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Biosurfactants produced by *Microbacterium* sp., isolated from aquatic macrophytes in hydrocarbon-contaminated area in the Rio Negro, Manaus, Amazonas

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ABSTRACT. Endophytic bacteria isolated from *Eichhornia crassipes* (Mart) Solms., collected in oil contaminated wastewater of effluent generated by Petrobras refinery in Manaus were investigated to determine their potential for producing biosurfactants. Assay with 2.6-dichlorophenol indophenol (DCPIP) indicator to verify hydrocarbon biodegradation activity; oil emulsification test; drop-collapse method; surface tension and growth curve of biosurfactant production. The M87 *Microbacterium* sp. strain chosen for this work was identified by the sequencing of the rDNA region and the chemical characterization was performed by FTIR, UFLC/MS and 1H RMN techniques. The selected bacterial isolate provided 3g L^{-1} of biosurfactant, using diesel oil as sole carbon source, being efficient in biodegrading oil as demonstrated by the DCPIP test. Fractions obtained by column chromatography were efficient in reducing water surface tension around 40 mN m^{-1}, especially fraction 1, which reduced it to 34.17 mN m^{-1}. The different techniques of chemical analysis used for the identification of the biosurfactant isolate indicated that this is probably a long - chain fatty acid lipid type, which may be used in the future as both biosurfactant in decontamination processes of hydrocarbon-polluted areas or as bioemulsifier in countless processes, since it exhibited no toxicity as determined by Alamar Blue assay.

Keywords: bioremediation, *Eichhornia*, diesel oil.

Biossurfactantes produzidos por *Microbacterium* sp., isolada de macrófita aquática em área impactada por hidrocarbonetos no rio Negro, Manaus, Amazonas

RESUMO. Foram investigadas bactérias endofíticas isoladas de *Eichhornia crassipes* (Mart.) Solms., coletadas em águas contaminadas com resíduos de petróleo em um afluente da refinaria da Petrobrás/Manaus, para avaliação da produção de biossurfactantes. Para selecionar o micro-organismos à produção e caracterização de biossurfactantes, foram realizados os seguintes testes: a descoloração do indicador 2,6 indofenol (DCPIP), emulsificação do diesel, colapso da gota, tensão superficial e curva de produção. A caracterização química foi realizada por meio das técnicas de FT-IR, UFLC/MS e RMN1H. A bactéria M87 *Microbacterium* neste estudo, foi identificada pelo sequenciamento da região rDNA e produziu 3g L^{-1} de biossurfactantes utilizando o diesel como fonte de carbono, mostrando-se eficiente na ação biodegradadora do petróleo, por meio do teste de Indofenol (DCPIP). As frações obtidas, mostraram-se eficazes na redução da tensão superficial da água abaixo de 40 mN m^{-1}, com destaque para a fração 1 que reduziu a tensão superficial para 34,17 mN m^{-1}. Pelas análises química utilizadas, pode-se inferir que, provavelmente, se trata de um ácido graxo de cadeia longa, que pode ser utilizado futuramente tanto como biossurfactante em processos de descontaminação de ambientes impactados por hidrocarbonetos, assim como bioemulsificante em inúmeros processos uma vez que não apresentou toxicidade por meio do teste realizado.

Palavras-chave: biorremediação, *Eichhornia*, diesel.

Introduction

Microbial biosurfactants have potential commercial applications within several industrial sectors. These products are effective in enhancing oil recovery and in the bioremediation of hydrocarbon-contaminated environments. Additionally, they may play a role in agriculture, in cosmetics,

pharmaceuticals, personal hygiene and food processing products, among others (Colla & Costa, 2003, Sourav, Susanta, Ghosh, Saha, & Saha, 2015). Moreover, they have advantages over synthetic surfactants, considering their biodegradability and low toxicity.

Biosurfactants can be biologically produced from various substrates, such as waste from tropical agronomic crops, fruit processing industries, oil processing and coffee processing industries, and others (Bento, Camargo, & Gaylarde, 2008, Sourav et al., 2015).

