

Lipopeptide biosurfactant produced by *Mucor Circinelloides* UCP/WFCC 0001 applied in the removal of crude oil and engine oil from soil

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ABSTRACT. Biosurfactants are molecules synthesized by a wide variety of microorganisms. They are characterized by the ability to reduce surface tension and act in the dispersion, solubilization, mobilization, removal and viscosity of petroleum and its derivatives. The aim of this study was to produce biosurfactant using the filamentous fungus *M. circinelloides* in cultivation with previously established conditions to evaluate its properties, characterize the chemical structure and investigate the application of the biomolecule in the hydrocarbon recovery. Biosurfactant yield was 6.0 g L⁻¹. The surface tension was reduced to 26 mN m⁻¹ with a critical micelle concentration (CMC) of 1.5%. The biosurfactant exhibited stability to surface tension under adverse environmental conditions. *M. circinelloides* surfactant is similar to chemical surfactants for presenting Oil displacement area of 50 cm² in water-in-oil dispersion, as well as reduce the oil viscosity from 843.6 to 14.7 cP. Physical-chemical analyzes showed that the biosurfactant produced is a lipopeptide. The cationic character was identified by performing the zeta potential. The biosurfactant demonstrated no toxicity and recovered 95.9% of motor oil adsorbed on a clay soil sample, presenting considerable potential for use in bioremediation processes, especially in the petroleum industry.

Keywords: biotensioactive; surface tension; hydrocarbon; bioremediation.

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Introduction

Biosurfactants are amphiphilic compounds that can reduce surface and interfacial tensions, accumulating at the interface of immiscible fluids and increasing solubility and mobility of hydrophobic or insoluble organic compounds (Singha, Van Hammeb, & Ward, 2007; Bezza & Chirwa, 2017). The molecular structure of biosurfactants consists of hydrophobic and hydrophilic moieties, wherein the former may be a long chain fatty acid, hydroxy fatty acid or α -alkyl- β -hydroxy fatty acid. Carbohydrates, phosphates, amino acids, peptides or alcohols, may form the hydrophilic part. Lipopeptides are a class of biosurfactants structurally constituted by fatty acids in combination with a peptide (Inès & Dhouha, 2015).

The main characteristics of lipopeptide biosurfactants are low critical micellar concentration, emulsification/demulsification capacity, dispersants, viscosity reducers and solubilizing agents, which allow their use in several industrial sectors (Inès & Dhouha, 2015). Furthermore, they are easily degraded by microorganisms in soil and water, besides having a low degree of environmental toxicity and high biocompatibility (Shah, Nikam, Gaikwad, Sapre, & Kaur, 2016). Advanced Microbial Oil Recovery (Meor) is based on the metabolic versatility of microorganisms that can synthesize surfactant molecules that will act on oil displacement.

Due to the presence of colloids and asphaltenes, the viscosity of petroleum and its derivatives is usually high. Biosurfactants are able to reduce the viscosity, increasing the mobility of the oil and its miscibility (Bezza & Chirwa, 2017). In addition, lipopeptide biosurfactants are used to clean oil storage tanks increasing their flow through ducts and are also capable of stabilizing water-oil emulsions (Almansoori, Hasan, Idris, Abdullah, & Anuar, 2015). This study was conducted in order to explore the potential of *M. circinelloides* in biosurfactant production due to the few studies in the literature using filamentous fungi, and characterize its chemical structure and investigate the applicability in mobilizing oil derivatives.

Material and methods

Microorganism and culture conditions

The pre-inoculum of *Mucor circinelloides* UCP/WFCC 0001 was performed on Kasvi-K25-610102 potato-dextrose-agar solid medium (PDA), grown for 4 days at a temperature of 28°C. Then, the inoculum was run with 1% spurious suspension containing 10^7 sporangiospores mL⁻¹. This suspension was then used as an inoculum in the medium containing corn steep liquor and frying oil for production of the biosurfactant.

Biosurfactant production

The biosurfactant was prepared in a pre-optimized medium consisting of corn steep liquor (8.82%) and frying oil (2%), at pH 5 for 96 hours, under orbital shaking at 150 rpm and 28°C.

