *Sphingomonas paucimobilis*, showing most significant results for polysaccharides in xylene and kerosene. These authors demonstrated EPS stability at different temperatures, pH, salinity and times. So far, several other papers have been reporting the characteristics of EPS produced by *Sphingomonas* strains [4, 11], including gellan gum production [5, 12–14]. However, there is a lack of researches on the emulsifying activity of EPS produced by *Sphingomonas*, as cited above.

In order to understand EPS importance and major applications, mainly those produced by *Sphingomonas* strains, which have been largely used in drug and food industries, our study sought to evaluate the ability of an exopolysaccharide and culture media produced by a wild-type strain of *Sphingomonas* sp. isolated from freshwater to stabilize emulsions with several hydrophobic compounds.

## Materials and Methods

### Microorganism

For this study, we used a wild-type bacterial strain isolated from stream freshwater, which is intensively used in crop irrigation. Aside from the agricultural activity influence, this stream also receives effluents from a zoo farm located in the city of Araçoiaba da Serra—SP, Brazil.

The isolate was previously identified by sequence analysis of the 16S rDNA gene and, a further BlastN analyses classified as *Sphingomonas* sp. those bacteria primarily designed as C7 (KT372350) (Fig. 1).

For routine isolate growth and evaluations of emulsifying activities, the bacterial isolate was streaked in a PGYA medium containing glycerol (10 g $L^{-1}$) as carbon source and incubated for 24 h at 30 °C. After 24 h, the inoculating strain was cultivated in a 250-mL flask (100 mL of medium) containing PGYL liquid medium on rotary shaker at 150 rpm for 24 h. At this time, a suspension with an optical density at 600 nm ($OD_{600}$) of 2.5 was obtained. The temperature was maintained at 30 °C. Aliquots of the corresponding culture were transferred to 1000-mL Erlenmeyer flasks containing 500 mL of modified half-liquid PSYL medium (registration PI0304053-4) containing glucose (10 g $L^{-1}$) as carbon source and incubated for 120 h at 150 rpm and 30 °C.

### EPS Production

For EPS production evaluation, 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 incubation at 30 °C, inoculated strain was cultivated in 125-mL flasks (20 mL of medium in each) with PGYL liquid medium and left on a rotary shaker (innova 4335, New Brunswick Scientific) at 140 rpm for 30 h. Then, we obtained a suspension with an optical density at 600 nm ($OD_{600}$) of 0.3 at a temperature remained at 30 °C. Subsequently, aliquots of the corresponding cultures were transferred into 1000-mL Erlenmeyer flasks containing 500-mL of half-liquid PSYL medium at a final concentration of 0.10% (v/v) and incubated for 48 h at 150 rpm and 30 °C.

### EPS Extraction

For EPS extraction, cold 96% ethanol was added to the supernatant obtained from centrifugation at a 1:3 (v/v) ethanol: supernatant ratio to precipitate the EPS [15]. At this stage, it was promptly possible to observe precipitate formation. This precipitate was washed several times with ethanol, which was subsequently evaporated. The solvent precipitation also achieved a partial polymer purification by eliminating soluble components from culture media [16].

EPS production was measured in a precision scale after drying the precipitated product in a Hetovac VR-1 lyophilizer until constant weight (grams of EPS per liter of culture medium). The results were presented as mean ± standard error.

### EPS Monosaccharide Composition by RP-HPLC

For EPS monosaccharide composition analysis, raw EPS preparation was identified by RP-HPLC using the 1-phenyl-3-methyl-5-pyrazolone monomer chemical identification methodology with modifications accordingly to Castellane et al. [17].

