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Modification of wettability and reduction of interfacial tension mechanisms involved in the release and enhanced biodegradation of heavy oil by a biosurfactant

Modificación de la mojabilidad y reducción de tensión interfacial involucrados en la liberación y biodegradación mejorada de un crudo pesado por un biosurfactante

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Abstract

Bioremediation of hydrocarbon contaminated soils requires releasing the oil from the porous matrix to render it bioavailable. This work aims at evaluating a biosurfactant produced by a *Bacillus subtilis* strain to improve the release and biodegradation of heavy crude oil contaminating limestone sand. The biosurfactant effect on biodegradation of crude oil was elucidated, using a *Rhodococcus sp*. strain. The CO_2 production rate was used as indicator of biological activity that improved by 32.84%. Moreover, at least 67% of the $>C_{20}$ fraction was degraded in the systems with biosurfactant, reducing aromatic and resin fractions (12.5% and 2%, respectively), whereas without biosurfactant only 37% of the $> C_{20}$ fraction was removed compared with the null reduction in aromatics and resin. The decrease in surface and interfacial tension, as well as the modification to wettability caused by biosurfactant activity, were the main mechanisms promoting the release of heavy oil, thus making it bioavailable.

Keywords: biosurfactant, heavy oil release, wettability modification, bioavailability, biodegradation.

Resumen

La biorremediación de suelos contaminados con hidrocarburos requiere su liberación de la matriz porosa para hacerlos biodisponibles. Este trabajo tiene como objetivo evaluar un biosurfactante producido por una cepa de *Bacillus subtilis* para mejorar la liberación y biodegradación de crudo pesado contaminando arena caliza. El efecto del biosurfactante sobre la biodegradación del crudo fue dilucidado usando una cepa de *Rhodococcus sp*. La velocidad de producción de CO_2, utilizada como indicador de la actividad biológica, fue mejorada en un 32.84%. Al menos el 67% de la fracción$> C_{20}$ se degradó en los sistemas con biosurfactante, reduciendo las fracciones aromáticas y de resina (12.5% y 2% respectivamente), mientras que sin biosurfactante solo se eliminó el 37% de la fracción $> C_{20}$ y la reducción de aromáticos y resinas fue nula. La disminución de la tensión superficial e interfacial, así como la modificación de la mojabilidad causada por la actividad biosurfactante, fueron los principales mecanismos que promueven la liberación de aceite pesado haciéndolo biodisponible.

Palabras clave: biosurfactante, liberación de aceite, modificación de la mojabilidad, biodisponibilidad, biodegradación.

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1 Introduction

Nowadays, soils contamination with hydrocarbons is an important environmental problem. Bioremediation is one of the most used soil-recovery techniques as it represents a green, low cost, attractive alternative (Montagnolli *et al*., 2015; Maliki *et al*., 2021). Bioremediation takes advantage of the microorganism metabolic capacities to either, diminish oil toxicity in soils, decrease its concentration or even exert complete mineralization (Silva *et al*., 2014; Varjani, 2017). Bioremediation displays various advantages over physicochemical techniques, although at times it is limited by the scant solubility of oil in water, high hydrophobicity and strong sorption to soil constituents (Liu *et al*., 2017). Other limiting factors include, in the case of heavy crude oils, their low or null dispersion in water due to their high viscosities and cohesive forces (Ron and Rosenberg, 2002; Abioye *et al*., 2010). All the above mentioned, contribute to poor oil bioavailability for degrading microorganisms, which curtails ensuing biodegradation processes (Li *et al*., 2015). Bioavailability can be improved using surface active agents such as biosurfactants that accelerate biodegradation processes, duly increasing biodegradation rates (Al-Bahry *et al*., 2013; Souza *et al*., 2014).

Biosurfactants are amphiphilic compounds with hydrophobic and hydrophilic portions that allow their insertion at the interface between fluids with different polarity degrees, concomitantly reducing both surface tension (ST) and interfacial tension (IFT) and promoting favorable changes in oil wettability over rock minerals and soil surfaces (Karlapudi *et al*., 2018). Consequently, repulsive forces between interfaces decrease allowing matter exchange between them (Kapellos, 2017). Due to these properties, biosurfactants are capable of releasing, solubilizing and/or dispersing hydrocarbon compounds in the aqueous phase (Wang and Mulligan, 2004; Mao *et al*., 2015).

Biosurfactants are produced from some species of bacteria, yeasts and fungi (Karlapudi *et al*., 2018); because of their biological nature, they have several advantages over chemical surfactants: are obtained from renewable sources (Silva *et al*., 2014), are biocompatible, have wide chemical and structural diversity, are stable to extreme environmental conditions and have low critical micellar concentrations. Because of their low toxicity and biodegradability (Deng *et al*., 2016; Mani *et al*., 2016), they are considered an ecofriendly alternative; currently, they have applications in contaminated water and soil bioremediation processes. Among the main biosurfactants producing microorganisms there are some species of genus *Bacillus* (Chen *et al*., 2015; Borah and Yadav, 2017; Karlapudi *et al*., 2018), *Pseudomonas* (Varjani, 2017) and *Rhodococcus* (Pi *et al*., 2017). The main concerns in biosurfactants production are low yields (Bouassida *et al*., 2018) and high production, separation and purification costs (Martinez-Trujillo *et al*., 2015), so they are preferably used raw for environmental applications, thus yielding an attractive low-cost alternative.

Biosurfactants have been used successfully in biodegradation of motor oils (Karlapudi *et al*., 2018), medium and light oils (Pi *et al*., 2017), petroleum fractions, diesel, kerosene and polyaromatic hydrocarbons (Bezza and Chirwa, 2017). However, reports on biodegradation of heavy and very heavy crude oils are scarce in literature (Tao *et al*., 2017). Biodegradation of the latter is difficult due to their inherent characteristics, such as high viscosity and high molecular weight compounds content, whose degradation depends on the proportion in which they are present (Montagnolli *et al*., 2015). Elucidation of the mechanisms involved in heavy oil release is scarcely approached in the literature and most works studying biosurfactants used in oil biodegradation apply surface and hydrocarbon models (Sharma and Pandey, 2020). Thus, studies with rocks and heavy crude oils are critically needed to understand the release mechanisms of this variety of oils in biodegradation systems.

