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Detecting microorganisms producing surface active compounds in mangrove sediments in São Luís, Maranhão

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ABSTRACT

Mangroves are areas of sediment transitions, favoring the cycling of materials associated with high concentration of microorganisms, presenting vulnerability to anthropogenic actions. The objective of this study was to evaluate the ability of microbiota in mangrove sediments of the Anil River, in the city of São Luís, Maranhão. To produce surface active compounds (SACs), samples were collected according to the EMBRAPA methodology by inoculating them in the Bushnell Haas medium, with carbon sources varying at 3% (v/v). From the production obtained, the supernatants of each culture were submitted to E24 analysis, drop spreading, thermal stability/pressure and pH variation. The SAC-producing species were identified using the MALDI-QTOF MS method. The producing species were identified as *Serratia marcescens* (2), *Ochrobactrum* spp. (3). E24 values ranged from 33% to 48% yield, with stability increase after autoclaving of 3% to 6% and of 17% for basic pH ranges, demonstrating the feasibility of using regional microorganisms present in mangroves to produce SACs from different carbon sources, as they have good rates of emulsification and stability to the diverse environmental factors.

Keywords: Biosurfactants; Hydrocarbons; Estuary

1 INTRODUCTION

The Brazilian coastal zone is considered one of the most extensive in the world, presenting a significant amount of distinct landscapes such as dunes, islands, reefs, and mangrove areas, with variations of physical chemical factors (PASQUAUD *et al.*, 2015). It is a unique ecosystem with extreme sensitivity and dependent on the variations of

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tides, salinity, temperature, and so on. The microflora present in these environments perform biochemical changes of nutrients, necessary for the consumption of organic matter and toxic media, and demonstrate a fundamental role in the ecosystem equilibrium (PRAMANIK; SENGUPTA; BATTACHARYYA, 2018). In the mangroves, the bacteria domain exerts significant functions in the decomposition, nutrient synthesis and other biogeochemical processes linked to the preservation of the soil production capacity in environmentally stressed situations (WANG *et al.*, 2018).

Important processes, such as the cycling of nutrients, common to the environment are directly related to the activities and diversities of soil microbial communities. Pollutants of anthropogenic origin may alter the structure of microbial communities and cause ecological imbalances that may lead to the extinction of important species or the decreasing of nutrient cycling to maintain the ecosystem (PAINGANKAR; DEOBAGKAR, 2018).

The molecules produced by microorganisms, such as primary and secondary metabolites, biosurfactants, and bioemulsifiers, are known as surface active compounds (SAC). These compounds play a key role in cycling organic matter present in the environment (ANTONIOU *et al.*, 2015). SAC present potential for biotechnological applications in several areas, such as agriculture, pharmacology, industry, environmental, among others (AMER *et al.*, 2015; ANTONIOU *et al.*, 2015). Biosurfactants have the capacity to reduce stress in air–water and oil–water interfaces, enabling insolvable phases to become soluble between them. In addition, biosurfactants can stabilize the oil in water in different types of emulsions (DANG; LANDFALD; WILLASSEN, 2016).

SACs produced by hydrocarbon-degrading microorganisms have different chemical and physical properties, forming micelles, increasing the apparent solubility of insoluble or poorly water-soluble compounds (DANG; LANDFALD; WILLASSEN, 2016). In the micelles, the biosurfactant molecules are oriented in such a way that the hydrophobic nonpolar regions are in maximum contact with each other, forming a hydrophobic core, and the hydrophilic polar regions are in contact with the aqueous phase (KARLAPUDI *et al.*, 2018). The biomolecules produced are capable

of increasing the availability of many water-insoluble substrates, the biodegradability of which is limited by their low solubility, such as petroleum derivatives (EL-SHESHTAWY *et al.*, 2017; KARLAPUDI *et al.*, 2018).

The presence of petroleum derivatives as an environmental contaminant in a region, causes serious damages to the microbiota of the site, besides promoting a natural selection of microorganisms capable of consuming and degrading such molecules (EL-SHAWSTAY *et al.*, 2017). The formation of micelle structures formed by the BS minimizes the repulsion of the hydrophobic groups present (KARLAPUDI *et al.*, 2018), requiring a minimum number of BS to allow the configuration of the critical micellar concentration (CMC) to occur (GARGOURI *et al.*, 2017). The CMC is the point where the aggregation of molecules to the formation of micelles is considered, considered a fundamental physical parameter to determine the performance of a SAC (GARGOURI *et al.*, 2017).