### Fourier Transform Infrared Spectroscopy (FTIR)

EPS characterization was finished by acquisition of FTIR absorption spectra. Pellets for infrared analysis were obtained by grinding 1 mg of EPS. The FTIR spectra were obtained with a Paragon 1000, Perkin–Elmer spectrometer (4000–400, 4 $cm^{-1}$ of spectral resolution, and 64 scans). The spectra were baseline and offset corrected from 3000 to 2800 $cm^{-1}$ and from 1800 to 900 $cm^{-1}$, and normalized by the intensity of the amide II peak (intensity = 100) at 1550 $cm^{-1}$ [18].

### Surface Tension Measurement

Surface tension measurements were performed by the du Nöuyring method using a Krüs Tensiometer K12 (Krüs,

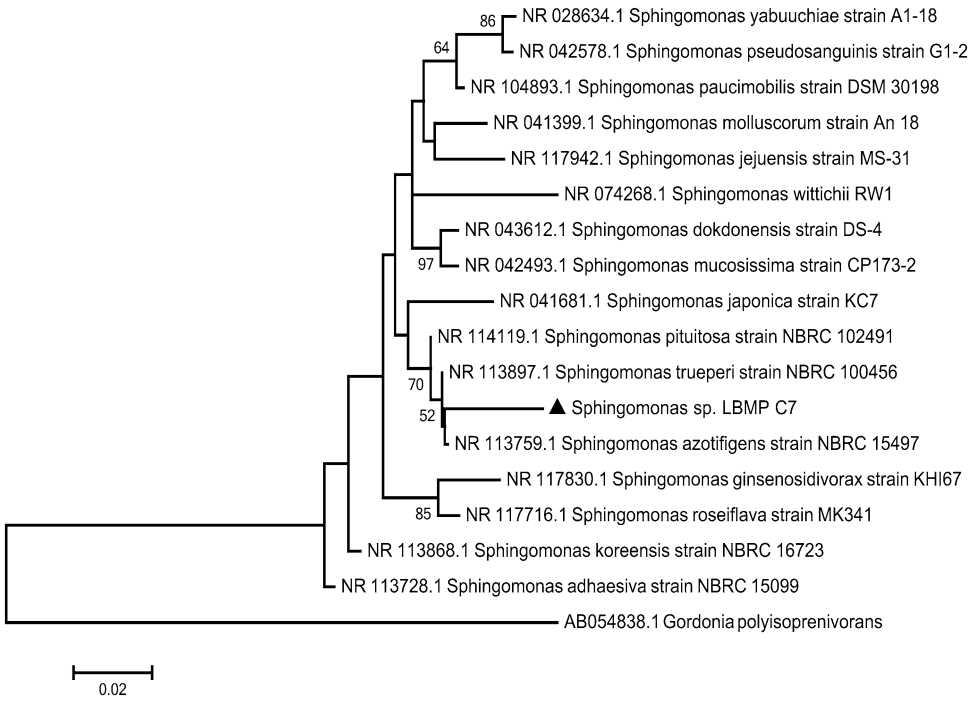


**Fig. 1** Classification of 16S rRNA gene sequences of the *Sphingomonas* sp. LBMP C7. The analyses were conducted using the Maximum Likelihood method, with the K2-parameter nucleotide substitution matrix and a bootstrap of 1000 replicates. The figure displays only bootstrap values of at least ≥50%. *Scale* 0.02 nucleotide substitutions per position

Helsinki, Finland) on EPS solubilized in ultra-pure water at concentrations ranging from 0 to 5.0 mg $mL^{-1}$. All determinations were performed in three replicates.

### Emulsifying Activity

EPS capacity to stabilize emulsions using various hydrophobic compounds was tested as described by Freitas et al. [9]. Emulsion formation and stabilization were assessed for four different EPS concentrations. Certain amounts of EPS aqueous solutions (0.5, 1, 2 and 3 mg $mL^{-1}$) were mixed with each hydrophobic compound (3:2, v/v ratio) and stirred in the vortex. After 24 and 168 h, we measured emulsification index using the following equation for both times: $E_x = (he/hT) \times 100$; wherein, $E_x$ is the emulsification index after stirring for 24 ($E_{24}$) and 168 ($E_{168}$) hours, *he* is the emulsion height (in mm), and *hT* is the mixture height (in mm). Tested compounds consisted of hydrocarbons as diesel oil, hexane, gasoline, kerosene, which were purchased at local market; and used frying oil, which was provided by a local restaurant. We also assessed emulsion formation and stabilization of culture media mixed with each hydrophobic compound (3:2, v/v ratio) and stirred in the vortex as described above.

