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Production and Application of No-purified Rhamnolipids in the Soil-washing of TPHs Contaminated Soils

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Authors' contributions

This work was carried out in collaboration between all authors, each one being responsible for one or more steps. The author LGT was the supervisor of the master dissertation of author RG that originated the scientific article and responsible for the development of the project that originated the dissertation, besides reviewing the written article, ordering the text. The author RG was responsable for the experiments. The author JG updated the bibliographic review and the bibliographical citations after the article, moreover the authos reviewed the work of master degree student autor RG. The authors LGT and JG formatted the article for publication, besides the revision of the translation into the English language.

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ABSTRACT

Aims: This work aimed to demonstrate the feasibility of producing (mono- and di-) rhamnolipids employing a strain of *Pseudomonas aeruginosa* strain ATCC 9027 employing olive oil as a substrate and some mineral salts. This rhamnolipid is a biosurfactants with multiple applications The CMC of this product under different conditions (filtered, unfiltered, in the presence and absence of Fe and Mg, at different pH values) was assessed. At the end, the UP was assessed in the washing of a **TPH** contaminated soil.
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Methodology: Rhamnolipids were produced with *P. aeruginosa* in olive oil, then by drying the culture broth was generated an unpurified product (UP) that contained 0.19% rhamnolipids. Critical micelle concentration CMC of UP products were evaluated in the presence of Ca^{2+} or Fe^{3+} from 0.5 to 2 mM, and pH values from 4 to 10. Finally, this surfactant was assessed in the washing of hydrocarbon-contaminated soils, and compared with other synthetic surfactants.
Results: It was found that CMCs were similar to those reported in the literature for pure rhamnolipids. The UP products have shown dynamic behavior in the soil washing at concentrations below 176 mg/L because removed 80% of 6,500 mg **TPH**/Kg from a gravel-sandy soil; the rhamnolipids could be removed **TPH** through mobilization mechanism.
Conclusion: It was possible to produce rhamnolipid using olive oil as carbon source and strain of *P. aeruginosa* ATCC 9027 to levels of 100 mg/L. It was feasible to produce a powder containing 1.19% of rhamnolipids. The UP had better properties as a surfactant than the purified product. The pH affects the CMC of the rhamnolipids in a way that promotes their behavior as ionic surfactant or nonionic surfactant. The ionic strength with Ca^{2+} and Fe^{3+} has an effect on the CMC of rhamnolipids so that the decreases in the range of 35 to 41 mg/L in the presence of 0.5 to 2 mM of metals. The UP rhamnolipids were employed for washing soil contaminated with 6,500 mg/kg increased **TPH** removal at low concentrations and to be as effective as chemical surfactants. **TPH** removal observed was about 80% for rhamnolipid with a CMC x 0.074 concentration.

Keywords: Biosurfactants; hydrocarbons; rhamnolipids; soil washing.

1. INTRODUCTION

In recent years, many research groups are developing new surfactants which cope with a wide range of necessities in oil, cosmetic, food and other industries [1]. Specifically, there are a lot of problems in an environment which need new sustainable, efficient and cost-effective products [2]. Soil contaminated by the oil spill is a common problem at zones where oil is extracted, processed, stored or transported [3]. Also, intensive industrial activity has resulted in the accumulation of high concentration of heavy metals in the soil [4]. These problems can be solved using surfactant assisted soil washing because that is a cost-effective technology to extract diverse types of pollutants from contaminated soil [5]. Due to surfactants enhance the efficiency to remove contaminants [6]. Surfactants are amphiphilic compounds that reduce the free energy of the system by replacing the bulk molecules of higher energy at an interface, hence it can mobilize or solubilize contaminants. There are many surfactants and biosurfactants according to their production processes, the last are extracellular metabolites of yeast or bacterial organisms. Rhamnolipids produced by *Pseudomonas aeruginosa* are a group of biosurfactants that has been studied extensively. Up to 28 homologues products have now been identified. Solutions with surface tensions of about 29 mN/m are characteristics of these compounds. Two types of rhamnolipids contain either two rhamnoses attached to ß-hydroxydecanoic acid or one rhamnose connected to the identical fatty acid [7]. Surface-active compounds commonly used are chemically synthesized. Some reports have been documented about of effect of rhamnolipids in remove hydrocarbons or metals showing promising results [8,9,10] and [11]. Hence, replacing the synthetic surfactants with biosurfactants could provide advantages such as biodegradability and low toxicity in the soil. Currently, the use of rhamnolipids has been limited by their relatively high production cost [12] and their downstream process of purification [13]. on the other hand, have reported the washing of soils contaminated with metals, trhough bioleaching with oxidizing bacteria and rhamnolipid biosurfactants, with excellent results. Zamudio-Perez et al. [14] reported the washing of a soil contaminated with **TPH** (31,900 mg/kg) employing synthetic and natural surfactants (including guar, mesquite and locust bean gums. Removal effieciencies obtained were between 15 (guar gum) and 55 % (Brij 35), respectively. On the other hand, Zacarias-Salinas et al [15] washed **TPH** contaminated soils (14,700 mg/kg) using a set of surfactants including synthetic products and natural products (locust bean, guar and mesquite gums), reaching high removals, up to 55% for the las group of vegetal products in comparison to the removal effieciency of just water (5%).