The existence of a wide functionality of the SACs is due to the chemical structures that form them, such as glycolipids, lipopeptides, polysaccharide complexes, proteins, phospholipids, fatty acids and neutral lipids present in biosurfactants (MNIF and GHRIBI, 2015). In this way, the SACs have attracted in recent years great interest of industry, among them the petroleum industry, because they are less toxic to the environment than the chemical additives commonly used in these processes (MNIF and GHRIBI, 2015).

The variability of functional groups presents in the SACs favor the diversity of physical and chemical properties present in these biomolecules. The SACs are shaped to suit different applications, varying their property according to the enrichment source of the microbial growth medium used in their production (PARASZKIEWICZ *et al.*, 2018).

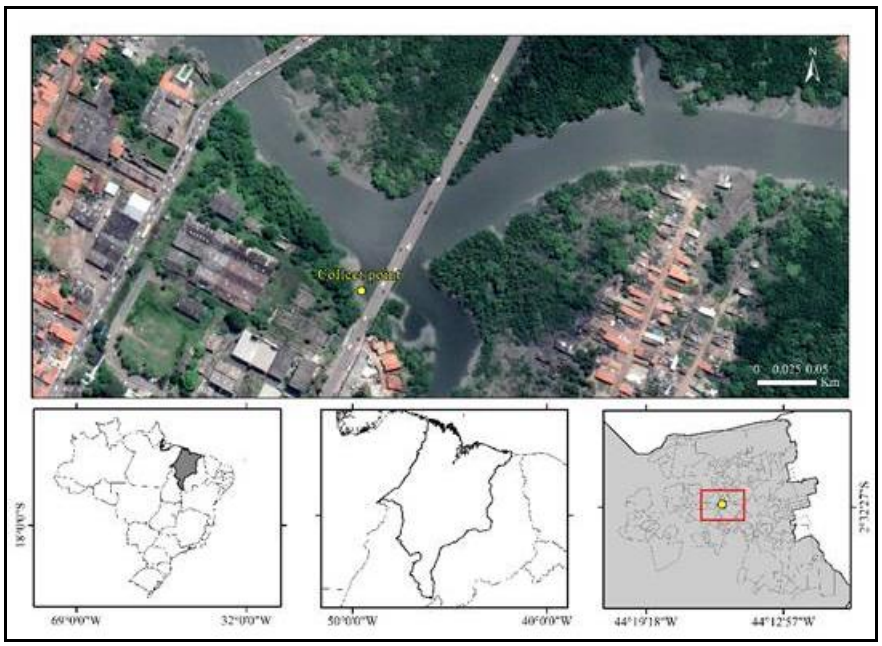
In this way, the SACs have the potential to act as biologically active compounds applicable in the fields of medicine and the petroleum industry. The present work proposes a study of microorganisms producing SACs present in mangrove environments in the city of São Luís of Maranhão, based on sunflower oil and kerosene.

2 MATERIAL AND METHODS

2.1 Point of collection

Samples of mangrove sediments were collected at a selected point in the Anil River estuary in the city of São Luís in the state of Maranhão, with coordinates, 2º 32 '19.1''S 44º 15 '46.6' 'W/–2.538639, –44.262944 (Figure 1).

Figure 1 - Collection point



To accurately represent the microbial population of the area, the samples were collected within a 20 × 20 m traverse at the selected point and stored in hermetically sealed containers placed in a thermal box, and transported to the Laboratory of Applied Microbiology located at CEUMA on 09/2018. Three collections were carried out within the plot (P. *et al.*, 2017). Subsequently, it was submitted to 2 mm mesh sieving, with the purpose of eliminating boulders and decomposed organic material (P. *et al.*, 2017). The soil sampling procedure followed the EMBRAPA methodology to characterize the soil microbiota, that is, to compose a single representative sample of the whole polygon, 10 g of soil was collected from each collection and mixed in a single container containing 30 g total weight of the samples (EMBRAPA, 2017).