### Thermal and pH Stability

Emulsions were heated in a water bath at 80 °C for 1 h to study the heat effects on EPS emulsifying activities. The samples were allowed to stand at room temperature for 1 h after treatment and, before index measurements, we compared these results to the corresponding values taken before heating.

Next, diesel oil was selected for pH analysis. Therefore, we prepared four EPS solutions of 1 mg $mL^{-1}$ at different pH values (3, 5, 9 and 11). All emulsions were mixed and measured as previously described and then compared with the corresponding values obtained prior to analysis.

### Data Analysis

All determinations reported here were performed in triplicate, and results were presented as mean values. Data underwent analysis of variance (ANOVA), and means were compared by the Tukey's test ($p < 0.05$). For the analyses, we used the R software (https://cran.r-project.org/).

## Results

EPS production by *Sphingomonas* sp. strain C7 was quantified by culture ethyl alcohol precipitation being expressed in lyophilized weight per liter. Interestingly, we obtained 3.55 g $L^{-1}$ of EPS in a final pH growth of 6.5, after 48 h. Then, the EPS was diluted (m/v) in ultra-pure water at different concentrations, in which we reached a pH around neutrality (pH 7.3).

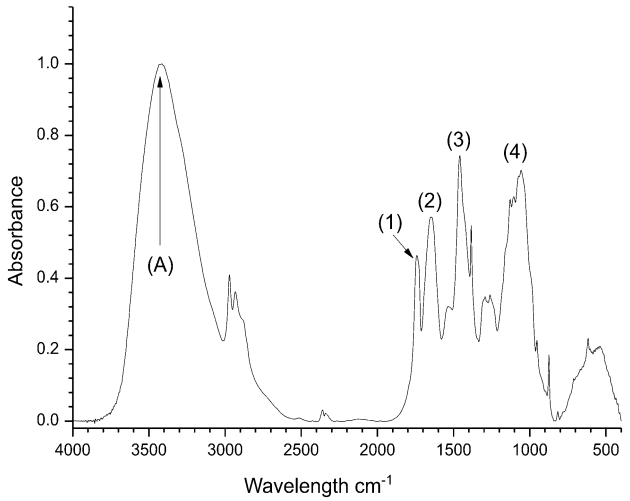


**Fig. 2** Gram-negative bacterium *Sphingomonas* sp. representative FT-IR absorbance spectrum. The figure displays the extracted exopolysaccharide intensities, the numbers highlighted represent the biomolecule biochemical bounds: (*A*) 3425 $cm^{-1}$ O–H bound, (*1*) 1730 $cm^{-1}$ C=O bound like acetate ester, (*2*) 1640 $cm^{-1}$ O–H bounds like carbohydrates, (*3*) 1455 $cm^{-1}$ COO– bounds (carboxylate) from pyruvate or glucuronic acids, (*4*) 1085 $cm^{-1}$ C–O bounds common for carbohydrates

From FT-IR spectroscopy, we could clearly demonstrate EPS high intensity of acetate and carboxylate groups (Fig. 2), at peaks of 1730 and 1455 $cm^{-1}$, respectively. In addition, HPLC outcomes revealed EPS is a heteropolysaccharide composed of mannose, rhamnose, glucuronic acid, glucose, galactose and xylose (Fig. 3). Among them, glucose was predominant representing 51%. However, there was no significant reduction in the surface tension of the EPS (data not shown). The surface tension of water (75 mN $m^{-1}$) was reduced around 10%, whereas no further decrease was observed.

