



# Optimization and characterization of biosurfactant production by *Bacillus subtilis* isolates towards microbial enhanced oil recovery applications



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## HIGHLIGHTS

- *Bacillus subtilis* isolates from Brazilian crude oils produce biosurfactants under reservoir conditions.
- Sucrose was found to be the best carbon source for biosurfactant production.
- Similar mixtures containing C<sub>13</sub>-, C<sub>14</sub>- and C<sub>15</sub>-surfactin were found for the biosurfactants produced.
- Biosurfactants have better interfacial activity and lower critical micellar concentrations than chemical surfactants.
- The produced biosurfactants are promising for Microbial Enhanced Oil Recovery applications.

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## ABSTRACT

Biosurfactant production by three *Bacillus subtilis* strains (#309, #311 and #573) isolated from Brazilian crude oils was optimized based on different carbon and nitrogen sources. The lowest surface tension values were obtained using sucrose containing media for the three isolates. Biosurfactants produced by each strain were characterized by Fourier transform infrared spectroscopy (FTIR), proton nuclear magnetic resonance (<sup>1</sup>H NMR), and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF). The chemical characterization showed that the three isolates produced very similar mixtures, containing C<sub>13</sub>-, C<sub>14</sub>- and C<sub>15</sub>-surfactin, although in different proportions. The structure of surfactins produced by the three strains was shown to be in good agreement with their surface-activities. The interfacial-activities of the biosurfactants were characterized and compared with chemical surfactants. Furthermore, the application of biosurfactants and chemical surfactants in oil recovery was evaluated. The results obtained showed that biosurfactants have better interfacial-activity and oil recovery efficiency than common chemical surfactants, thus being more attractive to be applied in Microbial Enhanced Oil Recovery.

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## 1. Introduction

Surface-active agents or surfactants are an important class of chemical compounds used in different sectors of modern industry, such as food, pharmaceutical, cosmetics and petroleum industries [1,2]. These compounds are able to reduce surface and interfacial tensions, as well as to form and stabilize oil in water or water in oil emulsions [3]. Structurally they are amphiphilic molecules that comprise both hydrophobic and hydrophilic moieties, being the

apolar component usually a carbon chain, whereas the polar part, more variable, can be ionic (anionic or cationic) or non-ionic [4]. Due to environmental issues and restrictive laws, the demand for biodegradable surfactants and naturally produced by microorganisms is increasing [5]. The use of these bio-compounds as an alternative to the traditional chemical surfactants has been impelled by the fast progress of biotechnology and also by their interesting features, including their lower toxicity, higher biodegradability and effectiveness at extreme temperature, salinity and pH conditions [2,6]. Furthermore, biosurfactants exhibit high surface-activities together with low critical micelle concentrations (CMCs), being in some cases even lower than most of the traditional chemicals surfactants [6].

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Despite all these advantages, only a few biosurfactants are produced at large scale for commercial applications, mainly due to their considerable production and recovery costs [7]. In the last years, aiming a large industrial application of biosurfactants, the use of novel resources, raw materials and new microorganisms has been explored to reduce the production costs and to increase their effectiveness [8–14]. Many biosurfactants have been described, being glycolipids [15,16] and lipopeptides [4,17] the most common. Lipopeptides are produced, among others, by several *Bacillus* species. They are constituted by a peptide (hydrophilic moiety) linked to a fatty acid (hydrophobic component) [4]. Surfactin, produced by *Bacillus subtilis* strains, is the best studied lipopeptide biosurfactant. It consists of a long-chain  $\beta$ -hydroxy fatty acid whose two functional groups close a short peptidic moiety composed by seven amino acid residues [18,19]. The surfactins are classified in three different types (A, B and C) according to their amino acid sequences [20]. *B. subtilis* strains do not produce a unique type of surfactin, and a natural diversity of homologues occur, which differ in the length and ramification of the fatty acid chains, and isoforms, characterized by some differences in the peptidic sequence [18]. Different isoforms and homologues exhibit different properties and activities. As the surfactin name indicates, this biosurfactant exhibits an exceptional surface activity, and is among the most effective biosurfactants known so far, being able to decrease the surface tension of water from 72 to 27 mN m<sup>-1</sup> at concentrations as low as 0.005% [21].

These compounds are considered powerful biosurfactants [22], and can be used by the petroleum industry in MEOR processes and bioremediation [2], as well as by many other industries such as the biomedical industry as therapeutic agents [19,20]. Among several potential applications of surfactin, its use in MEOR represents one of the most promising methods to recover substantial amounts of the residual oil entrapped in mature oil fields [23,24]. The replacement of conventional synthetic surfactants by these biocompounds appears to be a good and efficient approach; however it still depends on the strategy adopted. The use of biosurfactants in MEOR can be performed in two different ways. In the first one, biosurfactants are produced *ex situ*, and subsequently injected into the reservoir. The other option is to produce the biosurfactant *in situ* by stimulation and/or injection of indigenous microorganisms. The first approach is limited by the costs involved in the biosurfactant production and purification processes. On the other hand, the costs involved in the second approach are apparently lower; however it requires that the microorganisms used are properly stimulated and able to produce sufficient amounts of the biosurfactant [23].