2.2 Microbiological Growth Conditions in Minimum Mineral Environment

The representative sample, weighed in two beakers containing 15 g of the sediment in each, was taken to the Laboratory of Applied Microbiology at the CEUMA University. The sediment samples were added to 250 mL Erlenmeyer flasks to which were added 135 mL of the minimal mineral medium (MM) containing 1 KH_2PO_4;0,02 $CaCl_2$; 0,2 $MgSO_47H_2O$; 1 NH_4NO_3; 0,05 $FeCl_2$, 1 Na_2HPO_4 (g•L-1), Bushnell Hass broth, added 3% of sunflower oil or kerosene. The flasks were incubated for 72 hours at 32 °C. After the incubation period the samples were diluted decimally in sterile NaCl solution (0.9%) and about 50 µL was plated on Tripticasein soy agar (TSB, Himedia). To maintain the bacterial isolates, they were preserved in TSB broth plus 20% glycerol in cryotubes at –80ºC.

2.3 Detection of BAC Producing Bacteria

All the isolated morphotypeswere submitted to tests of biosurfactants and bioemulsifiers in minimal MM, in duplicate, with carbon sources added, 3% of sunflower oil and 3% of kerosene respectively, in 50 mL falcons (GEETHA, BANAT, JOSHI, 2018). After the inoculum of the selected bacteria in the minimal mineral media the flasks were incubated on a stirrer at 32 °C at 140 rpm for up to eight days (EL-SHESHTAWY *et al.*, 2017). After the incubation period, the cultures were centrifuged at 5,000 G for 15 min to remove the supernatant. The supernatant was applied directly in test tubes, in the proportion of 50% supernatant and 50% of kerosene (GEETHA; BANAT; JOSHI, 2018). The mixture was vortexed for 2 min to verify emulsion stability and emulsifying activity after 48 hours (EL-SHESHTAWY *et al.*, 2017).

2.4 Isolation Identification

Bacterial species were identified using the Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) system using the Biotyper system (Biotyper; BrukerDaltonics, Billerica, MA).

2.5 Droplet Spreading Method and IE24

For the drop spreading analysis, 1 mL of each bacterial growth supernatant was added to a hydrophobic surface to analyse the droplet dispersion diameter. All results were compared as a sample containing only the minimal MM (GEETHA, BANAT, JOSHI, 2018).

To determine the emulsification index, 3 mL of the culture medium and 1 mL of kerosene were added in a test tube. The tubes were shaken for two minutes in Vortex and the reading was performed after 24 hours.

The calculation of the emulsification rate was modified (VARJANI, 2017), according to equation (1);

$$Te_0 = \left(\frac{HE}{HT} \times 100\right) - Te_b \quad (1)$$

at where:

Teo - Emulsification rate in the oily layer (kerosene);

HE - Total height of the emulsification layer;

HT - Total height of nutrient broth and kerosene;

Teb - Emulsification rate of the blank assay (emulsification in the culture medium phase without bacterial growth).

2.6 Temperature, Pressure and pH Tolerance Test

The supernatants produced from the SAC solutions were subjected to autoclaving temperature and pressure in an autoclave for 30 min (VARJANI, 2017). After heat treatment, each solution was cooled to room temperature for repetition of the E24 emulsification rate calculation step.

The non-autoclaved supernatants were subjected to pH adjustment to the values of 3, 4, 5, 6, 7, 8, 9, 10 by the addition of HCl and 0.1N or NaOH 1 and 0.1N, and maintained in a water bath for 30 min at a temperature of 25 ± 1 ºC. All treatments were performed in triplicate and measurements of emulsification rates were repeated after each treatment.

3 RESULTS AND DISCUSSION

The average amount of bacteria per gram of sediments that showed development in the minimal MM containing the hydrocarbon sources kerosene (P1) and sunflower oil (P2) were respectively 506.19 × 108 and 210.85 × 108 CFU/gram of sediments. Growth rates in the minimal medium, with restricted carbon source, were of the order of 108 CFU/g of sediment. This value of density for microbial growth in mangrove sediments was similar to those observed in studies already described in the literature for the production of SACs (DEEPIKA *et al.*, 2016). An analysis of the growth curve for the samples P1 and P2 showed that in the first days of incubation 24 to 72 hours, a microbial population increase was observed preceding a stationary phase, which was determined by the presence of kerosene and sunflower oil in solution, resulting in a selection of hydrocarbon tolerant species (KHADEMOLHOSSEINI *et al.*, 2019). In the present work, the use of hydrocarbons in the presence of hydrocarbons in the presence of a hydrocarbon source is not necessary for the identification of CFUs (DEEPIKA *et al.*, 2016; KHADEMOLHOSSEINI *et al.*, 2019).