Bacteria are the main microorganisms that produce biosurfactants, among them the varieties of the genera *Bacillus* sp., *Pseudomonas* sp., *Rhodococcus* sp. are the most representative (Bezerra, Holanda, Amorim, Macedo, & Santos, 2012; Decesaro, Rigon, Thomé, & Colla, 2013).

Bacteria of the genus *Microbacterium* are reported to have resistance property and to absorb heavy metals like Zn (II) and Cd (II), in addition, some authors have already reported that some strains in this gender can grow in lubricating oil which indicates they are able to degrade hydrocarbons (Supaphol et al., 2013, Kim et al., 2013). Due to that characteristic, they may produce biosurfactants as a way of preliminary absorption of these compounds (Bento, et al. 2008). Hassanshahian, Giti, and Simone (2011), Kumar, Mamidyala, Sujitha, Muluka, and Akkenapally (2012) and Wongwongsee, Chareanpat, and Pinyakong (2013) reported the formation of emulsifiers for this genus, though they did not appraise the quantity or the type produced. Also Camacho-Chab et al. (2013) have isolated bacteria of the genus *Microbacterium* from marine environments and identified a compound classified as microbatan. However, there are no reports on biosurfactant production by bacteria of this gender in Amazonian freshwater environment, which are contaminated by hydrocarbons. Thus, the identification of new species that produce biosurfactants or emulsifiers in these environments is required.

This work represents an important contribution for the identification of bacterial isolates collected from oil-contaminated environments in the Amazon region. Those strains can further be exploited commercially, both for the production of new bioemulsifiers or biosurfactants and for the bioremediation of oil-contaminated environments. Furthermore, a range of new possibilities of research with those microorganisms may be explored, notably regarding their physiology in producing amphiphilic compounds.

In this work the production of biosurfactants by M87 *Microbacterium* sp. isolated from *Eichhornia crassipes* (Marts.) was evaluated and the chemical characterization of the biosurfactants was determined.

Material and methods

Microorganisms

M87 *Microbacterium* sp. strain isolated from *Eichhornia crassipes* (Marts.) collected in oil contaminated wastewater of effluent generated by the Petrobras refinery in Manaus (REMAN).

Molecular Taxonomy based in 16S rRNA gene sequence

The bacterium was identified following Isola, De Vries, and Chu (1994) and Chies, Dias, Maia, Braga, and Astolfi-Filho (2006) methodology. The bacterial genomic DNA was purified using a QIAGEN Genomic Tip 20/G (QIAGENGmbH), yielding 13 μg from 2 mL. The SSU rDNA was amplified 552 by 30 cycles of PCR using primers 530F (5' TGA CTG ACT GAG TGC CAG CMG CCG CGG 3') and 1492R (5' TGA CTG ACT GAG AGC TCT ACC TTG TTA CGM YTT 3') (Invitrogen - USA). The PCR mixture (50 μL) contained 1 μL of template DNA, 50 mM Tris pH 8.4, 500 μg mL^{-1} of BSA, 1.5 mM MgCl2, 250 μM dNTPs, 400 nM of each primer, and 2.5U of Taq DNA Polymerase (CenBiot Enzimas - BR). All reagents were mixed and heated at 94ºC for 2 min. Thirty-five cycles of PCR were carried out in a PTC 200 thermocycler (MJ Research, USA) as follows: 94ºC for 2 min, 50ºC for 30 seconds, and 72ºC for 2 min; followed by a final elongation at 72ºC for 2 min. The PCR products were directly sequenced with a DYEnamic ET Dye Terminator Cycle Sequencing Kit in the MegaBACE 1000 System (Amersham Pharmacia Biotech - UK), using 530F and 1492R primers. PCR amplification and amplicon DNA sequencing were repeated five times in order to confirm the results. The sequences were aligned and edited with MEGA6 software (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) and grouped by Neighbor-Joining method, and used to identify the isolates by comparing them with the patterns based on the results found in BLASTn.

Biodegradability test using redox 2.6-diclorofenol indophenol (DCPIP).

Biodegradability test was performed using an adaptation of redox 2.6-diclorofenol indophenol (DCPIP) indicator (Hanson, Desai, & Desai, 1997). The bacterium was inoculated in test tubes containing 2 mL of indophenol diluted at 0.010 mg L^{-1} in Bushenell Hass (BH) (Himedia), 10 µL of crude oil and 20 µL of the standard inoculum for

each of the three replicates. The total volume of the experiment was 20.03 mL. The evaluations were done after 24 and 48 hours, to detect biodegradation activity.