Moreover, taking into account the use of surfactins in *in situ* MEOR processes, the greatest challenge is to isolate microorganisms able to grow under anaerobic conditions, high salinities, temperatures and pressures. Several reports have described the isolation of *B. subtilis* strains from oil reservoirs [24–27], which suggest that these organisms can be successfully used under reservoir conditions. Recently, Gudiña et al. [28] isolated several *B. subtilis* strains from a Brazilian oil reservoir that were able to produce different biosurfactants under aerobic and anaerobic conditions. Among them, three different strains produced biosurfactants with interesting surface-activities and emulsifying properties.

Therefore, aiming at the further use of these *B. subtilis* strains in MEOR processes, the biosurfactant production yields must be improved which can be achieved through the optimization of culture media, specifically of the carbon and nitrogen sources. In this work, biosurfactant production by the three *B. subtilis* strains previously isolated [28] was optimized through a proper manipulation of carbon and nitrogen sources. The biosurfactants produced by each strain were characterized by <sup>1</sup>H NMR, FTIR and MALDI-TOF, and their abilities to influence the interfacial tension of water/oil

systems and enhance oil recovery were evaluated and compared with chemical surfactants.

## 2. Experimental

### 2.1. Microorganisms

Three biosurfactant-producing *B. subtilis* strains (#309, #311 and #573) previously isolated from crude oil samples obtained from a Brazilian oil field [28] were used. Isolates were stored at –80 °C in LB medium supplemented with 20% (v/v) glycerol solution. The composition of LB medium was (g l<sup>-1</sup>): NaCl 10.0; tryptone 10.0; yeast extract 5.0. The pH was adjusted to 7.0.

### 2.2. Chemical surfactants

Two chemical surfactants (Enordet and Petrostep) commonly used in chemical enhanced oil recovery (CEOR) were studied and their interfacial activities compared with biosurfactants produced by *B. subtilis* strains.

### 2.3. Effect of carbon and nitrogen sources on growth and biosurfactant production

Growth and biosurfactant production by the three isolates was evaluated using Mineral Salt Solution (MSS) with different carbon and nitrogen sources. The MSS consisted of (g l<sup>-1</sup>): NaCl 10.0; Na<sub>2</sub>HPO<sub>4</sub> 5.0; KH<sub>2</sub>PO<sub>4</sub> 2.0; MgSO<sub>4</sub> 7H<sub>2</sub>O 0.2. The carbon sources evaluated were: sodium acetate (AC), sodium citrate (CI), fructose (FRU), glucose (GLU), glycerol (GLY), *n*-hexadecane (HEX), lactose (LAC), meat extract (ME), paraffin (PAR), sucrose (SUC), tryptone (TRY) and yeast extract (YE), using ammonium nitrate as the nitrogen source (2 g l<sup>-1</sup>). The different carbon sources were added to the MSS at a concentration of 10 g l<sup>-1</sup>. The nitrogen sources tested were: ammonium citrate (AC), ammonium nitrate (AN), ammonium sulfate (AS), meat extract (ME), sodium nitrate (SN), tryptone (TRY), urea (U) and yeast extract (YE), keeping sucrose as the carbon source (10 g l<sup>-1</sup>). The different nitrogen sources were added to the MSS at a concentration of 2 g l<sup>-1</sup>. All media were adjusted to pH 7.0.

Assays were performed in 100 ml flasks containing 50 ml of the different media. Each flask was inoculated with 1% of a pre-culture grown in the same medium for 24 h. Cultures were incubated at 40 °C without shaking for 120 h. Samples were taken at different times during the fermentation to determine biomass concentration and biosurfactant production. Bacterial growth was determined by measuring the optical density at 600 nm. Afterwards, the samples were centrifuged (10,000g, 20 min, 20 °C) and the cell-free supernatants were used to measure surface tension and emulsifying activity. At the end of the fermentation, cells were harvested by centrifugation and dry cell weight (g l<sup>-1</sup>) was determined (48 h at 105 °C).

To recover the biosurfactants, cell-free supernatants were subjected to an acid precipitation. Briefly, the supernatants were adjusted to pH 2.0 with HCl 6 M and left overnight at 4 °C. Afterwards, the precipitate was collected by centrifugation (10,000g, 20 min, 4 °C) and washed twice with acidified water (pH 2.0). The precipitated biosurfactants were dissolved in a minimal amount of demineralised water and the pH was adjusted to 7.0 using NaOH 1 M. Finally, the biosurfactant solutions were freeze dried and the products obtained were weighed.