After the plating of the samples, ten bacterial colonies were isolated for the production of SACs. From the bacterial isolates tested, only the strains P23A, P23C, P211A, and P211C showed cell growth, representing 67% of the total bacterial isolates (Table 1). In the initial stage of selection of SAC bacteria, as biosurfactants and emulsifiers, isolates capable of consuming and developing the sources of kerosene and sunflower oil were identified. In general, the number of isolates with different morphotypes varied according to the source of carbon used in the enrichment (BARTAL *et al.*, 2018). Considering the interest and the potential for the application of microorganisms in the treatment of industrial waste based on hydrocarbons, the search for new SAC bioproducers is of great relevance, as there are currently few commercially available surfactant biological compounds such as surfactin, soforolipids, and rhamnolipids (BARTAL *et al.*, 2018; EL-SHESHTAWY *et al.*, 2016).

Table 1 - Growth of isolates with variation of carbon source

Strains	Hydrocarbon Sources	
	Kerosene	Sunflower oil
P13A	Positive	Negative
P13B	Positive	Negative
P13C	Positive	Negative
P13D	Positive	Negative
P23A	Positive	Positive
P23B	Negative	Positive
P23C	Positive	Positive
P211A	Positive	Positive
P211B	Negative	Positive
P211C	Positive	Positive

Source: Produced by the author

From the identification of the emulsifying activity of the supernatants of each culture, the strains that produced E24 activity were P13A, P13C, P23A, P23A *, P23B, P23C *, P211B; those that were not able to produce E24 activity were P13B, P13D, P23C, P23C *, P211A, P211A *, P211C, P211C *. The P1 isolates, which presented 33% to 38% and P2 values, with values from 33% to 47%, (Table 2 and Figure 2). The identification of SAC produced in strains P13A, P13C, P23B, P23A, P23C was considered to be high density because precipitation occurred in ethyl alcohol PA.

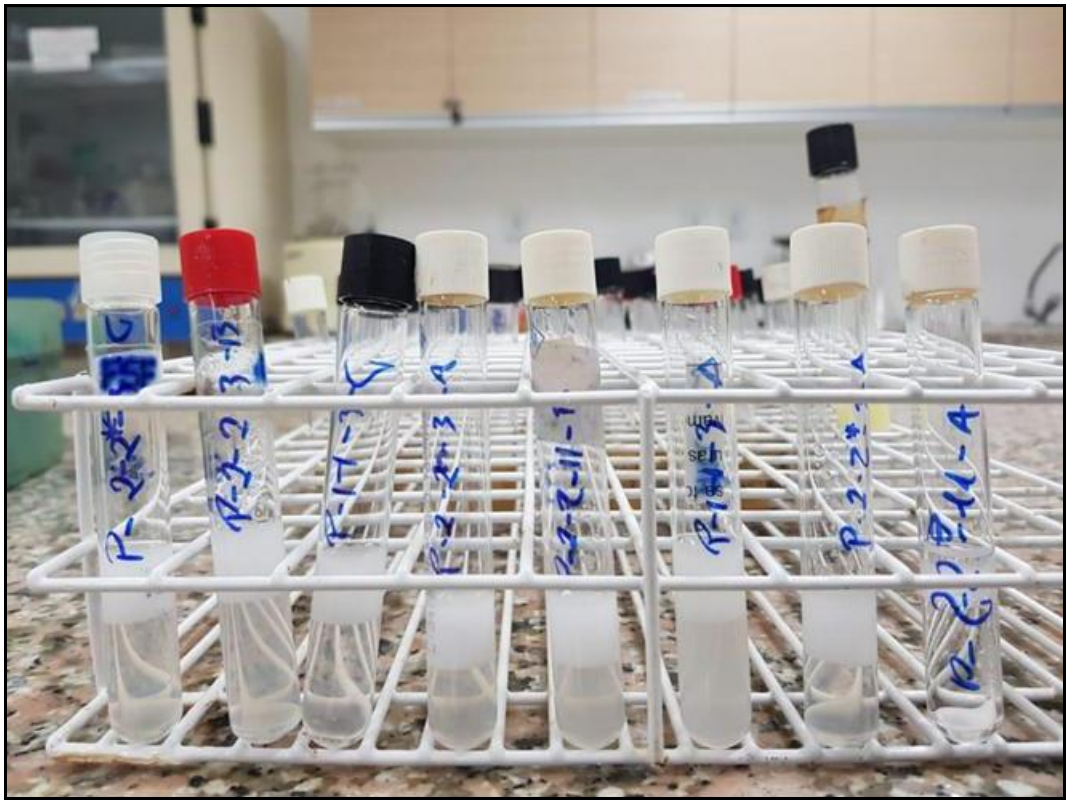
Table 2 - Emulsifying activity index of biosurfactant-producing isolates