Drop collapse qualitative test

The test was conducted in duplicates, in Petri dish containing 3.5 mL of growing bacteria and one oil drop, observing the following time intervals: 0, 1, 5, 30 minutes and 72 hours. The result was considered positive when dispersion and drop collapse occurred. As negative control it was added 3.5 mL from BH extract + oil, and the same volume of Sodium Dodecyl Sulfate (SDS) at 1 M was used as positive control.

Particular environment to produce biosurfactants

For the production of biosurfactants 20 μL of bacterial suspension, corresponding optical density OD 600 nm was cultured in 50 mL of growth medium containing MgSO4.7H2O 0.5 g L^{-1}, KH2PO4 1 g L^{-1}, NaNO3 3 g L^{-1}, 1 g L^{-1} of yeast extract and 0.3 g L^{-1} of peptone with pH at 6.8, modified Rapp and Backhaus (1992) and diesel oil at 1.0% (v/v) was used as the sole carbon source.

The microbial culture carried out in 125 mL Erlenmeyer flask at 30ºC in orbital shaker (New Brunswick Scientific) under constant 150 rpm agitation for 96 hours. Erlenmeyer flasks with 50 mL of growth medium and 1% of diesel oil (v/v), without bacterial inoculum were used as control. Experiments were conducted in triplicate. After the incubation period, the growth medium was filtered through a 0.22 mm porosity membrane coupled to the 20 mL sterile syringe (TPP, Europe, Switzerland), as described by Jackisch-Matsuura et al. (2014).

Analysis of biosurfactant production

To find out the most effective period for the production of biosurfactants a production curve was delineated. The biosurfactant production was calculated in centimeters for 24 hours during six days as described by Cooper and Goldenberg (1987). Statistical analysis was performed using arithmetic mean and standard deviation calculated using BioEstat 5.3 software (Ayres et al., 2007).

Extraction and purification of produced biosurfactants

The biosurfactants extraction was conducted using liquid-liquid method, chloroform and methanol solvents (2:1). The organic phase was separated in Erlenmeyer flask and the solvent was evaporated from 50 to 75ºC in rotary evaporator. The obtained extract was frozen and lyophilized following Jackisch-Matsuura et al. (2014).

Preliminary identification of biosurfactant

Crude extract (2.5 mg) was dissolved in 100 uL of methanol. Solution (10 μL) was plated on silica gel plate 60 Merck. The plate was eluted for 30 minutes with chloroform: methanol (1:1). Iodine vapors were utilized as developers to detect the presence of lipids (Neerati & Yanamala, 2013) and Rhodamine 6G spray (0.005%) visualized under UV lamp (Horowitz, Gilbert, & Grifin, 1990), 6N sulfuric acid heated at 100°C was used to verify the presence of amino acids and other organic compounds (Dittmer & Lester, 1964).

Isolation of biosurfactants using column chromatography

The substances in the lyophilized extract were purified in chromatographic column (20 x 2.7 cm) using silica gel 60 Merck (54 g) and 25 g of the lyophilized extracted with chloroform methanol in different ratios (2:1, 1:1, 1:2 v/v) to elute the column (Reis et al., 2015). The fractions were grouped by similarity after visualization with UV lamps.

Surface tension evaluation

Main fractions obtained via column chromatography (205, 358, 916, 135, and 144 mg) were diluted in 20 mL distilled water and the surface tension of the different fractions was measured using a Kruss Tensiometer (K-6 Model, Germany) following the ring method (Du Nouy method) at 25°C.

Biosurfactant structural identification

Infrared spectroscopy (IR)

The analysis of the characteristics of functional groups of surfactant agents was performed by Infrared Fourier Transformed Spectroscopy (FTIR). FTIR spectra were obtained using FTLA 2000-104 spectrophotometer ABB Bomem, and the infrared spectra showed absorption peaks in the range of 4000 to 400 cm^{-1} using KBr pellets.