The aim of this work is to produce a rhamnolipid using *P. aeruginosa* ATCC 9027 and employ a unpurified product that contain rhamnolipids in the washing of

contaminated soil with total petroleum hydrocarbons (**TPH**s) .

2. MATERIALS AND METHODS

2.1 Fermentation Media and Conditions

2.1.1 Inoculums preparation

Pseudomonas aeruginosa strain ATCC 9027 was used for the production of biosurfactants on nutrient agar, nutrient broth and mineral medium. The strain was grown in Petri dishes with nutrient agar (BD bioxon) for 48 h at 30ºC. Subsequently, the strain was transferred to 150 mL Erlenmeyer flasks containing 60 mL of nutrient broth (BD bioxon), the flasks were shaken at 150 rpm for 24 h in an orbital incubator (SEV, INO-650 M) at 30ºC. Biomass was monitored by optical density at 565 nm, once detected the exponential growth phase was transferred 1 mL of nutrient broth in 150 mL flasks containing 60 mL of mineral medium and olive oil as carbon source.

2.1.2 Fermentation development

The nutrient content of the mineral medium was (g/L): $MgSO_4$, 0.53; KCl, 1.34; NaCl, 1.34; $CaCl_2$, 0067; $NaNO_3$, 3.37 and H_3PO_4, 1151 (mL/L). The concentration of trace elements (g/L) was: $FeSO_4*7H_2O$, 0.00067; $ZnSO_4*7H_2O$, 0.0020; $MnSO_4*7H_2O$, 0.0020; H_3BO_3, 0.0004; $CoCl_2.6H_2O$, 0.0002; $CuSO_4*5H_2O$, 0.0002 and $NaMoO_4*2H_2O$, 0.00013 [16]. The medium pH was adjusted to 7 using NaOH and HCl solutions (both at concentrations of 0.5 N). The carbon source was olive oil in a C/N=16 equivalent to 19.9 g/L. Flasks with sterile mineral medium were inoculated with *P. aeruginosa*. These flasks were introduced to an orbital shaker at a speed of 150 rpm and 30ºC for 5 days. During the growth phase was determined rhamnolipids concentration, surface tension and biomass.

2.2 Biomass, Surface Tension and Rhamnolipids Determinations

Biomass was measured by using a gravimetric test described in [16]. The rhamnolipids content was determined by acid hydrolysis method, and determination of sugars hydrolyzed, as described briefly below. The culture broth was centrifuged at 6000 rpm for 15 min to remove cells. The rhamnolipids were extracted from the supernatant with ether (1:1, v/v). The organic phase was evaporated to dryness and the extract was dissolved in 0.25 volumes of demineralized water with respect to the volume of ether used in the previous extraction. Subsequently, the extract was added a solution of orcinol to 0.19% in 53% H_2SO_4 in a ratio 1:9 (v/v), the samples were heated at 80ºC for 30 min. After hydrolysis at room temperature, absorption was measured to 421 nm in a spectrophotometer (Thermo Scientific, Genesys 10S UV-Vis). The concentration of rhamnolipids was calculated by comparison with a standard curve of L-rhamnose from 0 to 50 mg/L (Aldrich), expressed as rhamnose equivalents [12]. The surface tension was measured with a semiautomatic tensiometer (Cole Parmer, Tensiomat 21) by Du Nouy method described [17].