Isolation and semi-purification of biosurfactant

The biosurfactant was extracted by the precipitation method from the cell-free metabolic fluid using ethanol (1:2 v v⁻¹), as proposed by Bueno, Silva, and Garcia-Cruz (2010). The precipitate was kept standing for 24 hours at 4°C, and then centrifuged at 5000 rpm for 15 min. Then, the precipitate was freeze-dried and the yield calculated gravimetrically. The crude biosurfactant sample was semi-purified by dialysis using a 2.5 cm-diameter membrane with pore size of 10 kDa with distilled water. The procedure was performed for 72 hours changing the water 3 times a day.

Determination of the Critical Micellar Concentration (CMC)

The critical micellar concentration of the semi-purified biosurfactant was performed at different concentrations (0.001, 0.01, 0.03, 0.05, 0.1, 1, 1.5, 2 and 2.5%) and surface tensions were measured on an automatic tensiometer (Model Sigma 70-KSV Ltd., Finland) using a Noüy ring by immersion in the liquid (Sheppard & Mulligan, 1987).

Stability of surface tension under different ambient conditions

The stability of the biosurfactant surface tension was evaluated under different adverse conditions, consisting of different pH levels (2, 4, 6, 8, 10 and 12), NaCl concentrations (0, 5, 10, 15 and 20%) and a heat treatment at 100°C for 20, 40, 100, 120 and 140 min. The measurements were made through surface tension (Sobrinho et al., 2008).

Biochemical composition of biosurfactant

The biochemical composition of the biosurfactant was investigated. To determine the total proteins, the Diagnostic S.A. Brazil kit was used. Total sugars were determined according to the methodology described by Dubois, Gilles, Hamilton, Rebers, and Smith (1956). The amount of lipids from the biosurfactant was determined using the method proposed by Manocha, San-Blas, and Centeno (1980).

Thin Layer Chromatography (TLC)

After extraction and semi-purification, the biosurfactant was resuspended in chloroform/methanol (1:1 v v⁻¹). A sample (100 µl) was placed on a silica gel plate (20x20) in a solvent system containing chloroform/methanol/acetic acid (65:15:2 v v⁻¹ v⁻¹), as proposed by George and Jayachandran (2013). Amino acids, lipids and glycolipids were detected after spraying the reagents ninhydrin (0.05%), rhodamine (0.25%) and anthrone (Yin et al., 2008) on the plates, respectively. Under UV light, the visualization of the bands corresponding to the biosurfactant components was detected and the retention factor (R_f) values were calculated.

Fourier Transform Infrared Spectroscopy (FTIR)

The functional groups of the biosurfactant were identified by the infrared spectroscopy technique. Infrared spectra were recorded on a Mattson 1000 FT-England FTIR system within the range of 500-4000 cm⁻¹ wave numbers.

Ionic charge of biosurfactant

The identification of the ionic charge of the biosurfactant molecule was initially investigated by the technique of diffusion in agar with low viscosity (1% solution) (Meylheuc, Van Oss, & Bellon-Fontaine,

2001) and confirmation was made by the zeta potential with the equipment Zeta-Meter system 3.0 + ZM3-DG direct imaging, Zeta Meter, Inc., USA.

Gas Chromatography coupled to the Mass Spectrum (GC-MS)

The analysis of the hydrophobic composition of the biosurfactant was performed using the GC-MS instrument (ITQ 700 Thermo Scientific) operated under the same conditions of gas flow, column type and slope used in gas chromatography (GC) analysis. Helium (purity 99.999%) was used as the carrier gas of the vaporized species.

Application of the biosurfactant in reducing the viscosity of petroleum derivatives

To investigate the effects of the biosurfactant on reducing the viscosity of petroleum by-products, a test with burned motor oil (6 mL) was conducted. Viscosity was measured using a standard viscometer (Brookfield-500TC) at 25°C. Then, 2 mL biosurfactant solution (1.5%) was added to the engine oil. Water, liquid detergent (Ype) and synthetic surfactant (Triton X 80) were used as controls. Samples were vortexed for 1 min and then their viscosities were measured again. The results were expressed in cP and % (Jara, Andrade, & Campos-Takaki, 2013).