The objective of this study was to assess the activity a biosurfactant produced from a strain of *Bacillus subtilis*, to enhance biodegradation of heavy crude impregnated in limestone sand and to elucidate the main mechanisms involved.

2 Materials and methods

2.1 Oil, rocks and sand

A Mexican heavy crude oil (14.85 °API) was used, density 0.964 kg·m^{-3} and viscosity 4236 mPa·s (both at 20 °C). The composition of this oil, by SARA analyses, was 16% saturated, 31% aromatics, 36% resins and 17% asphaltene compounds. For wettability determination, limestone rock plates (1 x 2 x 0.25

cm^3) were used. The sand was obtained from ground limestone rock, sifted (20/30 meshes) and washed with distilled water and dried at 50 °C for 72h.

2.2 *Biosurfactant production*

The biosurfactant production was carried out in a 3 L Applikon bioreactor with 1.5 L operating volume. The composition in gL^{-1} of culture mineral medium was: $NaNO_3$, 2; KCl, 1.0; $CaCl_2$, 0.01; $MgSO_4{\cdot}7H_2O$, 0.5; $MnSO_4{\cdot}H_2O$, 5×10^{-5}; K_2HPO_4, 1.0 and KH_2PO_4, 0.5. Additionally, 15 mL of trace element solution were added (in gL^{-1} $FeCl_2$, 0.116; H_3BO_3, 0.232; $CoCl_2{\cdot}6H_2O$, 0.41; $MnSO_4{\cdot}H_2O$ 0.008; $ZnSO_4$ 0.174). As carbon source, 10 gL^{-1} of unrefined sugar (muscovado) were used. The bioreactor was inoculated with 5 vol % of a 24-hour-old culture of a strain *Bacillus subtilis* CDBB-B-1015 from the CINVESTAV Microorganisms Collection. Production conditions were 30 °C, 180 rpm and aeration rate 0.7 vol/vol per minute. The maximum growth rate was obtained after fitting the biomass data to modified Gompertz equation (equation 1) (Heryani and Putra, 2017), using the Excel Solver function.

$$B(t) = B_{\max} \exp\left[-\exp\left(\left(\frac{\mu_{\max} * e}{B_{\max}}\right) * (\lambda - t) + 1\right)\right] \quad (1)$$

Where $B(t)$ is the time dependent estimated biomass ($g{\cdot}L^{-1}$), $B_{\max}$ is the potential maximum biomass concentration ($g{\cdot}L^{-1}$), $\mu_{\max}$ is the maximum growth rate ($g{\cdot}L^{-1}{\cdot}h^{-1}$) and λ is the time to exponential biomass formation (h).

Biosurfactant production was followed by measuring ST of cell free culture broth and foam (centrifuged at 10000 rpm for 10 minutes). For collecting the foam, the bioreactor was equipped with a tube connected to the air vent and then to a reservoir.

2.3 *Biosurfactant characterization*

The collected collapsed foam was centrifuged at 10000 rpm for 15 minutes in order to separate biomass. The cell-free supernatant was set to pH 2 using HCl 2.N, refrigerated overnight and extracted with methanol (Ghojavad, *et al*., 2008); the dried-off product was characterized by Fourier Transform Infrared Spectroscopy (FTIR) in order to elucidate the main functional groups, present in the raw biosurfactant. The FTIR spectra were obtained with a Thermo Scientific Nicolet FT-IR spectrometer operating with total attenuated reflection and 2 cm^{-1} scan rate in the 399 to 4000 cm^{-1} range.

Additionally, the biosurfactant was analyzed with an HPLC Agilent 1200 equipment fitted with a Zorbax SB-C18 column, coupled to a mass spectrometer and MALDI-TOF (Matrix-Assisted Laser Desorption Ionization-Time of Flight), under the following conditions: flow: 20μl min^{-1}, isocratic method, 80/20% acetonitrile /H_2O, polarity: positive; mass range: 900-1200 amu, 1mg mL^{-1}.

2.4 *Biosurfactant activity at different conditions of pH, temperature and salinity*

The activity criterion was the biosurfactant capacity for lowering ST under different environmental conditions. The reference condition to estimate the activity of the biosurfactant was the surface tension (at 25 °C) of cell free collapsed foam with salt absence and pH 6.5. Salinity stability was evaluated at NaCl concentrations 0, 2, 4, 6, 8 and 10% w/v. The thermostability was followed by incubating the biosurfactant at 4, 20, 40, 60 and 80 °C for 24 h. The effect of pH on biosurfactant activity was tested in the 5 to 9 range.

2.5 *Determination of the mechanisms involved in oil release and main environmental factors that affect them*

Interfacial tension and contact angle (CA) were evaluated to determine the main mechanisms involved in heavy oil release. To evaluate the effect of environmental factors on IFT and CA, a four-factor orthogonal Taguchi L_9 with three-levels experimental design (DOE) was performed; factors were: temperature, salinity, sand-oil contact time (aging) and SO_4^{-2} concentration (Table 1). The experimental set up comprised glass columns with 13 g of limestone sand impregnated with heavy crude oil at 300,000 ppm, 10 mL of biosurfactant (autoclaved collapsed foam, ST 27.78 mN m^{-1} at 25 °C) was added to each column, pH was set to 6.5 and then incubated for seven days. After the incubation period, the amount of oil released from the sand was measured, as well as the supernatant ST and IFT. The controls were run with distilled water instead of biosurfactant.

Table 1. Taguchi experimental design and results for biosurfactant activity evaluation: oil release, surface tension (ST), interfacial tension (IFT) and contact angle (CA).