2.3 Unpurified Product Preparation and Determination of Its Rhamnolipid Content

The culture medium after the growth was sterilized at 120ºC for 15 min at 1 atm of pressure, then it was subjected to drying in an oven (RIOSSA, B-51) at 75ºC for 96 h, the powder was labeled as unpurified product UP. Rhamnolipids content was determined by one gram of UP that was suspended in 50 mL of deionized water, after centrifuge 10,000 g for 15 min cells were separated, then the rhamnolipids were precipitated by acidification of the supernatant to pH 2 with concentrated HCl for 12 h at 4ºC. The rhamnolipids were recovered by centrifugation at 10,000 g for 1 h, the pellet was suspended in 50 mL of deionized water and then added two volumes of chloroform-ethanol (2:1) in a 250 mL Erlenmeyer flask with a lid to stir for 30 min at 120 rpm on an orbital shaker [12]. The organic phase was recovered and evaporated to dryness, the residue mass was calculated gravimetrically.

2.4 Determination of CMC for Different Up in Aqueous Systems

Sets of solutions were prepared from 0.1 to 800 mg/L of rhamnolipids, a set of samples were filtered with 0.45 µm membrane, each sample was measured by surface tension and CMC was calculated. Then sets of solutions were prepared from 0.1 to 400 mg/L of rhamnolipids, at several different sets of them 4 to pH 10 with NaOH or HCl both at 0.5 N, CMC was calculated for each set. Finally, prepared sets of solutions of 0.1 to 400 mg/L of rhamnolipids, to different sets of them vary the content of Ca^{2+} or Fe^{3+} in a range from 0 to 2 mM, was calculated CMC for each set.

2.5 Washing of Soil Contaminated with TPH Using Up and Surfactants

We used a gravel-sandy soil type from the former Azcapotzalco refinery located in the city of Mexico, which was previously characterized [18] (Table 1). The procedure for washing the contaminated soil was as follows: 6 g of soil were sieved in sieve number 10, which were added to 40 mL vials with 20 mL of washing solution. The vials were shaken in a thermo-shaker at 70 rpm for 23 h at room temperature, after this period, sedimentation was allowed for 1 h. The liquid phase was separated and the soil was dried at room temperature. We determined the content of **TPH** contaminated soil by Soxhlet extraction with hexane according to the NOM-138-SEMARNAT/SS-2003. Moisture was determined for each soil sample according to Fernandez et al. [19] to report the **TPH** content in dry basis. The differences in **TPH** before and after washing were expressed as percentage of removal.

The soil was washed using different extracting products and surfactants such as mesquite, carob and guar gum water solutions at 0.125% w/w concentration. Tween 80 and SDS were also evaluated at 0.125% w/w concentration. The UP rhamnolipid was evaluated at concentrations of 0.138, 0.130, 0.065, 0.013, 0.006 y 0.001 w/w. Some characteristics of these products are showed at Table 2.

3. RESULTS AND DISCUSSION

3.1 Production of Rhamnolipids

The profile of biomass generation is shaped like a typical bacterial growth curve with a time lag of 6 h, an exponential growth that culminates at 24 h and a maintenance phase that lasted until 100 h. The maximum biomass concentration was 2.76 g/L a low value compared with 7.1 g/L obtained in mineral medium with glucose. The limitations of nitrogen [20] or phosphorus [21] in cultures with *P. aeruginosa* affect cell metabolism, reducing reactions for biomass generation, diverted to energy production and other products such as

Table 1. Gravel-sandy soil characteristics

Parameter	Value	Metal	Concentration (mg/kg)	Standard deviation
TPH (mg/Kg)	31,902	As	0	0
pH	6.3	Cd	4.09	0.272
Moisture (%)	4.5	Cu	310.25	5.2
Particles <2 mm (%)	63	Zn	165.92	10.8
Particles >2 mm (%)	37	Pb	32,206.23	1435.5
TOC (%)	0.27	Ni	8,608	798
Soluble phosphorus (ppm)	0.84	K	1,376	259.5
Total nitrogen (%)	0.04	Ca	8,029.6	88
Cation exchange capacity (cmol(+)Kg^{-1})	16.54	Mg	4,298.8	163.7
Heterotrophic bacteria CFU/g	$2.3x10^7$			

Data taken from Bonfanti [18].TOC, total organic carbon; CFU, colony forming units; cmol (+) kg-1, centimoles of cations per kilogram of soil

Table 2. Type and concentration of extracting solutions for washing gravel-sandy soil contaminated with TPH

Extracting/surfactant	Concentration (%)	CMC (mg/L)	Nature of extracting or surfactant
Tween 80	0,125	65,4	Non ionic
SDS	0,125	400	Anionic
Mesquite gum	0,125	NR[a]	Non ionic
Carob tree gum	0,125	NR[a]	Non ionic
Guar gum	0,125	NR[a]	Non ionic
Rhamnolipid	0.138, 0.130, 0.065, 0.013, 0.006 y 0.001	175.8[b*]	Non ionic