Application of the biosurfactant in the dispersion of petroleum derivatives in water

In order to evaluate the potential of the biosurfactant in the dispersion of hydrophobic compounds in water, 20 µL motor oil was slowly added to Petri dishes containing 20 mL distilled water. This was followed by the addition of 1 mL crude biosurfactant. Commercial detergent and synthetic surfactant Triton X 80 were used as controls. Petri dishes were stored at room temperature (28°C) for 24 hours (Ali & Din, 2013). After this period, the diameter of the dispersed areas was measured in cm. The free zone diameter was calculated as an oil displacement area (ODA) according to the following Equation 1 (Urum & Pekdemir, 2004):

$$ODA = \pi (\text{radius})^2 \text{ cm}^2 \quad (1)$$

Application of biosurfactant in the removal of petroleum derivatives from soils

The capacity of removing hydrophobic compounds from the soil was performed using a solution containing 1.5% biosurfactant. Removal tests were carried out in 500 mL Erlenmeyer flasks, each containing 10 g clay and sandy soil impregnated with motor oil and petroleum. Then, 20 mL solution containing the biosurfactant was added. The flasks were subjected to orbital shaking at 200 rpm, 30°C, for 24 hours. The residual motor oil was extracted with hexane. The percentage removal of the hydrophobic compound was determined according to the following Equation 2 (Urum & Pekdemir, 2004):

$$\text{Removal \%} = \frac{(W_i - W_f)}{W_i} \times 100 \quad (2)$$

where:

W_i: initial weight of the Petri dish with the removal of oil by solvent;

W_f: final weight of the Petri dish with the oil resulting from the biosurfactant washes;

To calculate (W_i), the sand that had oil was not washed with the biosurfactant, being removed with Hexane.

Results and discussion

Biosurfactant yield

The results of this study showed a yield of 6.0 g L⁻¹ after 96 hours of *M. circinelloides* biosurfactant culture in a medium containing corn steep liquor and soybean oil waste. Similar studies on the culture of microorganisms were carried out by Sobrinho et al. (2008) with *Candida sphaerica* and by Silva et al. (2014) with the filamentous fungus *Cunninghamella echinulata* using corn steep liquor and residual frying oil for the production of biosurfactant. They obtained a final yield of only 4.5 and 4.0 g L⁻¹, respectively. Considering the studies above which used the same culture medium with agroindustrial residues, the *M. circinelloides* biosurfactant presented superior yield in the biomolecule extraction.

Surface tension and Critical Micelle Concentration (CMC)

Surface tension is an important property of surfactants. Water molecules are held together by cohesive forces; these intermolecular forces form the surface tension (Inès & Dhousha, 2015). Biosurfactants can be

characterized by their CMC above which their monomers come together and form non-covalent aggregates (called micelles). The hydrophobic part of the molecule forms a core that surrounds a drop of oil and a hydrophilic (ionic) part maintains contact with the external aqueous medium (Inès & Dhouha, 2015). The biosurfactant exhibited an excellent ability to reduce water surface tension (72 mN m^{-1}) to 26 mN m^{-1} , reaching a CMC of 1.5% (Figure 1). These results indicate that the increase in concentration higher than 1.5% of the biosurfactant solution synthesized by filamentous fungus *M. circinelloides* is not able to decrease even more the surface tension of the medium. The biotensioactive of *M. circinelloides* demonstrated a lower CMC than that produced by yeast *Candida glabrata* (Luna, Sarubbo, & Campos-Takaki, 2009).

Stability of the surface tension of the biosurfactant under different environmental conditions

The use of biosurfactants for remediation of sites contaminated with oil and by-products requires them to be stable within a large range of temperature, around $50\text{--}80^\circ\text{C}$, wide pH range and high salt concentrations, in order to ensure an efficient application in the recovery of several impacted environments (Al-Wahaibi et al., 2014). Preliminarily, the resistance of the biosurfactant to heat treatment at 100°C was investigated. Figure 2 shows the surface tension of the *M. circinelloides* biosurfactant varying according to the temperature of 100°C at different time intervals (20, 40, 60, 100, 120 and 140 min). The results of the surface tension stability of the biosurfactant against the heat treatment were very significant, evidencing that the biotensioactive remained effective until 120 min of exposure, but in the period of heat treatment up to 140 min, the surface tension of the biosurfactant remained between 30 and 40 mN m^{-1} , being still a significant result. In contrast, biosurfactants synthesized by *Enterococcus faecium* (Sharma, Saharan, Chauhan, Procha, & Lal, 2015) and *Nocardiosis sp.* (Khopade et al., 2012) exhibited low stability, being effective at 100°C for only 45 and 75 min, respectively.