### 2.4. Surface-activity determination

Surface tension measurements of culture broth supernatants and chemical surfactants solutions were performed according to

the Ring method described elsewhere [29]. A KRÜSS K6 Tensiometer (KRÜSS GmbH, Hamburg, Germany) equipped with a 1.9 cm De Noüy platinum ring was used. Whenever required, the culture broth supernatants were diluted 10 times ( $ST^{-1}$ ) or 100 times ( $ST^{-2}$ ) with PBS (10 mM  $KH_2PO_4/K_2HPO_4$  plus 150 mM NaCl with pH adjusted to 7.0) and the surface tension was measured as described above. The CMCs of chemical surfactants dissolved in demineralised water were calculated as described elsewhere [29]. To increase the accuracy of the surface tension measurements, an average of triplicates was determined. All measurements were performed at room temperature (20 °C).

### 2.5. Emulsifying activity determination

Emulsifying activity was determined by the addition of 2 ml of *n*-hexadecane to the same volume of cell-free culture broth supernatant in glass test tubes. The tubes were mixed with vortex at high speed for 2 min and subsequently incubated at 25 °C for 24 h. The stability of the emulsion was determined after 24 h, and the emulsification index ( $E_{24}$ ) was calculated as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm). All emulsification indexes were performed in triplicate.

### 2.6. Interfacial-activity determination

Concentrations ranging from 0.001 to 0.1 g l<sup>-1</sup> of crude biosurfactants recovered from the different isolates were prepared in brine solution (50 g l<sup>-1</sup> NaCl with pH adjusted to 6.0). These conditions were previously established as optimal for the activity of biosurfactants under study [28]. The chemical surfactants were dissolved in demineralised water at different concentrations up to 1 g l<sup>-1</sup>. Arabian light crude oil used in these experiments was kindly provided by GALP (Portugal). Interfacial tension of the water/oil system at different biosurfactant/chemical surfactant concentrations was measured using a Dataphysics contact angle system OCA-20. The method consisted in the analysis of the shape of a pendant drop by fitting to the Young–Laplace equation. A Hamilton DS 500/GT syringe connected to a stainless steel needle placed inside a glass chamber with the biosurfactant aqueous solution at room temperature was used to set a fixed drop oil volume (5.00 ± 0.15 µl). For each biosurfactant concentration, at least three independent measures were conducted. For each drop, data was recorded immediately after the drop volume was reached (5 µl) and continued during 1 min (an average of 80 images was captured per drop). The drop shape was analyzed with the software modules SCA 20, using the gravitational acceleration ( $g = 9.8018 \text{ m s}^{-2}$ ) and latitude (lat = 40°) values in accordance with the assay location. The density values required for the calculation of the interfacial tensions from the drop image data were acquired using a Viscometer Anton Paar (Model SVM 3000).

### 2.7. Extraction and purification of biosurfactants

Biosurfactants for chemical composition analysis were extracted from cell-free supernatants using the Folch extraction method that is commonly used to extract lipids from biomolecules. The Folch extraction procedure was performed as described elsewhere [30]. Briefly, a chloroform/methanol mixture (2:1) was added to the supernatant sample to a final chloroform/methanol/water ratio of 8:4:3. The mixture was centrifuged (9000g, 5 min), the organic layer was collected and the samples were evaporated to dryness under N<sub>2</sub> at 37 °C for 30 min. Prior to NMR spectroscopy and mass spectrometry analysis, the samples were re-dissolved in chloroform.

### 2.8. Fourier transform infrared spectroscopy

The solid biosurfactant extracts recovered from the supernatants of the three *B. subtilis* isolates were characterized by Fourier transform infrared spectroscopy (FTIR). The FTIR spectra, with a resolution of 4 cm<sup>-1</sup>, were collected from 400 to 4000 wavenumbers (cm<sup>-1</sup>), and is an average of 128 scans using a Tensor 27 Infrared Spectrometer operating in the attenuated total reflection (ATR) mode (equipped with a single horizontal Golden Gate ATR cell).

### 2.9. Nuclear magnetic resonance spectroscopy

The extracted biosurfactants were re-dissolved in deuterated chloroform (CDCl<sub>3</sub>) and the respective <sup>1</sup>H NMR spectra were recorded at 25 °C using a Bruker Avance 300 spectrometer operating at 300.13 MHz. Chemical shifts ( $\delta$ ) are given on the ppm scale relative to tetramethylsilane (TMS).

### 2.10. Mass spectrometry

The extracted biosurfactants were dissolved at a concentration of 2 g l<sup>-1</sup> in a solution composed of 50% acetonitrile (ACN), 50% water, and 0.1% trifluoroacetic acid (TFA). The samples were mixed (1:1) with a matrix consisting of a saturated solution of  $\alpha$ -cyano-4-hydroxycinnamic acid prepared in 50% ACN/0.1% TFA. An aliquot of 1 µL of each sample/matrix mixture was spotted onto the MALDI sample and slowly dried to allow matrix crystallization. Then, the extracted biosurfactants were analyzed by mass spectrometry (MS) using a MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Europe) in the positive ion reflector mode. Spectra were obtained in the mass range between 800 and 4500 Da with ca. 1500 laser shots, thus providing a mass resolution of about 18,000 (FWMH) and a mass accuracy of 20–25 ppm. MS data were processed using Data Explorer 4.4 (Applied Biosystems).