It is noteworthy mention that the emulsifying activities shown in Table 1 became consistent emulsions after 24 h for some of the hydrophobic substrates, such as gasoline, hexane, kerosene and used frying oil, either at an EPS concentration of 0.5 or 1 mg $mL^{-1}$.

We considered as best results those of emulsifying indexes $E_{24} \geq 70\%$. For gasoline, the best result of emulsion stability was reached after 168 h using 3 mg $mL^{-1}$ of EPS, having an index $E_{24} > 80\%$. Yet for hexane, it was reached an $E_{24} \geq 75\%$, using an EPS concentration of 2 mg $mL^{-1}$; however, after 168 h, this solution was no longer stable. Conversely, the same hydrocarbon obtained an $E_{24} > 90\%$ after 24 h using 3 mg $mL^{-1}$ of EPS; aside from that, after 168 h, this index raised to 100% ($E_{168} = 100\%$). For used frying oil, the best emulsions were reached for concentrations of 1 and 2 mg $mL^{-1}$ of EPS ($E_{24} > 80\%$), nevertheless being unstable. Finally, for kerosene, the best result ($E_{24} > 80\%$) was achieved using EPS concentrations of 1 and 3 mg $mL^{-1}$, though the first emulsion was improved after 168 h, while the second remained stable. Yet for 2 mg $mL^{-1}$ of the same solution, emulsion remained stable, with an $E_{24} > 90\%$ (Table 1).

Furthermore, heat treatments caused significant effects on bioemulsifier performances as seen in Fig. 4. According to our results, most of the emulsions were not stable; however, kerosene emulsions at 2 mg $mL^{-1}$ of EPS obtained the best results due to great stability with and without heating after both 24 and 168 h.

In addition, result comparisons, as a rule, showed no combinations of EPS concentrations and different pH values were significant for diesel oil, even with an index above 50% by concentration of 2 mg $mL^{-1}$. Once diesel has shown the shortest emulsifying index and as it is a major soil and water pollutant, we selected it to have its emulsifying activity improved. Thus, we altered pH values using an EPS concentration of 1 mg $mL^{-1}$ (Table 2). Hence, the smaller the EPS concentration is to emulsify an oil, the more economic and feasible it would be considered. By these modifications, we found the best

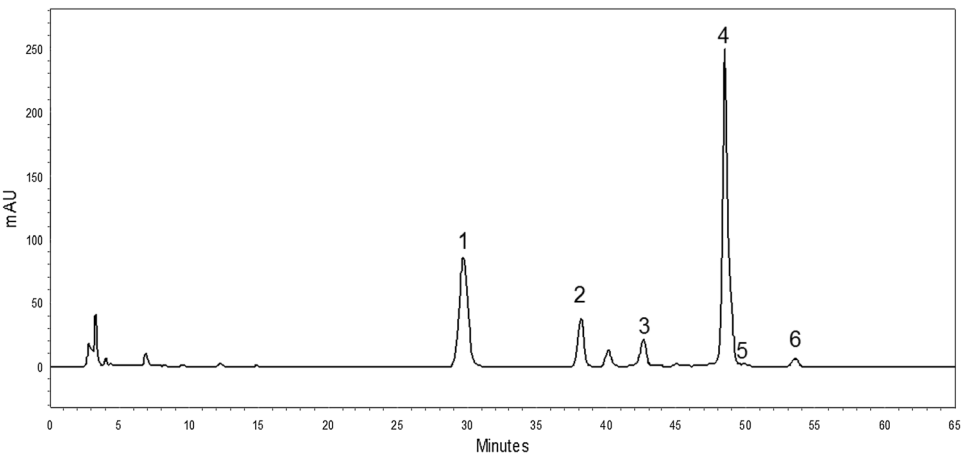


**Fig. 3** EPS sample monosaccharide analysis using the PMP derivative HPLC–UV method of the EPS acid hydrolysate. The chromatographs of the EPS from show peaks for (*1*) mannose, (*2*) rhamnose, (*3*) glucuronic acid, (*4*) glucose, (*5*) galactose and (*6*) xylose