	Factors/Levels										
Run	Aging (days)	Temperature (°C)	Salinity (%)	SO_4^{-2} (mol)	Oil release %	ST (mN·m^{-1}) Treatment	ST (mN·m^{-1}) Control	IFT (mN·m-1) Treatment	IFT (mN·m-1) Control	CA Treatment	CA Control
1	1	30	0	0	7.2 ± 0.8	27.7±0.1	70.9±0.7	1.5±0.2	12.6±0.2	51.9°	112.1°
2	1	40	5	0.01	41.3 ± 1.8	28.1±0.2	70.3±0.8	3.4±0.3	16.2±0.4	44.1°	127.1°
3	**1**	**50**	**10**	**0.02**	**83.4 ± 4.7**	**27.2±0.1**	**69.9±0.5**	**2.7±0.2**	**12.3±0.6**	**46.9°**	**113.4°**
4	4	30	5	0.02	30.7 ± 1.8	29.4±0.2	70.5±1.7	2.1±0.1	13.4±0.2	37.6°	127.2°
5	4	40	10	0	47.3 ± 2.8	28.9±0.1	69.9±2.2	3.4±0.1	13.1±0.3	60.1°	122.5°
6	4	50	0	0.01	45.3 ± 1.8	26.5±0.1	69.3±0.3	2.31±0.1	18.7±1.9	47.4°	130.2°
7	5	30	10	0.01	44.7 ± 2.8	30.3±0.2	71.6±0.8	2.5±0.1	11.4±0.1	43.8°	127.0°
8	5	40	0	0.02	30.7 ± 1.8	27.3±0.1	69.1±0.7	2.6±0.2	17.5±1.0	45.3°	127.0°
9	5	50	5	0	75.1 ± 4.2	27.4±0.1	69.2±0.4	1.2±0.1	13.8±0.9	46.9°	117.0°

Table 2. Experimental treatments for biodegradation of heavy crude oil in limestone sand.

Treatment	Sand (g)	Heavy crude oil (ppm)	Culture broth (mL)	Inoculum (mL)	Biosurfactant pretreatment
Abiotic control (AB)	10	50,000	50	0	yes
Treatment without biosurfactant (SL)	10	50,000	45	5	no
Treatment with biosurfactant (CL)	10	50,000	45	5	yes

Wettability was determined as follows: limestone plates were impregnated with crude oil for 72 h at 50 °C then the CA was evaluated. Subsequently, plates were immersed in test liquid mixes (from DOE) and incubated for seven days at the temperatures indicated in Table 1. After the incubation time, the CA was measured again in order to determinate wettability changes.

2.6 *Biodegradation of heavy crude oil*

Biodegradation assays were performed in 250 mL flasks fitted with *Mininert* valves for gas sampling. A strain of *Rhodococcus sp* was used as biodegrading microorganism. The inoculum was a culture of this strain aged 48 h (OD 1.24 at 620 nm, 10^6 CFU). A culture mineral medium based on reports by Karpenko *et al*. (2006), was used, setting pH to 6.7. The sand was impregnated with heavy crude oil at 50,000 ppm concentration and salinity (as NaCl) was set to 3.5%. Table 2 summarizes the experimental treatments; culture conditions were 30 °C and 150 rpm agitation rate.

Prior to inoculation, a pretreatment with raw biosurfactant was applied to the treatment identified as CL, that consisted of mixing oil-impregnated sand and 20 mL of autoclaved collapsed foam (ST 27.78±0.01 mN m^{-1}), incubated at 40±1 °C and 150 rpm for 72 h; biosurfactant foam was then removed (to avoid *Rhodococcus* growth inhibition and biosurfactant to be used as carbon source) to proceed with biodegradation.

Biodegradation was monitored by respirometry, 1 mL gas phase sample was taken periodically to determine CO_2 production, as indicator of the heavy oil components mineralization. After sampling, the flasks were aerated 1h under sterile conditions.

2.7 *Analytical techniques*

Surface tension was measured with a KRÜSS K100 tensiometer employing the Wilhelmy plate method. IFT was determined by the drop shape method with a KRÜSS DSA100 Drop Shape Analyzer; for ST and IFT determinations, temperature was set as required by the DOE. CA was evaluated at room temperature by the captive drop method using the DSA100 Drop Shape Analyzer.

SARA fractions (saturated, aromatic and resins) were determined according with method EPA 8015. The range of C-atom hydrocarbons in crude oil and residual oils was conducted according with standard methods ASTM D7169-05 for the boiling point

distribution.

Gas chromatography allowed CO_2 determination with a GowMac II Chromatograph running 65 mL·min^{-1} of helium as mobile phase and using a CTR column. The conditions were: column at room temperature; injector temperature 45 °C; detector temperature 100 °C; and 125 V detector potential. The cumulative CO_2 production plots were fitted with modified Gompertz equation (equation 2) (Castorena-Cortés *et al*., 2009).

$$P_{CO_2}(t) = P * \exp\left[-\exp\left(\left(\frac{r * e}{P}\right) * (\lambda - t) + 1\right)\right] \quad (2)$$

Where P_{CO_2}(t) is CO_2 cumulative production at time t (mg·g$^{-1}_{oil}$); P is maximum CO_2 production (mg·g$^{-1}_{oil}$); r is the CO_2 production rate (mg·g$^{-1}_{oil}$·h^{-1}) and λ is the lag phase duration (h). P and λ are calculated with iterative method using the Excel Solver function.

2.8 *Statistical analyses*

All experimental determinations were performed in triplicate; the mean and standard deviation were determined, and single tail variance analyzes were performed. For the mechanisms involved in oil release and main environmental factors that affect biosurfactant, contrast tests and means groupings were performed by Tukey test of honest significant difference (HSD) (Bezza and Chirwa, 2017). An α of 0.05 was used in all cases.