NR, not reported; a, surfactant extracted from plants; b, microbial surfactant
Source: Torres et al. [22]; except, determined in this work*

7,10-dihydroxy-8-(E)-octadecanoic acid [23], phenazyne pigments, exotoxins A, phospholipase C [24], 3-(3-hydroxyalkanoyloxy) alcanoic acids (HAAs), polyhydroxyalkanoates (PHAs), intracellular structures known as body-R [25] and rhamnolipids [20,8]. The evolution in the generation of rhamnolipids indicates that at the beginning of fermentation in the culture medium was about 36 mg/L of rhamnolipids perhaps because the inoculum was not washed using the mineral medium. After 24 h of production was decreased by 19.5 mg/L biosurfactants, attributable to the consumption of rhamnolipids as carbon source (Fig. 1). It has been reported the possibility of preferential consumption instead of rhamnolipids carbon source of the mineral medium [8]. Was detected a maximum of 100 mg/L of rhamnolipids at 100 h. The level production increased as the biomass became stationary growth phase; these observations are consistent with the results obtained by Benincasa et al. [26]. The surface tension profile began with a value close to that of water (72 mN/m) decreasing progressively to 33.9 mN/m at 24 h, which although it was not a high surface tension remained almost constant until the end of fermentation. The surface tension is related to the production of biosurfactants before the CMC of 15.5 mg/L, for this case. The determination of surface tension could be used to monitor the production of rhamnolipids without the need to quantify (Fig. 1). Finally, the initial pH of the culture medium was 7.02 and the end of fermentation increased to 9.19 (Fig. 1). Lee et al. [27] evaluated the growth conditions in a reactor fueled finding that at 25ºC and pH 7.0 to provide the best conditions to produce rhamnolipids, we can say that the pH used in this work was the most appropriate.

Most of the reports coincide with the fact that various strains *P. aeruginosa* are capable of producing from 71 to 12,470 mg/L. The carbon source used for their production can be a key element and generate products both water soluble or insoluble. For example *P. aeruginosa* PEER02 produced between 700 and 800 mg/L of rhamnolipids using glucose, while using soybean oil produced between 1,700 and 1,900 mg/L [12]. In the work of Guerra-Santos et al. [20] and Reiling et al. [28] rhamnolipid concentrations obtained were between 1,250 and 1,500 mg/L with the strain *P. aeruginosa* DSM2659 and glucose. In cultures with *P. aeruginosa* IFO 3924 and ethanol as carbon source, the amount of rhamnolipids detected exceeded 3,000 mg/L [29] its detection was high due to the purification process in thin layer chromatography. In general, when using soluble substrates such as n-alkanes and vegetable oils, the rhamnolipids concentration is an order of magnitude higher [27,30] and [31]. The biosurfactants reduce the surface tension in a range from 37.3 to 25.4 mN/m at with variable concentrations of rhamnolipids, perhaps because their quality is different in each case of production.. The final content of rhamnolipids obtained in this study was rather low, but their surface properties were very interesting. They were able to decrease the water surface tension from about 72 to 33 mN/m. This could be associated with proportions of mono-and di-rhamnolipids [26,32] and [33].

3.2 Non-purified Product Preparation and Determination of the Rhamnolipids Content

The UP consists of rhamnolipids, cell debris, residues of the carbon source of olive oil, proteins, salts, and sub-products from metabolism of the bacterium *P. aeruginosa* [34]. The quantification of rhamnolipids in the UP was of 1.19% (w/w), which is a low value, if compared with a product currently marketed by the company Jeneil biosurfactants which contains approximately 15% of rhamnolipids [9].

The separation and purification costs are 60% of the cost of production. Some authors have recommended using raw or unpurified biosurfactants for environmental applications [35]. Park et al. [36] recommend using crude preparations, provided they maintain the desired properties. In the present study, we attempted to influence the reduction of production costs, using no-purified rhamnolipids in remediation of contaminated soils. But as has been suggested by Park et al. [36] is crucial to study the characteristics of unpurified biosurfactants.