Mass spectrometry (HPLC/MS)

Analyses were performed using Ultra-Fast Liquid Chromatography (UFLC) system (Shimadzu) coupled with a tandem mass spectrometer (Bruker micrOTOF QII), equipped with a standard ESI source. The capillary voltage and ion collision energy ranged 4500 V and 650 Vpp, respectively.

Nuclear magnetic resonance (NMR)

Samples solubilized in deuterated methanol were analyzed by nuclear magnetic resonance spectroscopy of hydrogen (1H-NMR). Spectra were

obtained with a Varian Inova 500 equipment, operating at a frequency of 500 MHz.

Cytotoxicity test

Cell Culture

MRC-5 cells (human fibroblasts) were obtained from the Rio de Janeiro Cell Bank, Brazil and kept in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U ml^{-1}) and streptomycin (100 U ml^{-1}). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Cell viability assay:

Cytotoxicity was determined by Alamar blue method as described by Nakayama, Caton, Nova, and Parandoosh (1997). Briefly, adherent cells (5×10^3 cells $plates^{-1}$) were inoculated in 96 tissue culture plates and exposed to lyophilized and crude extracts (1.42 to 100 μg mL^{-1}) for 72 hours. After incubation, the solution of Alamar Blue (10 μL of 0.4% of Alamar Blue, suspended in water) was added and the cells were incubated for 3 hours at 37°C. The fluorescence was measured (excitation at 545 nm and emission at 595 nm). Assays were performed in triplicate. Doxorubicin (5 μg mL^{-1}) was used as a cell death positive control. Results were calculated considering the different absorbance of control cells.

Results and discussion

Bacterial strain investigated for the production of biosurfactants

The M87 bacterial strain, isolated from *E. crassipes* was morphologically identified as gram-positive cocci. The 16S rRNA gene was amplified and sequences were compared by BLAST analysis, with 99% of identity and query cover of 100% with *Microbacterium* sp. The sequence was deposited in the Genbank database under the access no. KX002029.

Evaluation of the M87 strain of *Microbacterium* sp. for biosurfactant production

The M87 *Microbacterium* sp. isolate showed positive results for oil biodegradation within 24 hours as demonstrated by the DCPIP test. Drop collapse occurred at 1.5 min and 72 hours, showing a satisfactory result since the drop remained dispersed for the whole period while it was being observed. It also demonstrated 1.7 cm or 52% of diesel oil emulsification index and breaking of water surface tension at 40.53 mN m^{-1} using the fermented cell-free extract for 96 hours (Table 1). These results are superior to those found for *M. aquamaris*, reported by Hassanshahian et al. (2011), of 5% emulsion and 43.54 mN m^{-1} surface tension break up. Moreover, Supaphol et al. (2006) and Kim et al. (2013) reported the biodegradation potential of hydrocarbon compounds and assimilation of heavy metals, such as cadmium, as well as growth in lubricating oil really showing, physiological characteristics for the use of hydrocarbons. Those authors emphasized the need and importance of further studies on the production and characterization of biosurfactants or bioemulsifiers in *Microbacterium*, due to the lack of reports referring this genus.

It is important to recognize that the use of M87 isolate crude extract produced superior diesel oil emulsion than surface tension reduction, and this may be related to the molecular weight of the biosurfactant isolated from the crude extract.

Production curve of biosurfactant from M87 *Microbacterium* sp. strain

To evaluate the biosurfactant production over time, a production curve was obtained (Figure 1) following method described by Bezerra et al. (2012). The production curve was plotted from the stationary phase, starting within 48 hours and remaining constant for up to 96 hours, this procedure was also followed by Pinto, Martins, and Costa, (2009) when using different microorganisms in mixed cultures of *Corynebacterium aquaticum* and *Bacillus* sp. and plain culture of *B. subtilis* and *B. pumillus*. The production curve allows the determination of the relationship between production period and the investigated biosurfactant.

The production curve data were used to continue production and subsequent isolation of biosurfactant within the period of 96 hours. The supernatant of the fermentation medium used for bacterium growth was extracted with chloroform: methanol. The formation of two distinct phases was observed and the organic phase was then collected and evaporated.

Table 1. Evaluation of M87 *Microbacterium* sp. isolate as a biosurfactant producer using different methods.