3 Results and discussion

3.1 *Biosurfactant Production*

Culture broth initial ST was 63.25 mN m^{-1} (Figure 1); foam formation began at 17 h, with ST 38.12 mN m^{-1}, from that moment onwards, ST determination was performed in culture broth and the collected collapsed foam. Biosurfactants separation and concentration in foam has been reported previously (Al-Bahry *et al*., 2013) and has been used as a primary recovery strategy and as a method to prevent growth inhibition by accumulation of this bioproduct (Chen at al., 2015; De Andrade *et al*., 2016). The lowest obtained ST, 27.89 mN m^{-1} remained constant until the end of the experiment. Since ST decreased as biomass concentration increased, the biosurfactant was product of primary metabolism as reported in the available literature (De Andrade and Pastore, 2016; Vaz *et al*., 2012; Houbron *et al*., 2021).

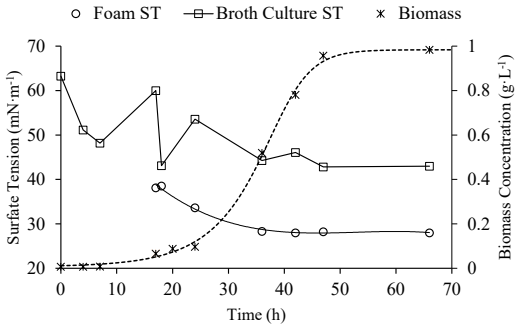

Figure 1. Growth kinetics and surface tension (ST) decrease during biosurfactant production.

The maximum growth rate μ_{max} was 0.043 g·L^{-1}·h^{-1}. Final biosurfactant concentration and yield were 1.047 g L^{-1} and 0.109 g g$^{-1}_{sugar}$, respectively. Biomass yield, 0.98 g L^{-1}, found in this work is similar to those reported in available literature (De França *et al*., 2015; Pereira *et al*., 2013). The lowest ST achieved in this work is comparable with that found in other reports: 30.9 mN m^{-1} by Montagnoli *et al*. (2015), 30.1 mN m^{-1} by Vaz *et al*. (2012), using similar carbon sources. The collapsed foam was dried, and its CMC was 0.684 g L^{-1}.

3.2 *Biosurfactant characterization*

The FTIR spectrum of dried foam showed the presence of aliphatic hydrocarbons and nitrogen compounds (Figure 2). The most relevant absorption bands were: 3320 cm^{-1} due to amino groups (NH) stretching attributable to peptide residues (Pereira *et al*., 2013); 1650 cm^{-1} by CO-N bond stretching; 590 cm^{-1} due to N-H bond deformation mode combined with stretching of C-N bond, 250 cm^{-1} due to deformation and vibration of C-O (Deng *et al*., 2016); 2930 cm^{-1}, 2850 cm^{-1} and 1370 cm^{-1} corresponding to radicals -CH_3, -CH_2 and -CH respectively; 1750 cm^{-1} due to carbonyl groups (Shao *et al*., 2015). These data revealed lipopeptide functional groups and demonstrated that lipopeptidic compounds were present in the collected collapsed foam. The spectrum obtained was very similar to those reported by Pereira *et al*. (2013), Shao *et al*. (2015), and Sousa *et al*. (2014); these authors worked with *B. subtilis* and concluded that the biosurfactant obtained was a surfactin like lipopeptide. Surfactin consists of 7 amino acids, but there may be variations in the seventh amino acid, since in some cases is leucine/isoleucine

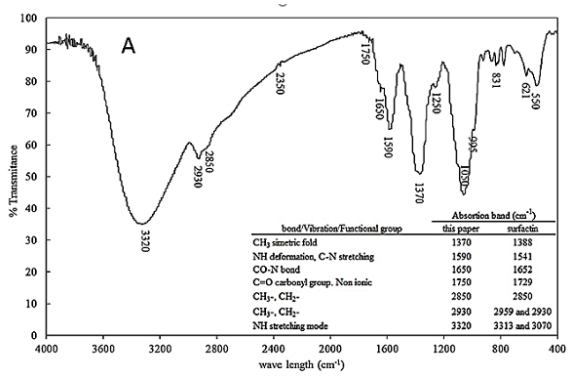

Figure 2. Biosurfactant characterization by Fourier Transform Infrared Spectroscopy (FTIR): main functional groups.

and in others it is valine (Tang, *et al*., 2010). Figure 2 also shows a comparison of the obtained bands in this work and those of surfactin.

The purified biosurfactant was characterized by high-performance HPLC coupled to mass spectrometry and MALDI-TOF (Figure 3) surfactin C_{14}, with a peak with a ratio of m/z 1,044, the peak with a ratio of m/z 1058.73, is explained considering that surfactin (m/z = 1036.7), which has a chain of 15 carbons and leucine at amino acid 7, is associated at the time of ionization to a Na ion with 22 m/z units, as published by Hoefler, *et al*. (2012). Peak with m/z = 1061.7 represents surfactin that has valine at amino acid 7 and a chain of 14 carbons and that is associated with a K ion with m/z = 39, according to Caldeira, *et al*. (2011). Iturine has chains C_{16} and C17 with m/z ratios of 1070 and 1084 respectively, when they are ionized, they associate with a potassium ion (39 m/z). Therefore, 1070 + 39 = 1109 and 1084 + 39 = 1123. The biosurfactant mixture is made up of three homologous series of peptides: the first of them corresponds to surfactin, which has valine as the seventh amino acid, the second series is also surfactin which has leucine, and the third series is represented by iturine. This conclusion is strongly supported by Pathak, *et al*. (2014) and by Wang *et al*. (2020), who have previously described that some species of *Bacillus subtilis* have produced a mixture of biosurfactants consisting of surfactin and iturine among other peptides.