The analysis of the stability of the biosurfactant obtained at different NaCl concentrations (5, 10, 15 and 20%) indicated a stable behavior in all saline concentrations tested (Figure 3). However, with the increase in salt concentration in the medium to 20%, the value of the surface tension suffered a small variation to 29 mN m^{-1} . These results were quite significant when compared to studies performed by Al-Wahaibi et al. (2014) using the biosurfactant produced by the bacterium *Bacillus subtilis*, with a surface tension of 30.07 mN m^{-1} and stable at 12% saline concentration.

Studies on *M. circinelloides* biosurfactant under different pH ranges as a function of the reduction in the surface tension of the medium (Figure 4) revealed that the best surface tension value (26 mN m^{-1}) was obtained in the presence of an acidic medium with pH between 4 and 6. However, in more acidic concentrations (pH 2) and in alkaline conditions, the surface tension of the biosurfactant was still with significant values of up to 40 mN m^{-1} . These results indicate that the decrease in pH leads to a positive effect on the surface tension efficiency of the biosurfactant. This may be caused by better stability of the micelles in the presence of acid and precipitation of secondary metabolites at pH below 5 (Wu et al., 2012).

Molecular characterization of biosurfactant

After extraction and dialysis, the biosurfactant synthesized by *M. circinelloides* was visualized using specific reagents on a TLC plate. The R_f of 0.5 confirmed the presence of lipids with the rhodamine reagent and positive reactions to the amino acids revealed by spraying the Ninhydrin reagent. These results were similar to those of *Kocuria marina* BS-15 lipopeptide biosurfactant with R_f of 0.6546 (Sarafin, Donio, Velmurugan, Michaelbabu, & Citarasu, 2014).

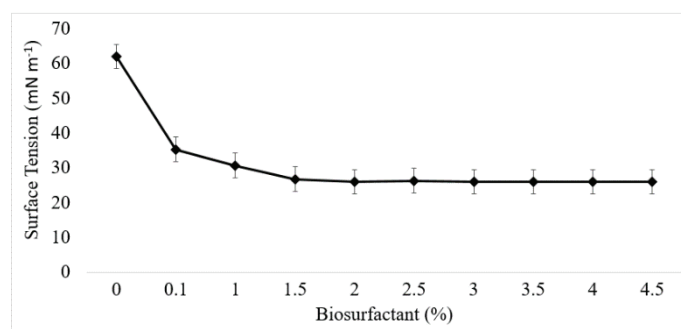


Figure 1. CMC of the biosurfactant produced by *M. circinelloides*.

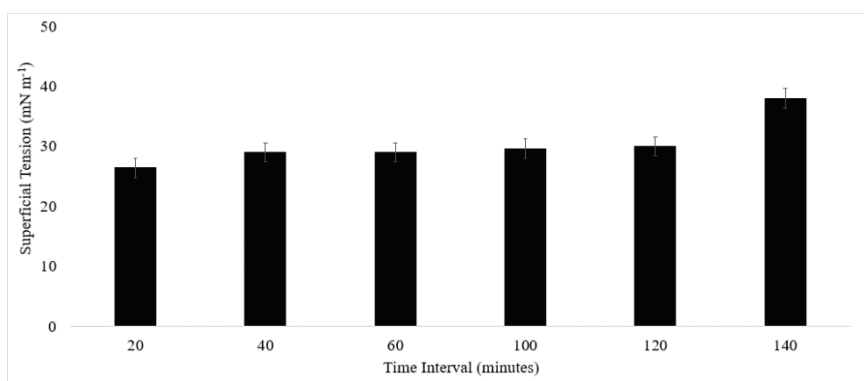


Figure 2. Stability of the surface tension of the biosurfactant produced by *M. circinelloides* at 100°C under different time intervals.

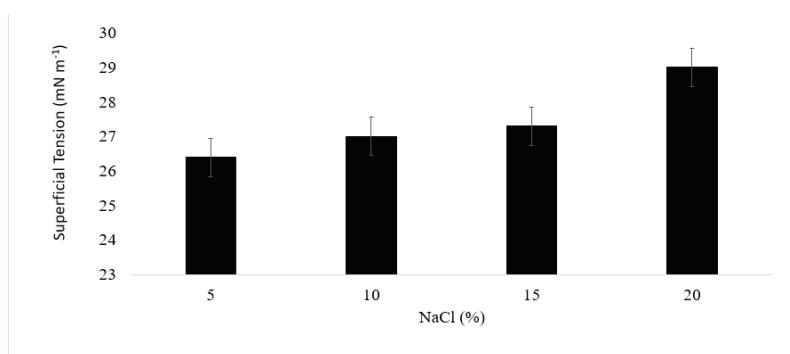


Figure 3. Stability of the biosurfactant surface tension under different salt concentrations.