### 2.11. Application of chemical surfactants and biosurfactants in removal of crude oil from sand

The applicability of biosurfactants produced by *B. subtilis* #309, #311 and #573 and the chemical surfactants Enordet and Petrostep in oil recovery was evaluated using artificially contaminated sand containing 10% (w/w) of Arabian Light oil. Samples of 40 g of sand were mixed with 4 g of crude oil in 100 ml Erlenmeyer flasks by shaking and allowed to age for 24 h. Afterwards, 40 ml of biosurfactants and commercial surfactants solutions at a concentration of 1 g l<sup>-1</sup> were added to each flask. The flasks were incubated at 100 rpm and 40 °C for 24 h. Afterwards, the oil removed was recovered from the surface and its volume was measured. Control assays were performed using demineralized water at the same conditions. All the experiments were carried out in triplicate.

## 3. Results and discussion

### 3.1. Effect of carbon and nitrogen sources on growth and biosurfactant production

To determine the optimal conditions that yield the highest biosurfactant production by *B. subtilis* #309, #311 and #573, the effect of various carbon and nitrogen sources was evaluated. The three isolates were able to grow in all carbon sources tested, except for *n*-hexadecane. When paraffin was used as the sole carbon source, a slight growth was observed for all isolates, however lower than the obtained with the water soluble carbon sources tested (Table 1). Several authors reported, with different *Bacillus* strains, that the use of hydrocarbons (including *n*-hexadecane and paraffin) as

**Table 1**  
Surface tension values (mN m<sup>-1</sup>), emulsifying indexes (%), biomass concentrations (g dry weight l<sup>-1</sup>) and surfactin concentrations (mg l<sup>-1</sup>) obtained for the three *Bacillus subtilis* isolates grown in MSS with different carbon sources (10 g l<sup>-1</sup>) at 40 °C for 120 h. Results represent the average of three independent experiments ± standard deviation.