**Table 1** Emulsifying activity after 24 h ($E_{24}$) and 168 h ($E_{168}$) using hydrocarbon (diesel, gasoline, hexane and kerosene) and residual oils (used frying oil) at different EPS concentrations. Data are presented as means and standard deviation

| Oil | EPS concentration (mg $mL^{-1}$) | $E_{24}$ (%) | $E_{168}$ (%) |
|---|---|---|---|
| Diesel | 0.5 | 17.7 ± 4 | 10.9 ± 3 |
| Diesel | 1 | 28.4 ± 13.9 | 18.9 ± 8.1 |
| Diesel | 2 | 61.6 ± 3.2 | 56.2 ± 0.9 |
| Diesel | 3 | 53.8 ± 1.8 | 52.7 ± 1.3 |
| Gasoline | 0.5 | 59 ± 5 | 51.4 ± 0.1 |
| Gasoline | 1 | 55.3 ± 1.3 | 53.3 ± 0.7 |
| Gasoline | 2 | 63.1 ± 0.8 | 57.9 ± 5.3 |
| Gasoline | 3 | **88.3 ± 5.3** | **84.3 ± 4.3** |
| Hexane | 0.5 | 26.3 ± 9.5 | 33.9 ± 14.5 |
| Hexane | 1 | 51.5 ± 6 | 61.8 ± 7 |
| Hexane | 2 | **75.5 ± 5.8** | 66.2 ± 13.1 |
| Hexane | 3 | **92.3 ± 6.9** | **100 ± 0** |
| Kerosene | 0.5 | 47.4 ± 7.9 | 44 ± 3.4 |
| Kerosene | 1 | **81.6 ± 1.3** | **92.9 ± 1.4** |
| Kerosene | 2 | **94.1 ± 1.8** | **93 ± 1.1** |
| Kerosene | 3 | **85.6 ± 5.8** | **76.1 ± 3.3** |
| Used frying oil | 0.5 | 55.7 ± 0.8 | 51.5 ± 1.5 |
| Used frying oil | 1 | **84.6 ± 13.1** | 65 ± 10.7 |
| Used frying oil | 2 | **89.5 ± 7.6** | 67.6 ± 21.6 |
| Used frying oil | 3 | 68.6 ± 10.6 | 51.5 ± 0 |

Values in bold: Emulsifying Index with values above 70%

Data are presented as means and standard deviation

emulsion results at a pH 11 for diesel oil, reaching an index of $E_{24} > 65\%$; however, such stability was not remained after 168 h.

The results on the use of culture media can be seen in Table 3. They show a great potential of emulsifying activity for all oils; however, the greatest potential was observed for hexane, once it achieved a complete emulsion and stability.

## Discussion

Several microorganisms are characterized by producing a wide range of bioemulsifiers of high or low molecular weight, such as EPS [19]. These substances have major

**Table 2** Emulsifying activity after 24 h (E24), 24 h at 80 °C (E24T) and 168 h (E168) using diesel and an EPS concentration of 1 mg $mL^{-1}$ at various pH values

| pH | $E_{24}$ (%) | $E_{24T}$ (%) | $E_{168}$ (%) |
|---|---|---|---|
| 3 | $25.99 \pm 4.46^{b}$ | $19.73 \pm 3.14^{b}$ | $25.29 \pm 1.27^{b}$ |
| 5 | $16.74 \pm 7.27^{bc}$ | $10.86 \pm 1.89^{c}$ | $18.93 \pm 0.17^{bc}$ |
| 9 | $13.32 \pm 4.17^{c}$ | $13.42 \pm 1.71^{bc}$ | $6.54 \pm 5.79^{c}$ |
| 11 | $67.06 \pm 1.04^{a}$ | $60.37 \pm 0.86^{a}$ | $61.94 \pm 2.08^{a}$ |