Isolate	Test with 2.6 indophenol (DCPIP)		Drop Collapse				Diesel oil Emulsion	Surface Tension
	24 hours	48 hours	0 s	1 min	5 min	72 hours	24 hours	
M87	+	+	-	+	+	+	1.7 cm	40.53 mN m^{-1}
Controls								
BH+DS*	-	-	-	-	-	-		
SDS**							2.0 cm	
MCS***								48.87 mN m^{-1}

Subtitle: (-) negative / (±) slow collapse / (+) rapid collapse, mN m^{-1} = milliNewton/meter. *BH + diesel oil with no bacteria used as negative control in indophenol and Drop Collapse tests. **Sodium Dodecyl Sulfate in concentration 1 M used as control in emulsion test. *** Growth medium without the bacteria used as control in surface tension test.

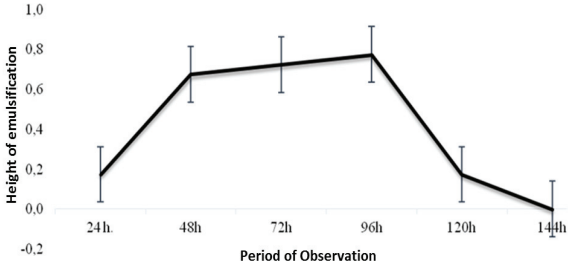

Figure 1. Production curve of biosurfactant produced by M87 *Microbacterium* sp. strain.

The material fermented for 96 hours was then lyophilized yielding 3 g L^{-1} biosurfactant. Sousa et al. (2014), using *B. subtilis* stationary culture, a known biosurfactant producer, obtained 3.47 g L^{-1} surfactant in 54 hours. Comparison between presented data demonstrates that the M87 *Microbacterium* sp. isolate used in this work is a potential source of biosurfactant.

Lyophilized extract (250 mg) was column chromatographed yielding 15 fractions ranging from 18 to 916 mg. The five most representative fractions were subjected to surface tension test to determine the production of biosurfactants. Fraction 1 was the most effective in reducing water surface tension of 34,17 Mn m^{-1} also fraction 3 presented significant results. That may be related to the concentration of low molar mass of the biosurfactant in those fractions, which according to Bento et al. (2008) may cause the water surface reduction. Except for fraction 4, all the others reduced surface tension to less than 40 mN m^{-1}. The biosurfactant effect exhibited by the fractions tested in this work was more significant than that showed by 80 tween commercial surfactant used as control and similar to the values recorded for the SDS (Sodium Dodecyl Sulfate) commercially used synthetic surfactant.

Preliminary structural characterization of the biosurfactant produced by the M87 strain

The chemical composition of the biosurfactant from fraction 1 was analyzed by three different techniques (UFLC/MS, 1H NMR and FTIR). Six peaks were observed in LC/MS spectrum being, the peak 1 the most representative at 6.5 min, followed by peak 4 at 10.5 min and peak 6 at 19.5 min retention (Figure 2).

LC/MS data showed in Table 2 along with those presented in Figure 3 indicated, considering the respective molecular weights, possible chemical structures with a predominant incidence of carbon-hydrogen bond or carbon-oxygen bond, possibly forming a long carbon chain. Considering Bento et al. (2008) findings, the biosurfactant produced by the M87 strain, matches the fatty acid lipid type classification. Those data were confirmed by FTIR and 1H NMR techniques.