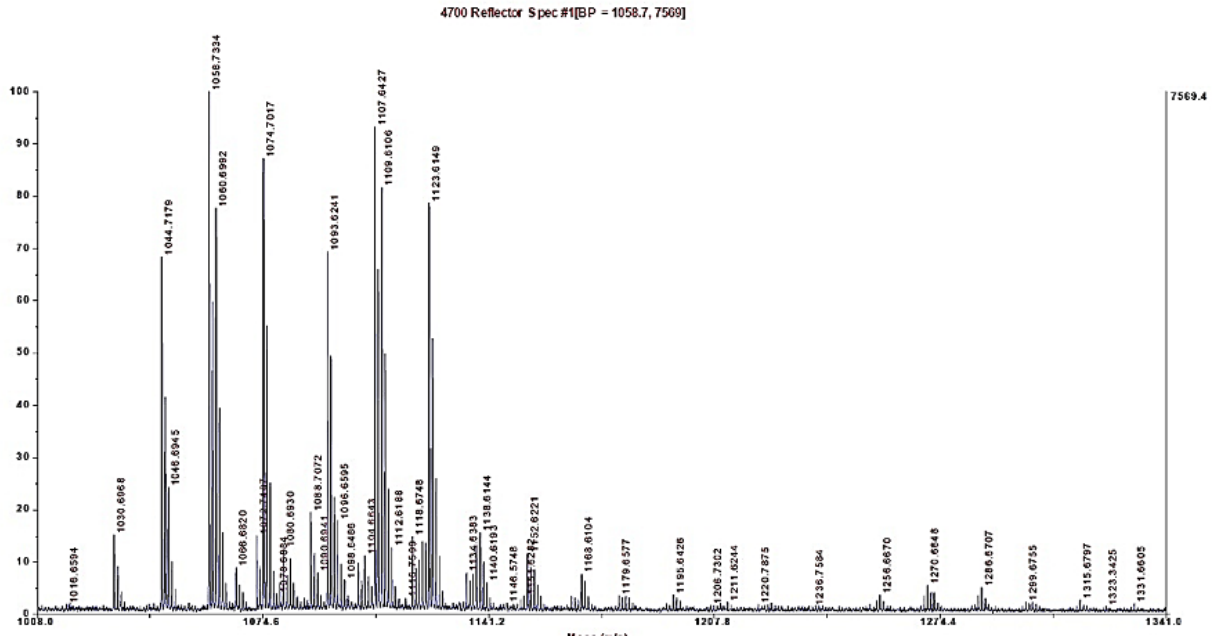

Figure 3. Biosurfactant characterization: Mass spectrometry.

Table 3. Biosurfactant activity results.

pH	ST ($mN{\cdot}m^{-1}$)	std. Dev
5	31.58	0
6	30.86	0.09
6.65	30.77	0.25
7	31.05	0.02
8	31.09	0.04
9	31.89	0.1
T °C	ST ($mN{\cdot}m^{-1}$)	std. Dev
4	31.04	0.09
20	31.01	0.1
25	30.78	0.25
40	30.76	0.07
60	30.27	0.13
80	30.31	0.01
NaCl (%)	ST ($mN{\cdot}m^{-1}$)	std. dev
0	30.78	0.25
2	32.08	0.29
4	31.56	0.26
6	33.5	0.21
8	34.31	0.96
10	34.71	0.06

Single tail variance analyzes were performed for the activity tests. Surface Tension (ST) remained almost invariable in the pH, Temperature and Salinity tested ranges.

3.3 Biosurfactant activity

The results of biosurfactant activity at different environmental conditions are shown in Table 3. In the literature available, a biosurfactant is considered stable when it retains its surface activity after subjecting it to different environmental conditions (Deng *et al*., 2016); surfactant activity has been evaluated by emulsion index E24, ST and IFT. The produced biosurfactant retained its activity at temperatures between 4 to 80°C, pH range from 5 to 9 and salinity between 0 to 4 % of NaCl.

The increase of surfactant activity with temperature has been previously documented (Karnanda *et al*., 2013). The biosurfactant stability at salinity could be due to salinity of aqueous phase modifying its relative solubility (Fernandes *et al*., 2016). Reports in literature available indicated that the biosurfactant produced by B. subtilis strains was stable at salinities up to 4% (Al-Bahry *et al*., 2013); the lipopeptide produced by B. subtilis W19 strain lost 50% of its surfactant activity at 5% salinity; Deng *et al*. (2016) reported that lipopeptidic compound produced by Achromobacter sp. had an activity loss greater than 50% at 6% salinity, while in this work, activity loss at such salinity was 8.88%. Regarding pH, the stability range reported for lipopeptides produced by B. subtilis was 6 to12 (Deng *et al*., 2016), with the maximum surfactant activity around the neutral pH (Varadavenkatesan and Murty, 2013), which is consistent with the results found in this study. These results show raw biosurfactant feasibility to retain its activity under several environmental conditions, a primary condition for been used for practical purposes in oil spills and soil recover.

3.4 Mechanism involved in the release of heavy crude oil and environmental factors that affect biosurfactant performance

The contact angle is an indicator of a system wettability; when the contact angle in a solid-oil-water system is smaller than 90°, solid is preferentially wet by water, but when it is bigger than 90° it is preferentially oil wet (Anderson, 1986). In this work, CA and IFT were evaluated as the main mechanisms in heavy oil release, previous assays showed that the emulsion with the heavy crude oil was not attained because of its high viscosity. The initial CA of oil impregnated limestone rock was 117°, meaning it was oil wet. After the rocks were treated with biosurfactant, the CA changed to 37.7° (Figure 4A and 4B), so rock wettability was modified to water preference. The heavy crude oil IFT was also reduced by biosurfactant action; in oil/water systems, the IFT was 27 mN m^{-1} while in oil/collapsed foam systems, the IFT was 0.8 mN m^{-1}.