	Emulsifying Strains			
Strains	He	HT	Teb	E24
P13A	1,5cm	4cm	0	38%
P13C	1,3cm	4cm	0	33%
P23A	1,3cm	3cm	0	43%
P23A*	1,4cm	3cm	0	47%
P23B	1,3cm	4cm	0	33%
P23C*	1,5cm	4cm	0	38%
P211B	1,4cm	3cm	0	47%

Source: Produced by the author

He - Emulsification height, HT - Total height, TEb - Emulsification rate of the white medium test, E24 - Emulsification rate of the oily layer.

Figure 2 - Strains with emulsifying activity



Source: Produced by the author

The use ofkerosene (3% V/V) as the main source of carbon and energy allowed the isolation of four (4) morphotypes but only two (2) were SAC producers. This justifies the use of hydrocarbons as a source of enrichment for selecting degrading microorganisms. The strategy of using different hydrocarbons as the only source of carbon and thus inducing the production of CAS is related to metabolic pathways of na inducible nature present in many bacterials trains (JOY; RAHMAN; SHARMA, 2017). P13A, P23A, P23B, P23C were identified by the MALDI-TOF MS system as *Ochrobactrum anthropi, Serratia marcescens, Serratia marcescens, Ochrobactrum anthropi* and *Ochrobactrum tritici*, respectively.

Emulsification indices (E24) for the supernatants of strains *O. anthropi* P13A, *S. marcescens* P13C, S*. marcescens* P23A, *Ochrobactrum anthropi* (P23B) and *Ochrobactrum tritici* P23C from culture in MM enriched with kerosene, presented a variation from 33% to 38%, and for the médium enriched with sun flower oil, the variation was from 33% to 47%. The E24 values were considered excellent: the literature reports that good emulsifying agents are those with E24

values higher than 50% (PANJIAR; SACHAN, SACHAN, 2015), being that the yield of the isolates of the work referent na emulsification index considered average for kerosene emulsification. However, studies already carried out with the strains *Ochrobactrum anthropi* HM-1 and *Citrobacter freundii* HM-2 presented negative results for the emulsification of kerosene, but covering a good yield in the emulsification of diesel oil, motor oil, crude oil, olive oil, and frying oil (GEETHA; BANAT; JOSHI, 2018). The bacterial genera *Ochrobactrum* and *Serratia* are widely distributed in the environment as soil in plant and in intestinal tract tissues of humans and animals (ALMANSOORY *et al.*, 2017), demonstrating their versatility and broad spectrum adaptive physiological and evolutionary according to the environment to which they belong, and are considered promising microorganisms for the production of biosurfactants and compounds with emulsifying activity.

Drop spreading was performed on strains that showed the highest emulsifying stability (3)—P13A with 8.0 mm, P13C with 7.8 mm, P23A with 7.9 mm, P23B with 8.7 mm, P23C * with 7.3 mm of the dispersion diameter—without discounting the droplet spreading only of the minimal MM, which presented a dispersion value of 4 mm in diameter for calculating the percentage of the increase of the droplet diameter for the strains were 100% for P13A, 96% for P13C, 97% for P23A *, 117% for P23B and 83% for P23C * as shown in table (3). The isolate P23B presented the highest index of dispersion diameter of the drop than the other strains. In this work, the results of the evaluation of the emulsification activity are presented in the following table (BARTAL *et al.*, 2018; GEETHA; BANAT; JOSHI, 2018). The *Ocrhobactrum anthropic* strain, P23B, grown in enrichment médium containing sunflower oil, presented results that confirm the ability of the lineage to produce extracellular biosurfactants. *O. anthropi* has a significant emulsifying activity on the growth of several carbon sources already documented, such as octane, toluene, and crude oil (IBRAHIM, 2018). In the present study, the results obtained show that the *O. anthropi* strains can produce biosurfactants and secrete them extracellularly in the in vitro culture medium (GEETHA; BANAT; JOSHI, 2018; IBRAHIM, 2018).