Parameter	AC	CI	FRU	GLU	GLY	HEX	LAC	ME	PAR	SUC	TRY	YE
<b>Isolate #309</b>												
ST (mN m <sup>-1</sup> )	30.5 ± 0.5	31.6 ± 0.9	28.4 ± 0.4	29.2 ± 0.3	29.7 ± 0.2	68.5 ± 0.1	30.7 ± 0.2	29.9 ± 0.2	32.6 ± 0.7	28.0 ± 0.3	29.8 ± 0.4	29.7 ± 0.2
ST <sup>-1</sup> (mN m <sup>-1</sup> )	48.9 ± 3.2	62.7 ± 3.8	31.0 ± 0.7	32.8 ± 0.7	34.8 ± 1.4	-	54.9 ± 3.8	33.1 ± 1.1	66.4 ± 1.9	31.3 ± 0.7	33.9 ± 1.5	32.5 ± 0.5
ST <sup>-2</sup> (mN m <sup>-1</sup> )	71.2 ± 0.8	71.2 ± 0.7	46.2 ± 5.1	62.6 ± 3.6	69.0 ± 2.9	-	72.7 ± 0.1	46.2 ± 2.7	-	42.3 ± 2.1	58.8 ± 5.1	61.3 ± 3.7
E <sub>24</sub> (%)	10.9 ± 2.1	12.7 ± 2.8	10.7 ± 5.3	35.3 ± 1.7	33.5 ± 2.7	0.0 ± 0.0	12.9 ± 3.0	47.8 ± 1.8	3.1 ± 0.0	39.7 ± 3.9	49.6 ± 0.7	52.7 ± 0.9
[Biomass] (g l <sup>-1</sup> )	0.808 ± 0.083	1.012 ± 0.021	1.150 ± 0.047	0.778 ± 0.091	0.568 ± 0.011	0.0 ± 0.0	0.628 ± 0.027	0.598 ± 0.019	0.195 ± 0.034	1.066 ± 0.092	1.238 ± 0.063	0.878 ± 0.021
[Surfactin] (mg l <sup>-1</sup> )	92.0 ± 22.1	107.1 ± 19.0	743.9 ± 129.5	325.1 ± 91.7	154.1 ± 71.3	0.0 ± 0.0	65.3 ± 5.1	744.6 ± 69.0	22.8 ± 4.7	844.1 ± 53.8	475.2 ± 131.5	410.8 ± 67.7
<b>Isolate #311</b>												
ST (mN m <sup>-1</sup> )	33.3 ± 1.5	35.6 ± 0.9	28.1 ± 0.2	29.0 ± 0.0	30.1 ± 0.1	68.2 ± 0.4	31.2 ± 0.5	30.2 ± 0.1	34.7 ± 1.0	27.9 ± 0.2	30.1 ± 0.1	30.4 ± 0.1
ST <sup>-1</sup> (mN m <sup>-1</sup> )	66.7 ± 1.9	71.5 ± 1.0	32.7 ± 0.9	33.1 ± 1.1	37.6 ± 0.7	-	52.6 ± 4.3	36.2 ± 1.1	71.0 ± 1.2	32.5 ± 1.2	34.8 ± 0.9	36.4 ± 1.3
ST <sup>-2</sup> (mN m <sup>-1</sup> )	71.8 ± 0.9	-	56.2 ± 2.7	68.5 ± 2.5	69.9 ± 2.9	-	72.3 ± 0.2	70.8 ± 1.0	-	46.5 ± 4.9	68.1 ± 1.8	71.1 ± 0.9
E <sub>24</sub> (%)	8.8 ± 3.7	9.4 ± 4.4	0.0 ± 0.0	29.8 ± 2.3	26.2 ± 0.6	0.0 ± 0.0	10.9 ± 2.2	40.3 ± 0.4	6.2 ± 0.0	37.3 ± 2.8	39.1 ± 2.2	26.8 ± 5.8
[Biomass] (g l <sup>-1</sup> )	1.038 ± 0.056	0.705 ± 0.043	1.042 ± 0.065	0.536 ± 0.026	0.660 ± 0.029	0.0 ± 0.0	0.758 ± 0.053	0.702 ± 0.033	0.201 ± 0.046	1.002 ± 0.092	0.924 ± 0.025	0.934 ± 0.041
[Surfactin] (mg l <sup>-1</sup> )	117.7 ± 21.4	12.4 ± 2.4	516.3 ± 68.1	233.0 ± 57.2	150.3 ± 70.5	0.0 ± 0.0	81.9 ± 24.1	141.5 ± 24.6	13.8 ± 3.1	765.8 ± 126.1	231.2 ± 30.1	135.7 ± 23.9
<b>Isolate #573</b>												
ST (mN m <sup>-1</sup> )	33.2 ± 1.5	36.4 ± 2.9	29.4 ± 0.3	29.5 ± 0.4	29.9 ± 0.3	68.1 ± 0.3	32.9 ± 0.9	31.1 ± 0.4	41.5 ± 1.8	28.4 ± 0.3	31.0 ± 0.1	30.4 ± 0.2
ST <sup>-1</sup> (mN m <sup>-1</sup> )	62.7 ± 2.2	71.6 ± 1.1	36.6 ± 1.5	35.0 ± 1.8	35.1 ± 1.2	-	66.2 ± 1.8	39.4 ± 1.6	72.2 ± 0.2	33.0 ± 1.3	38.6 ± 1.6	35.4 ± 1.3
ST <sup>-2</sup> (mN m <sup>-1</sup> )	72.3 ± 0.7	-	70.4 ± 1.0	66.9 ± 2.9	66.1 ± 3.4	-	72.2 ± 0.9	69.7 ± 1.3	-	46.3 ± 3.0	70.8 ± 0.9	66.0 ± 3.7
E <sub>24</sub> (%)	7.1 ± 1.6	4.6 ± 1.9	30.6 ± 2.6	27.1 ± 1.6	48.4 ± 2.2	0.0 ± 0.0	9.6 ± 3.1	34.0 ± 3.2	2.9 ± 0.0	38.8 ± 4.4	43.6 ± 4.3	40.3 ± 2.9
[Biomass] (g l <sup>-1</sup> )	0.848 ± 0.032	0.762 ± 0.021	1.150 ± 0.012	0.556 ± 0.048	0.870 ± 0.033	0.0 ± 0.0	0.780 ± 0.052	0.708 ± 0.010	0.221 ± 0.067	1.030 ± 0.057	1.184 ± 0.042	1.058 ± 0.047
[Surfactin] (mg l <sup>-1</sup> )	109.6 ± 60.5	24.8 ± 6.8	292.6 ± 47.9	725.9 ± 114.5	965.3 ± 89.3	0.0 ± 0.0	160.1 ± 40.2	323.6 ± 67.6	20.2 ± 7.3	2158.5 ± 240.2	284.0 ± 29.1	894.3 ± 110.5

the only carbon source inhibited bacterial growth and biosurfactant production [31–33], or resulted in poor growth and biosurfactant production [34].

The highest biomass production, expressed as dry cell weight, was obtained with tryptone and fructose for isolates #309 and #573, and with fructose and sodium acetate for isolate #311 (Table 1). Glucose and sucrose were reported as the best carbon sources for growth using different *Bacillus* isolates [31,32], although Joshi et al. [34] obtained better results with fructose.

The lowest surface tension values (ST<sup>-2</sup>) were obtained when sucrose was used as the sole carbon source for all the isolates. The surface tension values were between 27.9 and 28.4 mN m<sup>-1</sup>, and the ST<sup>-2</sup> values between 46.5 and 42.3 mN m<sup>-1</sup> (Table 1). Fructose also offered good results regarding surface tension reduction (ST<sup>-2</sup>) for isolate #309. The use of lactose as carbon source resulted in high surface tension values when compared with the other the carbohydrates tested.