For Taguchi DOE, the salinity and temperature factors and their levels were selected according with stability tests results. The effect of SO_4^{2-} was also evaluated since some reports indicated that this ion was able to change limestone rocks wettability from oil preference to water preference (Zhang and Austad, 2006). Table 1 shows the effects of environmental factors on biosurfactant activity. The controls remained oil-wet after the incubation period and both ST and IFT were high; likewise, no detectable crude release was found in systems with impregnated sand and without biosurfactant. In contrast, the nine systems containing biosurfactant exhibited oil release. Therefore, the release in these systems is attributable to biosurfactant action, as the change in wettability and decrease in both ST and IFT

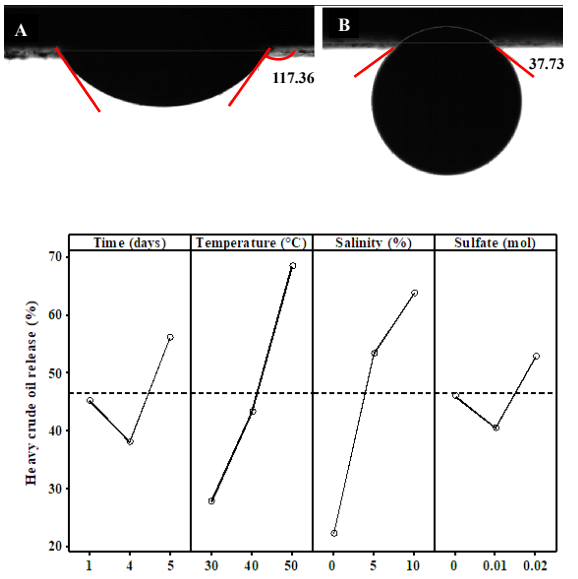

Figure 4. Oil release. [A] contact angle before and [B] after treatment with biosurfactant; [C] Effect of independent variables on biosurfactant activity during oil release: time, temperature, salinity, and sulfate ion concentration. Dotted line represents the global oil release average.

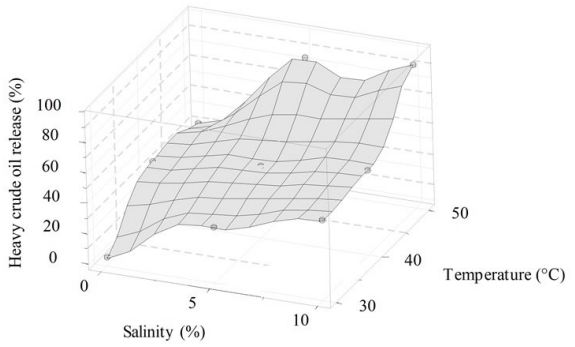

Figure 5. Oil release response surface as function of combined effect of temperature and salinity in sand systems impregnated with heavy crude oil using biosurfactant. Points represent experimental data.

were achieved after incubation with biosurfactant. The degree of oil release was observed to be dependent on environmental conditions since it was observed within a wide range (from 7.2% to 83.4%). The factors that showed greater effect on oil release were temperature and salinity, as shown by Taguchi DOE statistical analysis for the systems with biosurfactant (Figure 4C). These variables were also analyzed by the response surface technique and then plotted (Figure 5).

As can be seen, an increase in temperature promoted greater oil release, which agrees with stability tests results that showed that high temperatures favored biosurfactant activity; also, an increase in salinity resulted in greater release. The treatment yielding the highest release was number 3, with 83.42%, which was performed at 50 °C and 10% salinity. In this treatment the lowest ST value was obtained (27.27 mN m^{-1}) and its IFT was 2.7 mN m^{-1}, which represented a 96.14% decrease respect to its own control, added to the fact that treatment 3 wettability changed from oil to water preferent, as CA decreased from 113.47° to 46.98°. To determine relevant differences between the treatments, the means grouping analysis was performed by Tukey's DHS based on oil release, it was obtained that only treatment 3 showed no significant differences with treatment 9.

The forces that retain oil in a porous matrix depend, among other factors, on oil viscosity, interfacial tension between oil and water, and solid matrix oil wettability. Wettability in contaminated soils impacts pollutants adsorption and determines how easily they can be removed from soil (Andersson *et al*., 2016). The interaction with the biosurfactant modified the carbonate rock/sand wettability, from oil-wet to water-wet, making the surface more hydrophilic, weakening hydrocarbon adsorption, and increasing its bioavailability. By reducing the interfacial tension and/or modifying wettability, oil can be released (Johannessen and Spildo, 2013).

As pointed out, oil release from porous media is subject to decrease in water/oil and sand/oil interfacial forces (Law and Zhao, 2016). IFT decrease is mediated by biosurfactants in the salts presence, as has been reported previously by Karnanda *et al*. (2013); some studies found that salinity favors oil release by acting as biosurfactant coadjutant (Al-Sulaimani *et al*., 2012); De Andrade and Pastore (2016), reported studies conducted with surfactin in contact with different cations that led to conclude that the presence of Na+ resulted in lowest ST values. Fernandes *et al*. (2016), pointed out that salinity affects biosurfactant partition since salt concentration increase in an aqueous phase promotes the migration of biosurfactant molecules to oil phase. Optimum salt concentration for oil release is the one that promotes accumulation of biosurfactant in the water-oil interface, which generally corresponds to lowest ST and IFT values, as those reached in treatments 3 and 9 of this work. Regarding the effect of temperature, it has been suggested that high temperatures can affect biosurfactants aggregation forms and then modify

their tensioactivity (De Andrade and Pastore, 2016).

The results suggested a synergistic effect between temperature and salinity on biosurfactant activity: treatment 6 at high temperature and non-salinity, had oil release 45.38%, hence temperature by itself was not enough to promote oil release above the global average 45.12%; on the other hand, treatment 1, at low temperature and non-salinity, gave only a 7.25% oil release, even though it showed that changes in wettability and both its ST and IFT were low. Conversely the treatments at high levels of these two factors presented the very best results according to DHS test. It has been suggested that salinity promotes biosurfactant molecules migration to water-oil interface (Fernandes *et al*., 2016), while temperature promotes convection (Sousa *et al*., 2014), combination of these two factors would yield a greater outcome in biosurfactant transport rates from liquid phase to water-oil interface, thus explaining the good results obtained at high temperature and salinity levels. In view of the results obtained in this work, it is preliminary correct to state that oil release was diffusion controlled, through a temperature dependent transport mechanism.