Figure 3 - Drop spreading method. (a) Drop scattering of the 5 strains, (b) Drop spreading method P13A, (c) Drop spreading method P13C, (d) Drop spreading method P23A *, (e) Drop spreading method P23B , (f) Drop Spreading Method P23C *

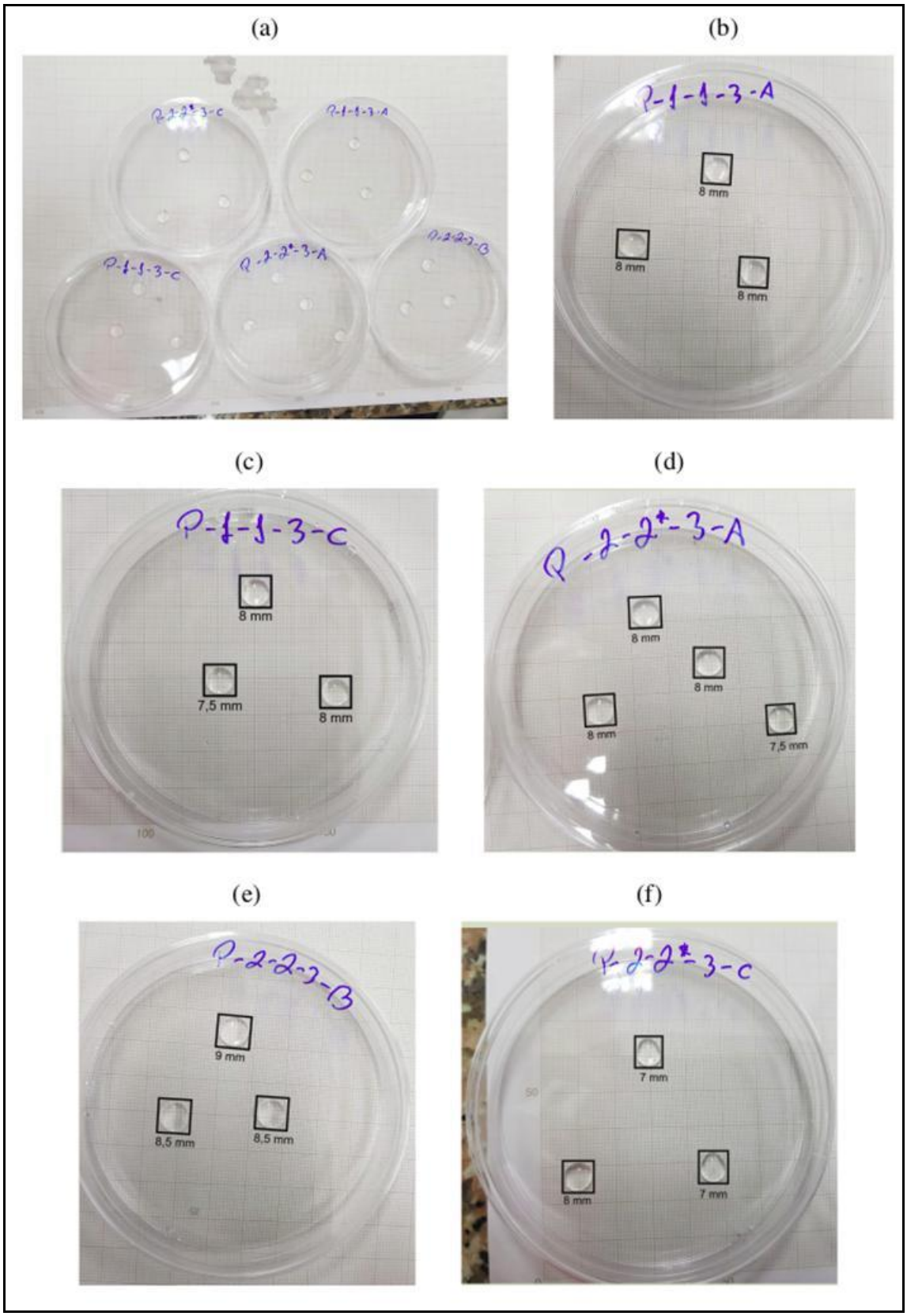


Table 3 - Table for the dispersion diameter and percentage of the increase of the diameter