3.3 Determination of CMC for Different UP Rhamnolipids in Aqueous Systems

Bio-surfactants can be characterized based on their properties, such as the CMC it is specific to each surfactant and aqueous system conditions where they are dissolved [37]. In this part of the paper first assessed the effect of UP filtered and unfiltered in the profile of surface tension and the

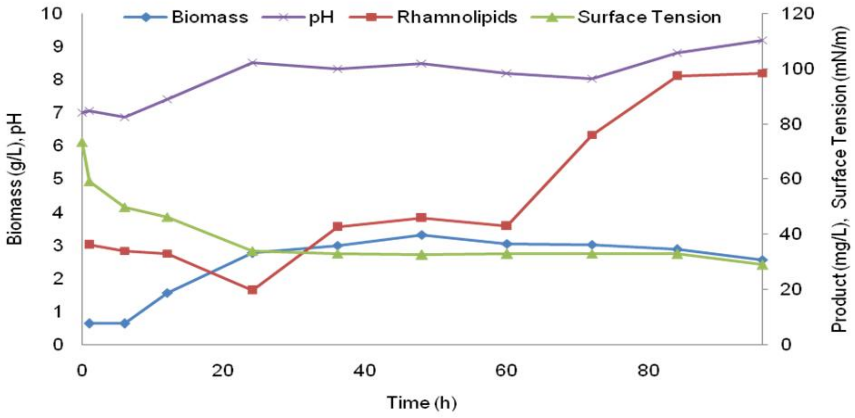

Fig. 1. Rhamnolipids production using olive oil in mineral medium. The error bars represent the standard deviation of triplicate samples

CMC. A rhamnolipids concentration around 10 mg/L promote surface tension close to 72 mN/m and with increasing rhamnolipids concentration up to 100 mg/L decreased to 45.7 mN/m for UP-filtered and 40.5 mN/m for the UP-unfiltered (Fig. 2). The values of CMCs were 175.7 and 25.7 mg/L for both unfiltered and filtered UP, respectively. The low value of the unfiltered UP CMC may indicate the presence of different metabolites besides rhamnolipids; fewer molecules are needed to form micelles. These results are similar to those by Zhu et al. [38] who reported a CMC of 63.3 mg/L for rhamnolipids in culture medium free of cells and 160 mg/L rhamnolipids extract in distilled water. From the above, we can say that the UP solution in water seems to have greater potential as compared to a surfactant solution containing rhamnolipids with greater purity. Perhaps because of the presence of some proteins, glycolipids or polysaccharides that could help to reduce surface tension and promote the formation of micelles at low concentrations [38].

Plotting the logarithm of the concentration of rhamnolipids was observed that at low concentrations the differences between the profiles of surface tension were higher (data not shown). For example, at concentrations near the CMC of the unfiltered and filtered UP, 215 mg/L and 53 mg/L, respectively, had greater decreases in the average surface tension was 17 mN/m (Fig. 2). The plateaus in the surface tension constant, prior to the CMC of the system, given the increase in the concentration of rhamnolipids propose changes in the organization of biosurfactants. For example, for UP unfiltered concentrations between 1 and 6 mg/L there is a decrease in surface tension followed by a constant plateau between 6.5 and 13.5 mg/L, which does not belong to the CMC of the solution (25.7 mg/L). One possible explanation for these variations have been reported in studies of Raza et al. [39] who determined the surface tension profile for pure surfactant solutions in the results found before the CMC decay of the solution, which were attributed to the micellization of different types of rhamnolipids, such as a decline in the first profile surface tension at 10 mg/L corresponded to di-rhamnolipid and a second decay of 40 mg/L to mixtures of rhamnolipids.

CMCs values data from literature were compiled for di-rhamnolipids, mono-rhamnolipids and mixtures. On average, for di-rhamnolipids the CMC value was 84.5 mg/L, 50.4 mg/L for mono-rhamnolipids and 43.14 for the mixture of rhamnolipids, which could mean that: $CMC_{di\text{-}rhamnolipids} < CMC_{mono\text{-}rhamnolipids} < CMC_{Mix}$, this can be used to explain the results of this study. In the surface tension profile of UP unfiltered can be see a decay between the concentrations from 1 to 10 mg/L and constant value up to 13.5 mg/L, may be due the micellization of mono or di-rhamnolipids. For the surface tension profile filtering UP, there is a decline from 10 to 1000 mg/L which could be corresponds to the CMC of the system (175.7 mg/L) and perhaps is associated with the micellization of di-rhamnolipids, prior to CMCs this value could be attributed to $CMC_{Mono\text{-}rhamnolipids}$ or rhamnolipids-$CMC_{Mix.}$

For the remainder of the aqueous systems studied only shows the value of the CMC in