The presence of SO_4^{-2} was not relevant in oil release although some authors suggest that this ion may modify limestone rocks wettability (Zhang and Austad, 2006). Regarding oil-sand contact time, no significant differences were obtained in the tested periods, so it would be interesting to evaluate longer aging times.

3.5 Effect of the biosurfactant in the biodegradation of heavy crude oil

Abiotic control (AB), treatment with biosurfactant (CL) and treatment without biosurfactant (SL), was plotted versus incubation time (Figure 6). In hydrocarbon biodegradation, CO_2 production is also associated with complete compounds mineralization (Castorena-Cortés *et al*., 2009), so, a higher CO_2 production indicates greater crude oil biodegradation. The SL CO_2 production plot showed that the microorganisms metabolic action led to crude oil biodegradation having a total CO_2 production of 55.88 mg at the end of the experiment; this treatment was used as reference of biodegradation behavior with no biosurfactant pretreatment. CL treatment showed biosurfactant effect on oil biodegradation, with final CO_2 production 74.23 mg, which was 32.84% higher than SL.

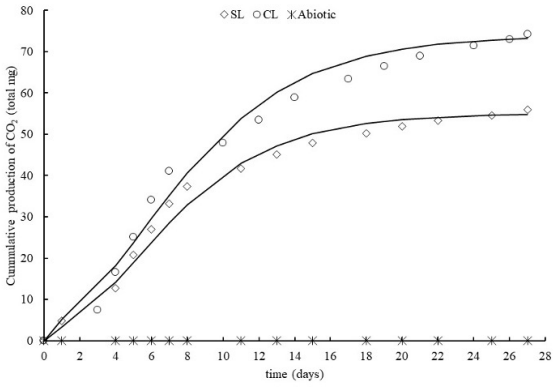

Figure 6. CO_2 cumulative production during biodegradation of heavy crude oil in limestone sand. [AB] abiotic control; [SL] treatment without biosurfactant; [CL] treatment with biosurfactant.

Net CO_2 production per gram of oil was 41.26 mg g_{oil}^{-1} and 76.72 mg g_{oil}^{-1} for SL and CL, respectively, so biosurfactant pretreatment improved net oil biodegradation related with CO_2 production by 85.94%.

Greater CO_2 production rate is linked to higher biodegradation rate: the maximum CO_2 production rate of CL treatment was 5.32 mg g_{oil}^{-1} h^{-1}, 73.38% higher than SL, which was 3.06 mg g_{oil}^{-1} h^{-1} estimated with equation 1. Higher CO_2 production on CL treatment (with biosurfactant) suggested that oil components mineralization was easier and that biosurfactant pretreatment had a positive effect, by improving the biodegradation process and CO_2 production rate, as suggested elsewhere (Borah and Yadav, 2017). The *B. subtilis* biosurfactants exerted positive effects on crude oil, diesel and kerosene biodegradation when it was used mixed with the hydrocarbons as reported by Montagnolli *et al*. (2015), in that study crude oil biodegradation (oil type was not indicated) showed that biosurfactant enhanced cumulative CO_2 production by 24.4% during the whole process, using a microbial consortium; in this work it was improved by 32.84%.

SARA analysis of both, initial crude oil and residual oils indicated that oil fractions biodegradation varied depending on treatment (Table 4): CL treatment (with biosurfactant), achieved 17.66% saturated compounds reduction, while in SL treatment (no-biosurfactant) it was 11.08%; CL also was able to reduce aromatic and resin fractions by 12.54 and 2.62%, respectively, whereas with SL, the reduction of these compounds was null, so the biosurfactant has a crucial role on biodegradation of these fractions.

Table 4. SARA analysis of the original oil, oil treatment without biosurfactant (SL) and oil treatment with biosurfactant (CL).

Fraction	Initial content g per100gcrude	Degradation%	
		CL Treatment with biosurfactant	SL Treatment without biosurfactant
Saturated	16	17.66	11.08
Aromatics	31	12.54	0
Resins	36	2.62	0

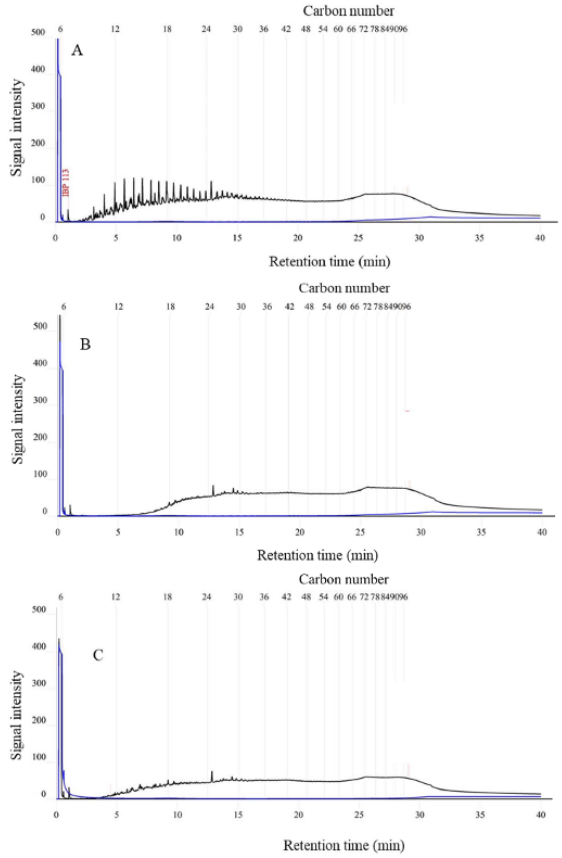

Figure 7. Hydrocarbon chromatograms of [A] Original crude oil, [B] residual oil from CL treatment and [C] residual oil from SL treatment.