Means with the same letter are not significantly different within columns

Data are presented as means and standard deviation

**Table 3** Emulsifying activity using hydrocarbon and residual oils with bacterial culture media and emulsion stabilization after 24 ($E_{24}$) and after 168 h ($E_{168}$)

| Oil | $E_{24}$ (%) | $E_{168}$ (%) |
|---|---|---|
| Diesel | 54.2 ± 3.1 | 55.1 ± 2.7 |
| Gasoline | 71.2 ± 2.7 | 75.9 ± 3 |
| Hexane | 100 ± 0 | 100 ± 0 |
| Used frying oil | 87.5 ± 17.7 | 85.9 ± 20 |
| Kerosene | 75.2 ± 7.7 | 78.1 ± 5.7 |

Data are presented as means and standard deviation

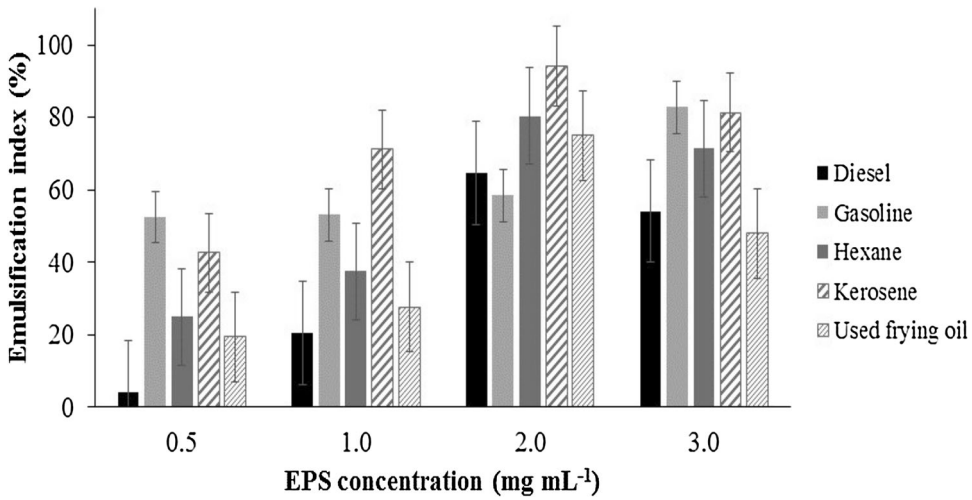


**Fig. 4** Effects of heat (80 °C) on stabilities of the hydrocarbon and residual oils esmulsification capacities of the EPS. Different EPS concentrations were used to emulsify diesel oil, gasoline, hexane, kerosene and used frying oil

physico-chemical properties like tolerance to extreme conditions of pH, temperature and salinity, besides of low toxicity and high biodegradability, being thus suitable for various environmental applications as enhancer of hydrocarbon biodegradation and bioemulsification, for example [1, 19].

We selected hydrocarbon and residual oils because of their impact on the environment worldwide. Furthermore, our research group has studied emulsification activity of some of these oils using EPS from rhizobia strains.

Interestingly, from the HPLC analysis, we found glucuronic acid, which is one of the components of sphingans [4, 20]. This fact may also explain the strong carboxylate peak in the EPS through the FT-IR analysis. In addition, we detected mannose, which is an usual component commonly found in other sphingans like S-88 and S-198 [7, 21, 22]. By the above mentioned characterization, we may infer that the studied EPS show a composition similar to those belonged to sphingan family, being produced by several *Sphingomonas* strains [4, 7]. However, there are variations in the repeating structure of sphingans, which produce distinct differences in rheology [7]. Accordingly to Fialho et al. [5], sphingans share the same linear tetrasaccharide backbone structure (X-glucose-glucuronic acid–glucose-X, where X is better L-rhamnose or L-mannose) to which distinct side groups are attached.