Table 3, the CMC at pH 4 and 5 were similar (40 and 69.5 mg/L, respectively). It has been reported that the CMC for systems with pH 4 or 5 is low because rhamnolipids induce them to behave as nonionic surfactants and causes at low concentrations (low CMCs) to pass from the air-solution interface to form micelles [39]. Similarly, there are similarities in the CMCs at pH 6, 8 and 10 (128, 113 and 102 mg/L, respectively). The high values of CMCs indicate that micelle formation requires high concentrations of rhamnolipids. Lovaglio et al. [40] reported pKa for rhamnolipids of 5.6 and that above this value, the anionic form of surfactants prevails and their functional groups are protected by Na^{2+} ions of the electrical double layer in the presence of NaCl. The carboxyl groups of rhamnolipids molecules are sensitive to pH, a pH greater than 5 dissociate into its conjugate base as carboxylic anions, while at pH less than or equal to 5 is protonated and exhibit nonionic behavior in aqueous solution [39]. According to Aranda et al. [41] at pH 7.4, 98.4% di-rhamnolipids molecules carry a negative charge, while at pH 4.0, 97.5% are neutral.

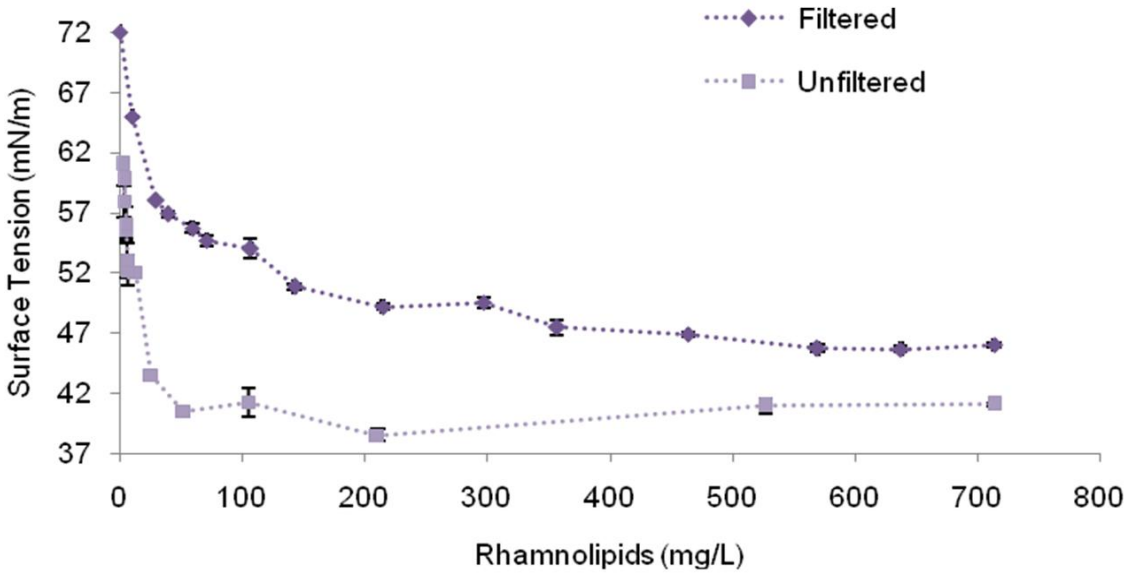

Fig. 2. Effect of filtration process of UP rhamnolipids on surface tension profile, error bars represent the standard deviation of three replicates

Table 3. CMC value for UP rhamnolipids different aqueous systems

Condition evaluated	Level	CMC (mg/L)
Non-filtered	-	25,7
Filtered	0.45 µm membrane	175.8
pH	4.0	40.0
pH	5.0	69.5
pH	6.0	128.0
pH	8.0	113,0
pH	10.0	102.5
Ca^{2+}	0.5 mM	35,2
Ca^{2+}	1 mM	40.8
Ca^{2+}	2 mM	40.8
Fe^{3+}	0.5 mM	41,2
Fe^{3+}	1 mM	37.7
Fe^{3+}	2 mM	41.0

The most predominant cations in contaminated soils are Ca^{2+}, Mg^{2+}, Na^{2+} and K^{+}. While Fe^{3+} is one of the 20 metals of greatest concern to the USEPA for its potential health risk [42]. Therefore, we evaluated the effect of ionic strength with Ca^{2+} or Fe^{3+} in the CMC of rhamnolipid UP solutions in water. There was a low variation in the values of the CMCs, these fell in the range of 35.2 to 41.0 mg/L, showed no significant variation. In general, we can say that the presence of ions affects the CMC decrease, though not have a significant effect when the concentration of ions increases from 0.5 to 2 mM under the conditions studied.