The tests performed to determine the biochemical composition of the biomolecule revealed the presence of 49 protein, 48 lipids and only 3% carbohydrates, evidenced by TLC. These studies suggest that the biosurfactant synthesized by *M. circinelloides* probably classified as a lipopeptide. The zeta potential determines the function of the particle surface charge to predict and control the stability of colloidal emulsions and suspensions (Cortés-Camargo, Pérez-Rodríguez, Oliveira, Huerta, & Domínguez, 2016). Under these conditions, the cationic character of the biosurfactant produced was confirmed.

In Figure 5, the FT-IR plot analysis shows bands at 3384 (NH) and 1656 cm⁻¹ (C=O), which means a possible existence of a secondary amide, apparently being a peptide bond or other amide function. The peak at 1395 cm⁻¹(OH) evidences the presence of the acid function and may represent the carboxy-terminal portion of its protein. These results indicate that the hydrophilic portion of the biosurfactant molecule is likely to be peptidic in nature.

The analysis by GC-MS identified the fatty acids present in the lipid portion of the biosurfactant. Figure 6 indicates that the peak with retention time (RT) of 21.18 is possibly a methyl stearate having a mass of 298. The RT of 19.27 suggests the presence of the methyl palmitate compound with a mass of 270 (Figure 7). The GC-MS techniques were used to elucidate the chemical structure of the lipopeptide biosurfactant BS-1547 from *Kocuria marina* (Sarafin et al., 2014).

Effect of biosurfactant on the dispersion of petroleum derivatives in water

A dispersant keeps the insoluble particles in suspension, preventing them from aggregating with each other. Lipopeptide biosurfactants are characterized by their ability to disperse layers of oil into water (Inès & Dhouha, 2015). The study using the biosurfactant of *M. circinelloides* as a dispersant of hydrophobic compounds in water (Figure 8C), presented a significant result with a high dispersion capacity of motor oil in water of ODA 50 cm². The positive control assay using the synthetic surfactant Triton X was able to obtain an oil displacement area in water of 46.5 cm² (Figure 8B). These results suggest that the action of surfactants of microbial origin may be similar or more efficient than synthetic surfactants of petrochemical origin.

Biosurfactant study on the reduction of viscosity of petroleum products

Due to the presence of colloids and asphaltenes, the viscosity of an oil layer is usually high (Inès & Dhouha, 2015). Analysis performed using motor oil exhibited an initial viscosity at 843.6 cP. The addition of a solution containing biosurfactant (1.5%) to the oil was able to reduce its viscosity

effectively to 14.7 cP (Figure 9). These results showed that the biosurfactant of *M. circinelloides* was superior to the effect of the commercial detergent (84.7 cP), and similar to the synthetic surfactant Triton X (13.07 cP) in reducing the viscosity of the motor oil. According to the established results, fungal surfactants are as efficient as petrochemical surfactants. Thus, lipopeptide biosurfactants can contribute to the mobilization of oil trapped in reservoirs, increasing its recovery (Inès & Dhouha, 2015).

Removal of hydrocarbons from soil by the action of biosurfactant

Pollution of diverse ecosystems is a consequence of industrialization and human activities. Soil is often contaminated with oil and its derivatives, which makes its treatment difficult due to the low solubility of hydrocarbons in water, because these compounds tend to be adsorbed on the porous matrix (Rosa, Freire, & Ferraz, 2015). Table 1 lists the results of the removal of crude oil and motor oil from soil by the action of the biosurfactant of *M. circinelloides* and the control being water without addition of the biosurfactant. The biomolecule demonstrated removal capacity of 80.2 for petroleum and 95.9% for motor oil adsorbed on clay soil. In addition, in sandy soil, removal values were 92.8 and 77.2%, respectively.

Fungal biosurfactants are poorly studied due to their long production time compared to bacterial biosurfactants. However, they exhibit better efficiency during the process of removal of petroleum and its by-products from soil compared to most bacterial biosurfactants (Qazi, Kanwal, Jadoon, Ahmed, & Fatima, 2014). This characteristic makes them promising biosurfactants for the development of technological processes used in the bioremediation of sites contaminated with hydrocarbons.