This is in agreement with the results reported by other authors for different *B. subtilis* isolates. Several authors obtained the lowest surface tension values using glucose or sucrose [31–35] as the sole carbon sources. Abdel-Mawgoud et al. [31] also found a poor biosurfactant production using lactose as the sole carbon source. On the other hand, the use of hydrocarbons as the sole carbon source usually resulted in no biosurfactant production [31,32,34]. In the case of *B. subtilis* #309, #311 and #573, the combination of a water soluble carbon source (sucrose) and a hydrocarbon (*n*-hexadecane) did not have a negative effect on the biosurfactant production [28].

Regarding the emulsifying activity, the complex carbon sources tested (meat extract, tryptone and yeast extract) were amongst the most favorable. For isolate #309, the highest emulsifying activity was obtained with yeast extract (52.7%), and similar results were obtained with tryptone and meat extract (49.6% and 47.8%, respectively). For isolate #311, the best result was obtained with meat extract (40.3%), followed by tryptone (39.1%) and sucrose (37.3%). For isolate #573, the highest emulsifying activity was obtained with glycerol (48.4%), followed by tryptone (43.6%) and yeast extract (40.3%). Raw glycerol, a by-product from biodiesel production plants, can be used as a low-cost carbon source for biosurfactant production [36,37]. Furthermore, Dastgheib et al. [33] found the highest emulsifying activity using yeast extract as the carbon source, followed by sucrose, fructose and glucose, whereas the use of lactose resulted in poor emulsifying activity. For the three isolates, the highest amount of biosurfactant produced (expressed as mg of biosurfactant per liter) was obtained when using sucrose as the carbon source. The amounts of crude biosurfactant produced were 844, 755 and 2158 mg l<sup>-1</sup> for isolates #309, #311 and #573, respectively. The highest amount of biosurfactant produced is in agreement with the lowest surface tension values (ST<sup>-2</sup>) obtained for all the isolates, but curiously not with the highest emulsifying activities, thus suggesting that different types of biosurfactants with different properties are being synthesized depending on the carbon sources used.

Regarding the nitrogen sources, the highest biomass production was obtained using meat extract as the sole nitrogen source for isolates #309 and #311. For isolate #573, yeast extract and meat extract gave a similar result (Table 2).

The lowest surface tension values (ST<sup>-2</sup>), which corresponded to the highest biosurfactant productions, were obtained with yeast extract for isolate #309 (38.3 mN m<sup>-1</sup>); ammonium sulfate and ammonium nitrate for isolate #573 (44.6 and 46.3 mN m<sup>-1</sup>, respectively); and for the isolate #311, yeast extract and ammonium sulfate gave similar results (39.2 and 37.0 mN m<sup>-1</sup>, respectively). The highest biosurfactant productions were 931, 980 and 2288 mg l<sup>-1</sup> for isolates #309, #311 and #573, respectively. Other authors reported crude biosurfactant productions between 720 and 1120 mg l<sup>-1</sup> [31,32]. Isolate #573 produced higher amounts

**Table 2**

Surface tension values ( $\text{mN m}^{-1}$ ), emulsifying indexes (%), biomass concentrations ( $\text{g dry weight l}^{-1}$ ) and surfactin concentrations ( $\text{mg l}^{-1}$ ) obtained for the three *Bacillus subtilis* isolates grown in MSS with different nitrogen sources ( $2 \text{ g l}^{-1}$ ) at  $40^\circ\text{C}$  for 120 h. Results represent the average of three independent experiments  $\pm$  standard deviation.