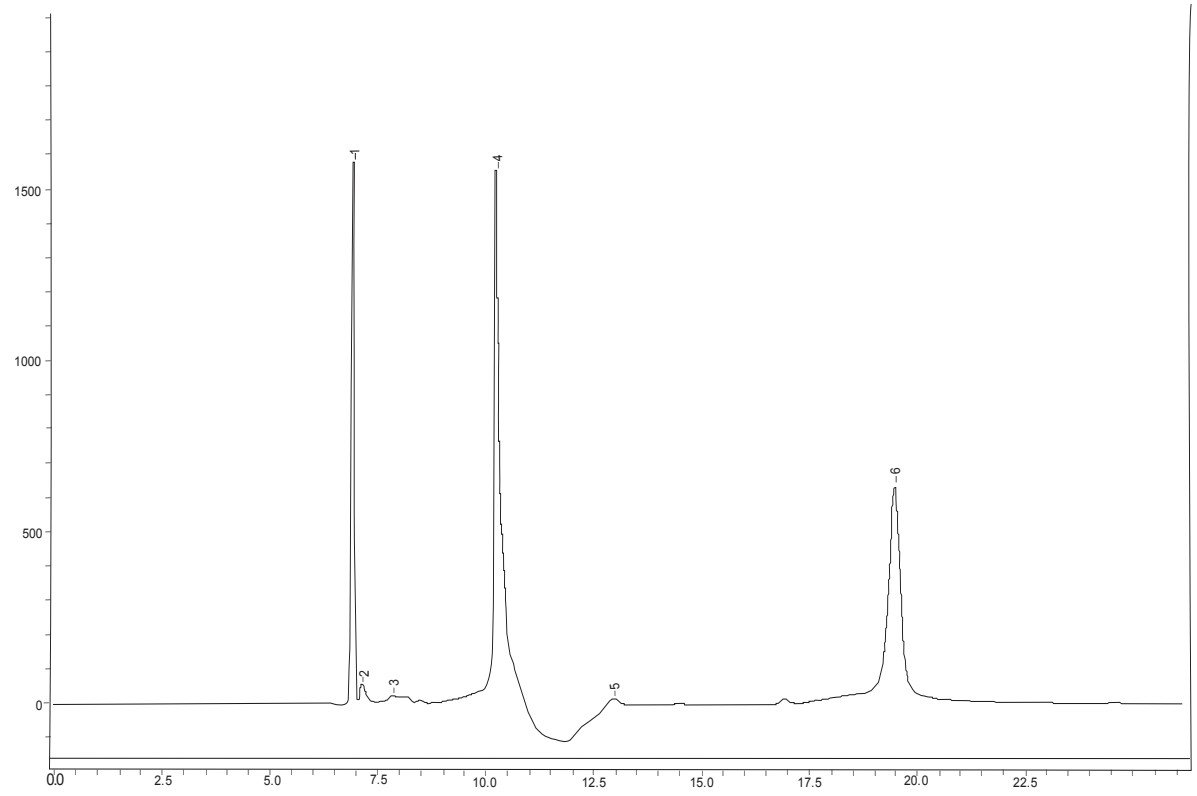

Figure 2. HPLC/MS spectrum of fraction 1.

Table 3. Chemical characterization of the biosurfactant produced by M87 *Microbacterium* sp.

Peaks	Molecular weight (m/z)	Chemical Structure	Retention Time (minutes)
1	1106.8051	$C_{60}H_{114}O_{17}$	6.5
2 and 4	1133.8	$C_{69}H_{113}O_{12}$	6.5, 10.5
3, 5 and 6	761.5944	$C_{39}H_{85}O_{13}$	7.5, 13.0, 19.0

The infrared Fourier Transform Spectroscopy (FTIR) spectra of the biosurfactant produced showed absorption signals at 1136 cm^{-1} corresponding to the region (CH), at 1383 cm^{-1} corresponding to the aliphatic region (-CH 3 -CH 2) and 1638 cm^{-1} corresponds to the C = O bond at 3448 cm^{-1} represents the elongation of the CH bond or the OH elongation. No NH elongation was observed in this spectrum, which indicates that the biosurfactant probably has no peptide residues present in its structure (Figure 4).

The absorption peaks were similar to those observed by Jara, Andrade, and Campos-Takaki (2013) and Sousa et al. (2014) when characterizing a tensoactive agent produced by *Geobacillus stearothermophilus* and a biosurfactant produced by *B. subtilis*, respectively. Camacho-Chab et al. (2013) studying the genus *Microbacterium* isolated from marine environment reported the production of glycoproteins able to emulsify aromatic hydrocarbons and oils. The emulsifier isolated from the marine environment differs from the biosurfactant isolated in this work as showed by the absorption bands obtained by FTIR, probably due to the environment differences. Possibly, the freshwater-isolated surfactant produces a distinct compound, tough with similar emulsifying activity. On the other hand, it is known that microorganisms from oil-contaminated environments have physiological mechanisms that actually help them in capturing hydrocarbons used as carbon source and can convert these substrates into several by-products, such as the biosurfactant identified in this study.