The unexpected failure of EPS to reduce the surface tension of water can indicate that the main stabilizing effect of EPS was not due to its surface-active properties, as highlighted by [10] that obtained similar results from a viscous EPS produced by *S. paucimobilis*.

It is clear that the EPS tested in this study produced stable emulsions at different concentrations of EPS; however, this statement relies on the oil type. Surprisingly, kerosene emulsification achieved good results. As mentioned in the prior section, the EPS concentration of 2 mg $mL^{-1}$ as hydrocarbon bioemulsifier showed great stability, being similar to the findings of Ashtaputre and Shah [10], who used EPS from *S. paucimobilis*. Nevertheless, the EPS from *Salipiger mucosus* A3, used as bioemulsifier for the same hydrocarbon by Llamas et al. [23], showed a lower emulsifying index than the one we found; even though the authors obtained an $E_{24} = 70\%$; it was not stable after 24 h.

Good results could be found for hexane, for two tested concentrations, similar to results found by Freitas et al. [9]. Differently, comparing our results with the bioemulsifiers studied by Moretto et al. [8] at 1 g $L^{-1}$ of EPS, we can note that their emulsifying indexes ($E_{24}$) ranged around 20%.

Even though we reached emulsifying indexes above 50% with 2 mg EPS per mL of diesel oil, other authors such as [8, 24, 25] had difficulties to emulsify the same hydrocarbon. On the other hand, Wu et al. [26] observed that sphingan Ss, produced by *Sphingomonas sanxanigenens* NX02, promoted formation of 100% emulsified layers stably with diesel when its concentration exceeded 1000 mg $L^{-1}$ (0.1%, w/v) at room temperature. Aiming to achieve optimal results, changes in pH, made in our study, were valuable, observing the best result for diesel oil at a pH of 11, which was similar to results found by Moretto et al. [8].

The emulsion stability under different pH is dependent on the chemical composition, structure and the compound to be emulsified [9]. For example, the exopolysaccharide produced by *S. paucimobilis* has shown to be able to emulsify kerosene, being the emulsions stable for the pH range 2–10 [10]. On the other hand, the exopolysaccharide produced by *Bacillus megaterium* formed emulsions whose stability increased with pH range from 4 to 8 [25].

As mentioned in the results section, we also tested culture media to analyze its influence on biomass cell. An unanticipated finding was regarding hexane, we can hypothesize that the used *Sphingomonas* sp. strain is able to degrade this chemical; however, specific studies must be carried out to confirm such statement. Sun et al. [3] reported a high chlorine-resistant capacity of a *Sphingomonas* strain isolated from a model drinking water distribution system, which can confirm the great environmental application of this bacterial group. The previously cited paper and our study are two of several studies found in literature on *Sphingomonas* biotechnological applications and evidence its importance on this subject.

As we saw here, culture media had a great potential for hexane emulsion; nevertheless, we want to highlight the great potential of EPS as hydrocarbon bioemulsifier, mainly for kerosene given its emulsifying index and stability. According to these results, we can suggest the studied EPS in biological applications as a safe alternative to chemical emulsifiers.

## Conclusion

A few EPSs from *Sphingomonas* strains have been studied as emulsifiers for hydrocarbon and residual oils. The one studied here can be used as emulsifier for solutions with diesel, gasoline, hexane, kerosene and used frying oil, reaching different degrees of stability. Among our findings, kerosene emulsion had remarkable results, since it showed a good emulsifying index and emulsion stability for a long-term period, as well as thermal stability. Furthermore, our findings promoted significant contribution by showing EPS potential as a bioemulsifier agent that would enhance hydrocarbon degradation and emulsification effects in environmental biotechnology.

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