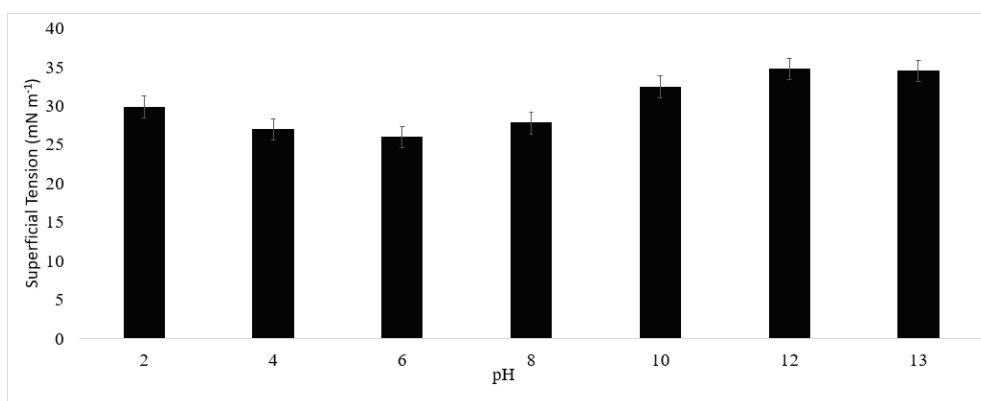


Figure 4. Stability of the surface tension of the biosurfactant against different pH levels.

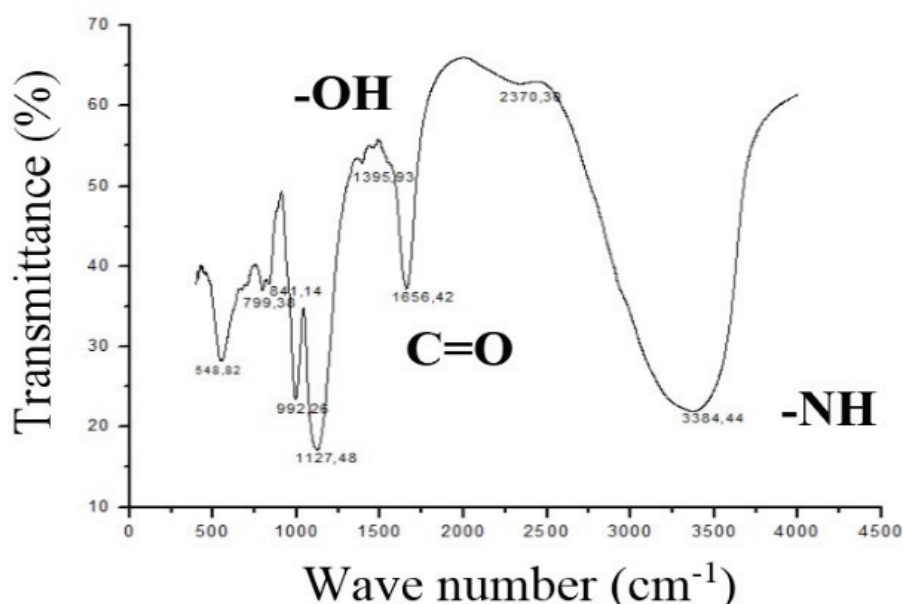


Figure 5. FT-IR analysis of the lipopeptide biosurfactant of *M. circinelloides*.

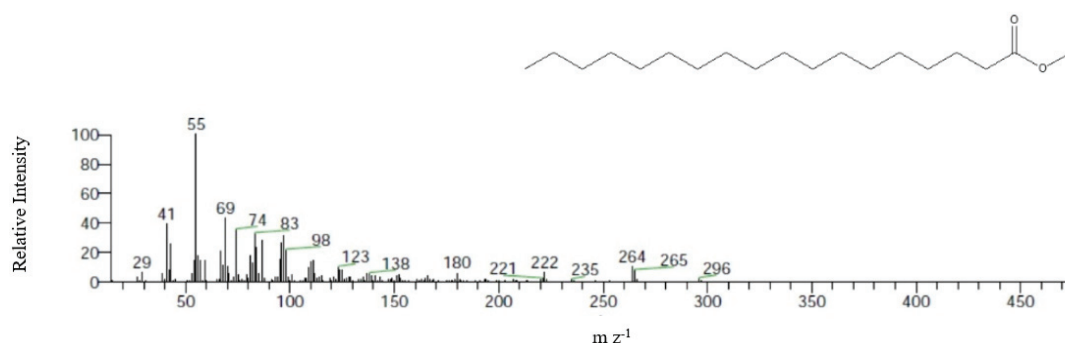


Figure 6. GC-MS analysis of methyl stearate of the biosurfactant.