The hydrogen spectrum shown in Figure 4 confirmed the presence of bond between hydrogen and carbon due to the chemical shift. The shift at 3.66 ppm indicates the presence of structural CH_3O, followed by 1.25 - 1.55 ppm showing presence of CH_2 aliphatic groups and in 5.3 ppm the presence of CHO groups; these shifts and the presence of the respective groups corroborate the work of Liu, Yang and Mu (2008) in the identification of a lipopeptide isolated from *B. subtilis*. Considering the chemical shifts, it is possible to infer that the M87 isolate probably has a carbonic str ucture similar to that of the biosurfactant produced by *B. subtilis* often described as a surfactin producer.

The biosurfactant isolated from lyophilized extract fraction is probably of long-chain fatty acid anion type, since it has not shown the presence of structures other than carbon and hydrogen throughout different techniques employed.

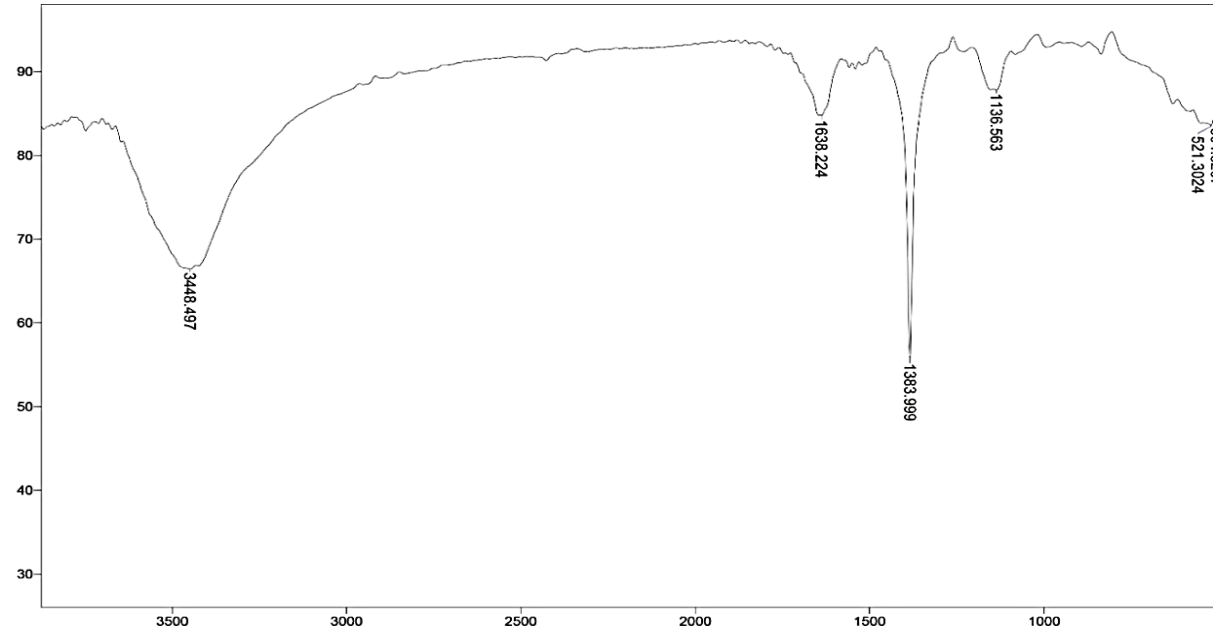

Figure 3. Fourier Transform Infrared (FTIR) Spectroscopy of the biosurfactant produced by the M87 *Microbacterium sp*. isolate.