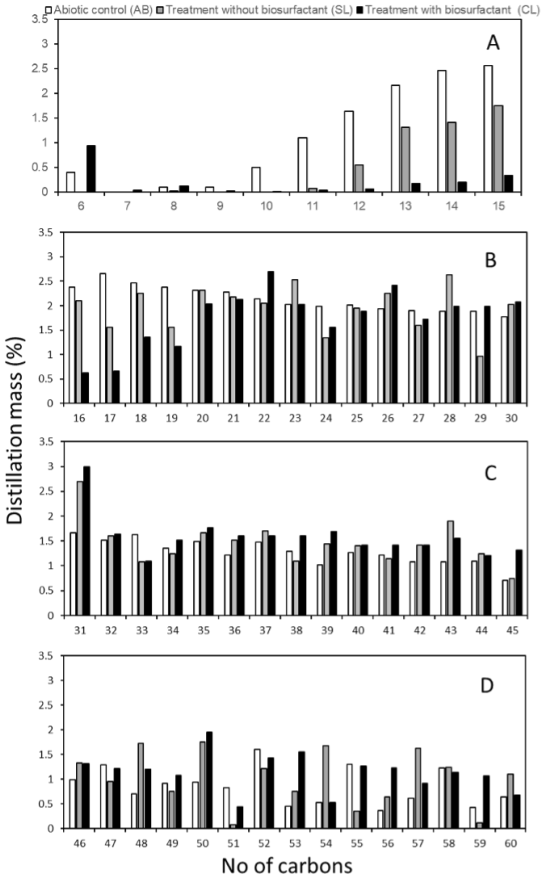

Figure 8. Distribution of C-atom hydrocarbon compounds of residual oil from control and treatments. [A] C_6-C_{15}, [B] C_{16}-C_{30}, [C] C_{31}-C_{45}, [D] C_{46}-C_{60} factions. Bars represent distilled mass content (%).

The total SARA fractions reduction was 32.83% in CL and 11.08 % in SL. This result was also corroborated by Carbon Chromatography on residual oils (Figure 7), it was observed that CL treatment depleted signals between C_6 and C_{15}, while in range C_{16} - C_{24} signals were lower than SL's chromatogram. Figure 8 shows the C-atom hydrocarbon distribution (distillation mass %) of control and treatments. A redistribution of hydrocarbons was detected in the different fractions analyzed. Biodegradation caused a decrease in $<C_{20}$ fractions, which shows that the microorganism can degrade different hydrocarbons,

but preferably using the light and medium fractions. The system with biosurfactant showed a notably higher degradation in the C_7-C_{20}, fractions. At least 67% of $>C_{20}$ fractions were degraded in CL systems, while in systems without biosurfactant only 37% of the same fraction was removed. The increasing C_6 fraction in biosurfactant systems is attributed to biodegradation of heavier fragments, such as heavy alkanes, aromatics, and resins. These modifications can in turn cause changes in oil viscosity. Oil degradation studies found that n-alkanes are the most susceptible fraction to biodegradation, followed by aromatics fraction, while the branched alkenes, cycloalkenes and poly aromatics fractions, are most resistant, presenting low degradation rates (Head *et al*., 2010). Results found in this work show that the degradation was favored by the presence of the biosurfactant, even in complex fractions. *Rhodococcus* strains have the ability to metabolize different hydrocarbons including aromatics (Kumar *et al*., 2020). However, in the present study, the SARA analysis of the SL systems did not detect degradation in the aromatic fractions, while in CL the reduction of this fraction was 12.54%. The different degradation degrees of the saturated, aromatic and resins compounds indicated that the pretreatment with biosurfactant changed the bioavailability and therefore facilitated their degradation.

Additionally, it was observed that during first four incubation days, microorganism in SL grew in suspended form, forming orange-colored flocs that caused broth culture turbidity, while in CL treatment the microorganisms grew stuck to crude oil as white biomass aggregates (Figure 9). Microorganisms adherence to oils has been attributed to biosurfactant migration to oil surfaces, forming an interface that favored microorganism-oil contact and nutrients uptake (Kapellos, 2017).

The addition of exogenous surfactants above CMC concentration, has been reported to be beneficial to oil biodegradation. However, it has also been reported that in case of hydrophobic microorganisms as genus *Rhodococcus*, the surfactants are detrimental because they modify surface oils hydrophobicity, which is necessary for adhesion of such kind of microorganism to oils (Kapellos, 2017). In contrast, results in this work showed that adding the exogenous biosurfactant produced by *Bacillus subtilis*, proved to be beneficial for *Rhodococcus sp* strain; this fact represents an advantage over the current commercial surfactants and dispersants, demonstrating its feasibility in oil spills and soil recovery using hydrophobic microorganisms.

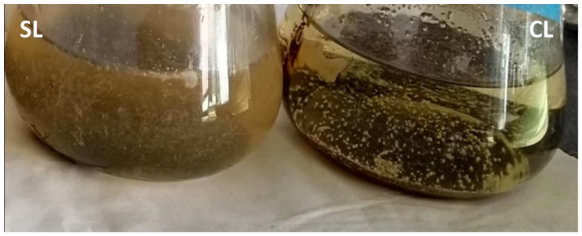

Figure 9. *Rhodococcus* sp. strain growing in suspended form in treatment without biosurfactant (SL) and stuck to oil in treatment with biosurfactant (CL).

Conclusions

The biosurfactant proved to have the capability to release heavy oil from sand and to promote its greater biodegradation. Decreases in surface and interfacial tension, as well as the modification of wettability caused by biosurfactant activity, were the main mechanisms. The biosurfactant activity releasing crude oil depends on the environmental conditions such as temperature and salinity, having a synergistic effect between them.

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Nomenclature

CA	Contact Angle
FTIR	Fourier Transformed Infrared Spectroscopy
IFT	Interfacial Tension
SARA	Saturates, Aromatics, Resins and Asphaltenes compounds analysis
ST	Surface Tension

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