The CMC values found in the present study are consistent with those reported in literature for pure rhamnolipids in the range from 50 to 200 mg/L [41]. The CMCs presented can serve as a guide for using the rhamnolipids in a specific problem of contamination.

3.4 Washing of Soil Contaminated with TPH Using Rhamnolipids

The water in this experiment (See Fig. 3.) had a high removal percentage (50.13%) compared with other values reported in literature [11,22,43] and [44]. Water can extract semi- soluble fractions of **TPH** in water as the surface tension of aqueous medium decreases promoting solubilization of some petroleum compounds. Torres et al. [6] used sea water to wash soil contaminated with hydrocarbons from three different crude oils and found that sea water can be removed about 20% of **TPH**, the salt content in sea water surface tension decreased to 56 mN/m. In the present experiments demineralized water was used, but there is a possibility that the salts in the soil are hydrated and passed to the aqueous phase by decreasing the surface tension [45]. The washing with guar gum did not draw oil from the ground, while mesquite and carob removed only 23 and 32%, respectively **TPH**. The low capacity to remove hydrocarbons from natural gums is associated with high molecular weights which range from $2.5x10^5$ to $6x10^6$ UMAS. Breure et al. [46] reported that high molecular weight surfactants could hardly spread and adsorbed in small pores of soil particles, reducing the possibility of solubilizing hydrophobic compounds. Additionally, the viscosity of natural gums in water affects the removal of HTP. When washed using the 0.130% rhamnolipids could remove 15.8% of **TPH**, this value is the second lowest compared to water, probably due to soil pH (6.3), not less the pKa of rhamnolipids (5.6), promotes the biosurfactants are in anionic form in the presence of high content of Ca^{2+} and Mg^{2+}, the biosurfactants precipitate. Billingsley et al. [47] studied the removal of PCBs by washing a sandy loam soil using LSS Nansa 38/AS a 1% anionic surfactant, they consider that the content of Ca^{2+} at concentrations of 4620 mg/g was sufficient to decrease the capacity for removal of PCBs, as a result of precipitation of surfactants. Kuyukina et al. [43] have reported that non-ionic surfactants in nature can be absorbed into soil particles, especially clay minerals and organic matter, limiting the application of surfactants on the recovery of crude oil. The best agents to extract hydrocarbons were Tween 80 and 0.125% SDS either with removals above the water reaches (75 and 69%, respectively). Although SDS and Tween 80 have been widely used in soil washing [4,6,10] and [22] has discussed its toxicity to soil organisms or other organisms [48] and low biodegradability [7]. While the biosurfactants have proven to be biodegradable and have low levels of toxicity. The review of Rouse et al. [49] has compiled the information that shows the toxic effect of ionic surfactants and to a lesser extent also of the nonionic surfactants chemical synthesis.

Additionally, it was investigated the effect of the concentration of rhamnolipids on the removal of **TPH** from a sandy-gravel soil. It was observed that at concentrations less than or equal to 0.074 times the CMC **TPH** removals were high (79%), although an increase in rhamnolipids concentration up to 7.4 times the CMC reduced the removal of **TPH** (16%) and finally a change in removal trend was observed at 7.9 times the CMC where it increased to 40.8% (Fig. 4).

Urum and Pekdemier [9] have described two mechanisms that explain the removal of soil hydrophobic compounds when using surfactants, mobilization and solubilization, these mechanisms are a function of the concentration and nature of the surfactants. It has been reported that the mechanism of mobilization, occurs at concentrations below the CMC and is associated with the reduction of surface tension, interfacial tension, the force of capillarity, wettability and contact angle [9]. One could hypothesize that at concentrations less than or equal to 0.37 times the CMC mobilization mechanism is responsible in promoting the removal of **TPH**. However, it should be noted that the CMC in aqueous solution is equal to the resulting CMC after contacting the surfactants

with half adsorbent. Urum and Pekdemir [9], reported that the CMC in aqueous solution to rhamnolipids was 0.02%, while the CMC for a rhamnolipids solution in contact with soil was calculated to be 0.008% adsorption was 75%. The soil used for Urum and Pekdemir [9], has features similar to the gravel-sandy soil and pH of 7.43. Perhaps with the increased concentration of rhamnolipids, the decrease in the removal was due to biosurfactants absorption in the ground.