Genus	Scatter diameter	Increase in diameter
O. anthropicP13A	8,0mm	100%
S. marcescens P13C	7,8mm	96%
S. marcescens P23A*	7,9mm	97%
O. anthropiP23B	8,7mm	117%
O. triticiP23C*	7,3mm	83%

The surfactant activity identification was determined by increasing the droplet diameter of supernatants from each source, P1 and P2, in hydrophobic Petri dishes. The increase in droplet diameter in strains P13A, P13C, P23A, P23B, and P23C was respectively of 100%, 96%, 97%, 117%, and 83%. The isolate P23B presented the highest index of dispersion diameter of the drop compared with the other strains. The results of the evaluation of the emulsification activity were evaluated.

The *Ochobactrum anthropic* lineage, P23B, cultivated in enrichment medium containing sunflower oil, presented results that confirm the ability of the lineage to produce extracellular SACs. *O. anthropi* strains have a significant emulsifying activity in the growth of several carbon sources already documented, such as octane, toluene and crude oil (IBRAHIM, 2018). In the present study, *O. anthropi* strains of the genus *O. anthropi* produced biosurfactants and emulsifiers and excreted them in the culture medium used for microbial growth (CATTER *et al.*, 2016; IBRAHIM, 2018).

The application of SACs as biosurfactants and bioemulsifiers depends on the stability of the molecules under extreme environmental and operational conditions, pH and temperature (IBRAHIM, 2018). The results on the stability of the emulsion by varying the temperature showed that the SACs produced by the bacterial strains are thermostable, since E24 showed an increase in their maximum value of 3 to 4% for the supernatants of the lineages P23A and P23B, respectively (Table 4). Antagonistic results were found by IBRAHIM, 2018, who reported the activity of a SAC produced by the strain *O. anthropi* HM-1, and E24 values showed a decrease in their emulsifying activity after heating at 100 ºC.

Table 04. Emulsification rate of the thermal stability test of the supernatants of bacterial cultures

Strain	He	HT	E24
S. marcescens P23A	2,4cm	5,0cm	48%
O. anthropi P23B	2,8cm	5,3cm	53%

Source: Produced by the author

The SACs produced by *S. marcescens* P23A P23A and *O. anthropi* P23B P23B showed emulsifying activity only at neutral and basic pHs 7–10 (Table 5), demonstrating an increase in the emulsifying activity as the medium becomes more basic. With variation of pH, precipitation of the active compound, alteration of the structure of the molecule or even complete degradation of the biomolecule can occur (IBRAHIM, 2018). In this study the absence of the emulsifying activity was observed in acid pHs 3 to 6. Discordant results in relation to this work have already been reported for SAC with distinct chemical and physical properties produced by microorganisms grown in specific carbon sources (AMER *et al.*; BARTAL *et al.*, 2018).

Table 5 - Emulsification rate of the pH stability test

Strains	pH	He	HT	E24
S. marcescens P23A	7	2,0 cm	3,5 cm	57%
	8	2,0 cm	3,2 cm	63%
	9	2,0 cm	3,2 cm	63%
	10	2,0 cm	3,1 cm	65%
O. anthropi P23B	7	1,8 cm	3,4 cm	56%
	8	1,8 cm	3,2 cm	56%
	9	1,7 cm	3,0 cm	57%
	10	1,7 cm	3,1 cm	55%

4 CONCLUSIONS

The work demonstrates the presence of bacteria in mangrove sediments capable of producing SACs. The results reinforce the capacity of the microorganisms present in mangrove ecosystems of the Anil River in the city of São Luis to show variability in the SAC properties. Such representativeness of this characteristic is due

to the presence of diverse micro and macro nutrients present in mangrove environments. In addition, the collection region undergoes anthropogenic influences, it becomes selective for microorganisms that produce hydrocarbon degrading biomolecules.

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