Parameter	AC	AN	AS	ME	SN	TRY	U	YE
<i>Isolate #309</i>								
ST ( $\text{mN m}^{-1}$ )	$28.5 \pm 0.5$	$28.0 \pm 0.3$	$28.4 \pm 0.2$	$29.0 \pm 0.2$	$29.3 \pm 0.2$	$30.2 \pm 0.2$	$29.6 \pm 0.2$	$28.7 \pm 0.3$
ST <sup>-1</sup> ( $\text{mN m}^{-1}$ )	$31.1 \pm 0.7$	$31.3 \pm 0.7$	$31.3 \pm 0.2$	$32.0 \pm 0.6$	$31.9 \pm 0.8$	$39.9 \pm 1.9$	$31.9 \pm 0.9$	$30.8 \pm 0.4$
ST <sup>-2</sup> ( $\text{mN m}^{-1}$ )	$42.3 \pm 5.3$	$42.3 \pm 2.1$	$44.0 \pm 2.7$	$47.8 \pm 3.4$	$45.6 \pm 5.4$	$70.4 \pm 0.8$	$44.4 \pm 2.4$	$38.3 \pm 1.8$
E <sub>24</sub> (%)	$32.3 \pm 1.5$	$39.7 \pm 3.9$	$11.2 \pm 1.8$	$0.0 \pm 0.0$	$41.0 \pm 2.8$	$0.0 \pm 0.0$	$31.2 \pm 8.8$	$43.3 \pm 6.7$
[Biomass] ( $\text{g l}^{-1}$ )	$1.270 \pm 0.044$	$1.066 \pm 0.092$	$1.184 \pm 0.065$	$1.662 \pm 0.044$	$1.243 \pm 0.032$	$1.396 \pm 0.074$	$0.997 \pm 0.032$	$1.534 \pm 0.094$
[Surfactin] ( $\text{mg l}^{-1}$ )	$789.3 \pm 107.6$	$844.1 \pm 53.8$	$799.6 \pm 67.9$	$703.2 \pm 85.6$	$758.2 \pm 138.5$	$110.5 \pm 25.0$	$790.7 \pm 62.2$	$931.8 \pm 42.7$
<i>Isolate #311</i>								
ST ( $\text{mN m}^{-1}$ )	$28.5 \pm 0.4$	$27.9 \pm 0.2$	$28.5 \pm 0.2$	$28.5 \pm 0.2$	$29.5 \pm 0.3$	$29.1 \pm 0.1$	$29.4 \pm 0.1$	$28.7 \pm 0.4$
ST <sup>-1</sup> ( $\text{mN m}^{-1}$ )	$31.3 \pm 0.2$	$32.5 \pm 1.2$	$31.3 \pm 1.8$	$30.6 \pm 1.2$	$33.0 \pm 0.6$	$31.5 \pm 0.7$	$35.0 \pm 0.7$	$31.2 \pm 0.8$
ST <sup>-2</sup> ( $\text{mN m}^{-1}$ )	$44.1 \pm 4.0$	$46.5 \pm 4.9$	$37.0 \pm 1.7$	$43.2 \pm 2.8$	$55.4 \pm 4.2$	$54.0 \pm 2.6$	$68.7 \pm 3.3$	$39.2 \pm 6.1$
E <sub>24</sub> (%)	$4.7 \pm 2.2$	$37.3 \pm 2.8$	$0.0 \pm 0.0$	$0.0 \pm 0.0$	$37.2 \pm 3.2$	$0.0 \pm 0.0$	$25.0 \pm 7.1$	$32.8 \pm 2.2$
[Biomass] ( $\text{g l}^{-1}$ )	$1.404 \pm 0.038$	$1.002 \pm 0.092$	$0.934 \pm 0.041$	$1.632 \pm 0.023$	$1.080 \pm 0.062$	$0.954 \pm 0.032$	$0.874 \pm 0.088$	$1.242 \pm 0.024$
[Surfactin] ( $\text{mg l}^{-1}$ )	$828.9 \pm 103.1$	$765.8 \pm 126.1$	$980.7 \pm 45.4$	$851.0 \pm 73.0$	$537.1 \pm 107.5$	$574.6 \pm 66.2$	$196.6 \pm 85.8$	$980.1 \pm 46.7$
<i>Isolate #573</i>								
ST ( $\text{mN m}^{-1}$ )	$30.3 \pm 0.9$	$28.4 \pm 0.3$	$29.6 \pm 0.7$	$30.0 \pm 0.1$	$29.9 \pm 0.6$	$32.7 \pm 0.6$	$30.1 \pm 0.5$	$29.5 \pm 0.5$
ST <sup>-1</sup> ( $\text{mN m}^{-1}$ )	$34.3 \pm 2.5$	$33.0 \pm 1.3$	$32.6 \pm 0.3$	$36.3 \pm 1.1$	$35.2 \pm 2.0$	$53.2 \pm 2.4$	$32.6 \pm 0.5$	$33.6 \pm 1.5$
ST <sup>-2</sup> ( $\text{mN m}^{-1}$ )	$54.4 \pm 5.4$	$46.3 \pm 3.0$	$44.6 \pm 2.7$	$57.9 \pm 4.1$	$57.5 \pm 5.5$	$71.7 \pm 0.2$	$51.6 \pm 5.1$	$56.7 \pm 5.1$
E <sub>24</sub> (%)	$33.3 \pm 1.5$	$38.8 \pm 4.4$	$35.0 \pm 2.3$	$0.0 \pm 0.0$	$41.7 \pm 1.8$	$0.0 \pm 0.0$	$27.3 \pm 5.6$	$25.0 \pm 7.1$
[Biomass] ( $\text{g l}^{-1}$ )	$1.154 \pm 0.050$	$1.030 \pm 0.057$	$1.466 \pm 0.065$	$1.708 \pm 0.030$	$1.020 \pm 0.044$	$1.562 \pm 0.015$	$1.500 \pm 0.029$	$1.762 \pm 0.080$
[Surfactin] ( $\text{mg l}^{-1}$ )	$1500.2 \pm 251.5$	$2158.5 \pm 240.2$	$2288.9 \pm 210.5$	$1369.9 \pm 169.9$	$1129.5 \pm 266.7$	$157.8 \pm 16.8$	$1853.5 \pm 305.7$	$1496.9 \pm 300.6$

of biosurfactant when compared with isolates #309 and #311. The critical micelle concentrations for these crude biosurfactants were determined in a previous work:  $20 \text{ mg l}^{-1}$  for isolates #309 and #311, and  $30 \text{ mg l}^{-1}$  for isolate #573 [28]. Therefore, biosurfactants produced by isolates #309 and #311 are more efficient than the ones produced by isolate #573, which explains why isolate #573 although producing higher amounts of biosurfactant provides similar (or higher, i.e. less active surfactant) surface tension values than the obtained with the other two isolates.