TPH removal varied regarding the concentration of rhamnolipids up to 7.9 times the CMC of UP rhamnolipids in water (Fig. 4). Lai et al. [11] reported that the removal was directly proportional to the concentration of rhamnolipids in the range of 16.12 to 64.51 times the value of the CMC (0.0031%) did not evaluate concentrations below 1 times the CMC, as a performed in this study. Biosurfactants probably will be absorbing the particles of gravel-sandy soil in two ways: 1) by precipitation due to the presence of metal ions or 2) by the negatively charged surfactant [43] this in turn have an effect on the ability of association of hydrophobic compounds in soil, as observed by Abu-Zreig et al. [50] who found that the use of non-ionic surfactants reduces the contact angle of hydrophobic compounds, while it increases an anionic surfactant, probably because the orientation of surfactants adsorbed to the soil is different. Maybe the rhamnolipids at low concentration are as nonionic surfactants and due to the presence of metal ions, the contact angle is increased, promoting the mobilization mechanism of hydrocarbons. By increasing rhamnolipids concentration the biosurfactants adsorbed to soil due to attraction between molecules (hydrophobic Interactions) to form aggregates (hemimicells), which prevents **TPH** strongly adsorbed leaving the soil particles. There is a limit of adsorption of rhamnolipids to soil; it is determined by its affinity to the hemimicells, by the soil adsorption sites and by the decrease of ion of Ca^{2+} and Mg^{2+}.

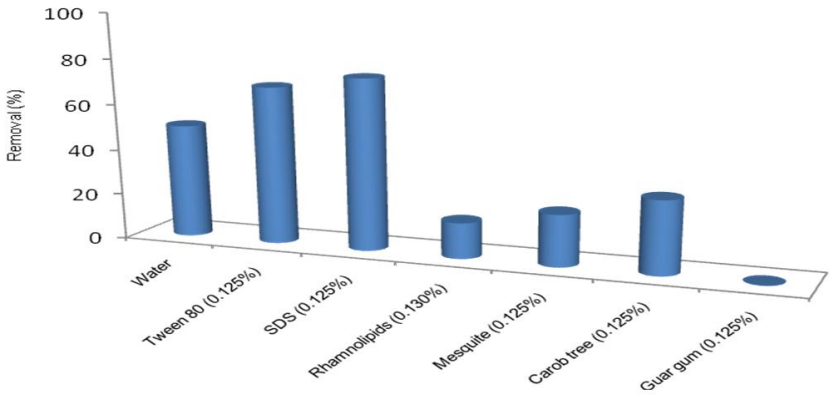

Fig. 3. Hydrocarbon removal rates obtained with rhamnolipids, Tween 80, SDS and natural gums at concentrations of 0.125%

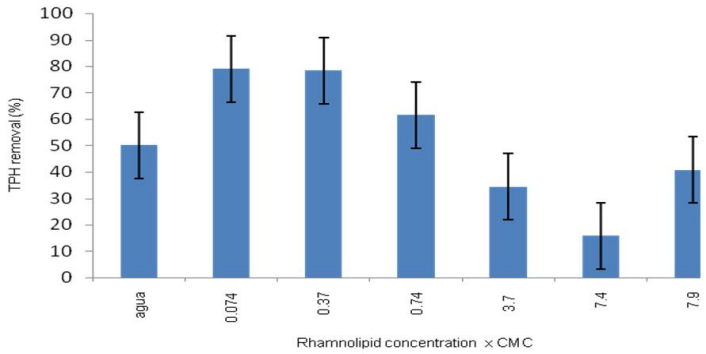

Fig. 4. Effect of rhamnolipids concentration on the removal of TPH-sandy gravel soil. The error bars represent the variation of duplicate samples

4. CONCLUSIONS

According to data obtained and in accordance with the objectives proposed in this research have led to the following conclusions. It was possible to produce rhamnolipid using olive oil as carbon source and strain of *P. aeruginosa* ATCC 9027 to levels of 100 mg/L. It was feasible to produce a powder containing 1.19% of rhamnolipids, although the concentration was low it was not very different from a product that currently markets (15%) and their properties as surfactants are very similar. The UP unpurified had better properties as a surfactant than the UP purified. The pH affects the CMC of the rhamnolipids in a way that promotes their behavior as an ionic surfactant or nonionic surfactant. The ionic strength with Ca^{2+} and Fe^{3+} affects the CMC of rhamnolipids so that the decreases in the range of 35 to 41 mg/L in the presence of 0.5 to 2 mM of metals, all values are similar to those reported in literature for pure rhamnolipids. The UP rhamnolipids were employed for washing soil contaminated with 6,500 mg/kg increased **TPH** removal at low concentrations and to be as effective as chemical surfactants. **TPH** removal observed was about 80% for CMC x0.074 concentration.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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