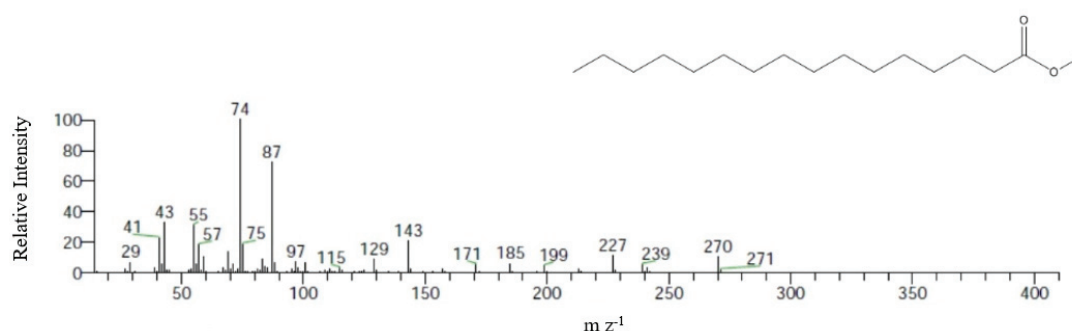


Figure 7. GC-MS analysis of methyl palmitate of the biosurfactant.

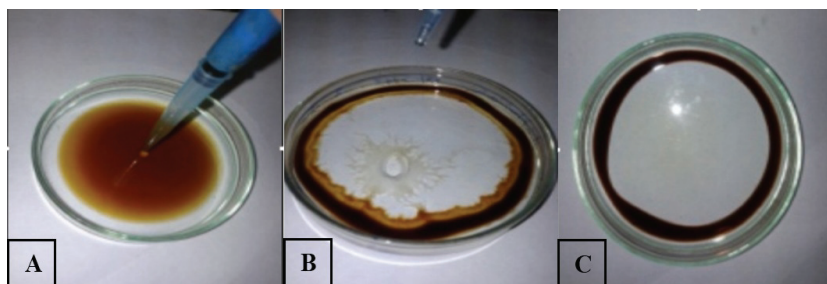


Figure 8. Dispersion of motor oil in water: (A) oil with water (control), (B) oil with chemical surfactant Triton X and (C) oil with biosurfactant of *M. circinelloides*.

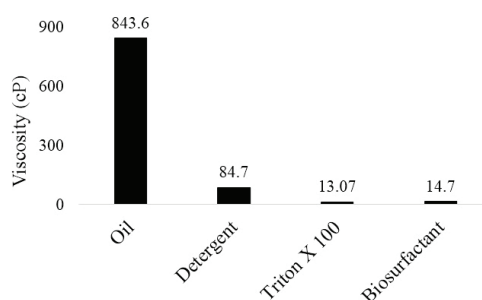


Figure 9. Reduction of oil viscosity using detergent, chemical surfactant and biosurfactant of *M. circinelloides*.

Table 1. Removal of petroleum and engine oil adsorbed on sandy and clay soils by *M. circinelloides* biosurfactant.

Removal agents	Clay soil		Sandy soil	
	Petroleum removal (%)	Engine oil removal (%)	Petroleum removal (%)	Engine oil removal (%)
Biosurfactant	80.2	95.9	92.8	77.2
Water	44.3	40.1	70.0	35.6

Conclusion

The filamentous fungus *Mucor circinelloides* UCP/WFCC 0001 presents a high biotechnological potential as a producer of natural surfactant. The biosurfactant presented good yield and excellent ability to reduce

the surface tension of the medium. It was able to remain stable in acidic and alkaline environments, with high temperatures and high concentrations of NaCl, being able to be applied in the bioremediation of several environments. The results obtained from the physical-chemical structural characterization of the biomolecule point to a cationic biosurfactant belonging to the class of lipopeptides. The biosurfactant application tests on hydrocarbon bioremediation were satisfactory, making these biomolecules possible before synthetic petrochemical surfactants.

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