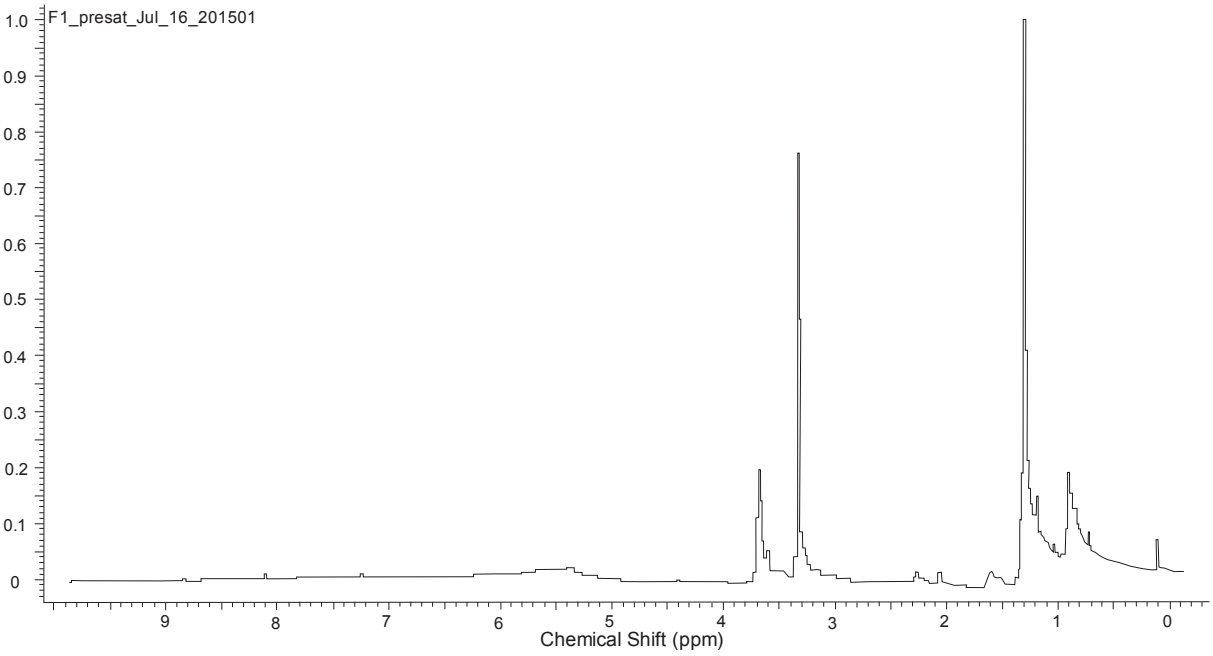

Figure 4. 1H NMR spectrum of the biosurfactant produced by M87 *Microbacterium sp*. isolate.

Citotoxicity test

Results of cytotoxicity assays with different concentrations of aqueous and lyophilized extracts of M87 *Microbacterium sp*. plotted in Figure 5 indicate that no toxicity was exhibited by the extracts. Decesaro et al. (2013) also reported no toxic activity exhibited by fungi or bacteria bioemulsifiers investigated in their work.

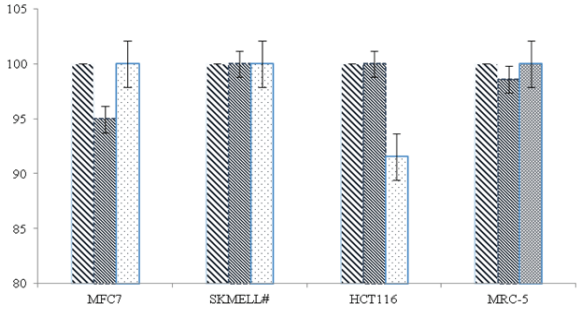

Figure 5. Cytotoxicity test of aqueous and lyophilized extracts from M87 *Microbacterium sp*. isolate.

Conclusion

This work reports for the first time the chemical characterization of a biosurfactant produced by the genus *Microbacterium* from oil-contaminated environment in the Amazon. The production of 3 g L^{-1} of microbial biosurfactant in flasks under agitation having diesel oil as the sole carbon source was considered satisfactory. HPLC, FT-IR and 1H-NMR analyses evidenced aliphatic CH groups (-CH2-CH3) and O-H stretching, indicative of the presence of fatty acids. Further studies must be conducted with the selected strain, to optimize methods to lower costs and enhance biosurfactant production. Our results validate the bioremediation potential of the obtained biosurfactant and its prospective use in cosmetics or food segments, since it did not show toxicity as determined by the Alamar Blue assay.

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