Abdel-Mawgoud et al. [31] reported the highest biosurfactant production using sodium nitrate and ammonium nitrate as nitrogen sources. Other authors reported the highest biosurfactant production using urea [32,35]. Some *Bacillus* isolates do not use ammonium sulfate for growth or biosurfactant production, but they can use ammonium nitrate, potassium nitrate or sodium nitrate [32].

The highest emulsifying indexes were obtained with sodium nitrate for isolate #573 (41.7%); while for isolate #309, similar results were obtained using yeast extract and sodium nitrate (43.3% and 41.0%, respectively); and for isolate #311, the highest emulsifying indexes were obtained with ammonium nitrate and sodium nitrate (37.3% and 37.2%, respectively). Dastgheib et al. [33] reported that sodium nitrate was the best substrate for emulsifier production, followed by urea, yeast extract and peptone.

Taking into account the amounts of biosurfactant produced with the different carbon and nitrogen sources, and in order to standardize the medium for all isolates, sucrose and ammonium nitrate were selected for the production of biosurfactants to be further used in the chemical characterization experiments. Moreover, the use of sucrose and ammonium nitrate represents a good compromise between biosurfactant yields and the costs associated with their production. More complex nitrogen sources provide higher emulsification indexes; however their cost is also higher which makes a potential application in MEOR economically unfeasible. Also, this medium was found to be adequate for the three isolates (i.e. could be considered as standard medium) and nitrate is crucial for microbial growth under the low oxygen concentrations present in reservoirs.

## 3.2. Chemical characterization

### 3.2.1. FTIR-ATR

Firstly, fast and direct characterizations of the biosurfactants produced by each isolate were performed using a FTIR-ATR analysis

(Fig. 1). The spectra obtained for the three isolates showed a great similarity between them. It can be clearly observed characteristic absorbance bands of peptides at  $3305 \text{ cm}^{-1}$  (NH-stretching mode); at  $1643 \text{ cm}^{-1}$  ( $1645 \text{ cm}^{-1}$  for isolate #311) resulting from the stretching mode of the CO-N bond; and at  $1543 \text{ cm}^{-1}$  from the deformation mode of the N-H bond combined with C-N stretching mode. In addition, it is also clear the presence of aliphatic chains ( $-\text{CH}_3$ ;  $-\text{CH}_2-$ ), represented by the bands between  $2957$ – $2855 \text{ cm}^{-1}$ , and  $1465$ – $1368 \text{ cm}^{-1}$ . The band observed at  $1734 \text{ cm}^{-1}$  is characteristic of a carbonyl group. This first FTIR-ATR analysis indicates the presence of aliphatic hydrocarbons combined with a peptide moiety that is characteristic of lipopeptide biosurfactants, as it has been previously described in the literature [34,36].

### 3.2.2. <sup>1</sup>H NMR spectroscopy

The characterization of biosurfactants produced by *B. subtilis* strains using NMR spectroscopy has been described in the literature [38–40]. Therefore, the composition of biosurfactants obtained from isolates #309, #311 and #573 was probed by <sup>1</sup>H NMR analysis (Fig. 2).

The three spectra showed two main regions corresponding to resonance of  $\alpha$ -carbon protons (3.5–5.5 ppm) and side-chain protons (0.25–3.0 ppm) [41,42]. The differences observed in the spectra are in chemical shifts from the region correspondent to the amide protons (6.5–10 ppm), in which the NMR spectrum is less defined. All spectra showed some similarity with the standard surfactin (spectra for standard surfactin reported by Wei and Chu [43]). However, it is clear the presence of distinct regions relatively to the standard molecule, as well as among the three biosurfactant extracts herein studied. The intense singlet at 3.47 ppm observed in the biosurfactants extracted from isolates #311 and #573 is similar with that observed in <sup>1</sup>H NMR spectra of lipopeptide monoesters previously reported in literature [38–40], thus suggesting the presence of one  $\text{OCH}_3$  group in those biosurfactants. The presence of the methyl esters in the structure of surfactins has been related with an increase of its hydrophobicity and, consequently an increment of their surfactant powers, antifungal and hemolytic activities [39]. On the other hand, for the three extracts, an intense singlet at 5.28 ppm (5.30 ppm for isolate #309) was observed. This resonance probably corresponds to the H-3 (terminal proton) of the fatty acid chain, which has also been previously observed by other authors [39,40].

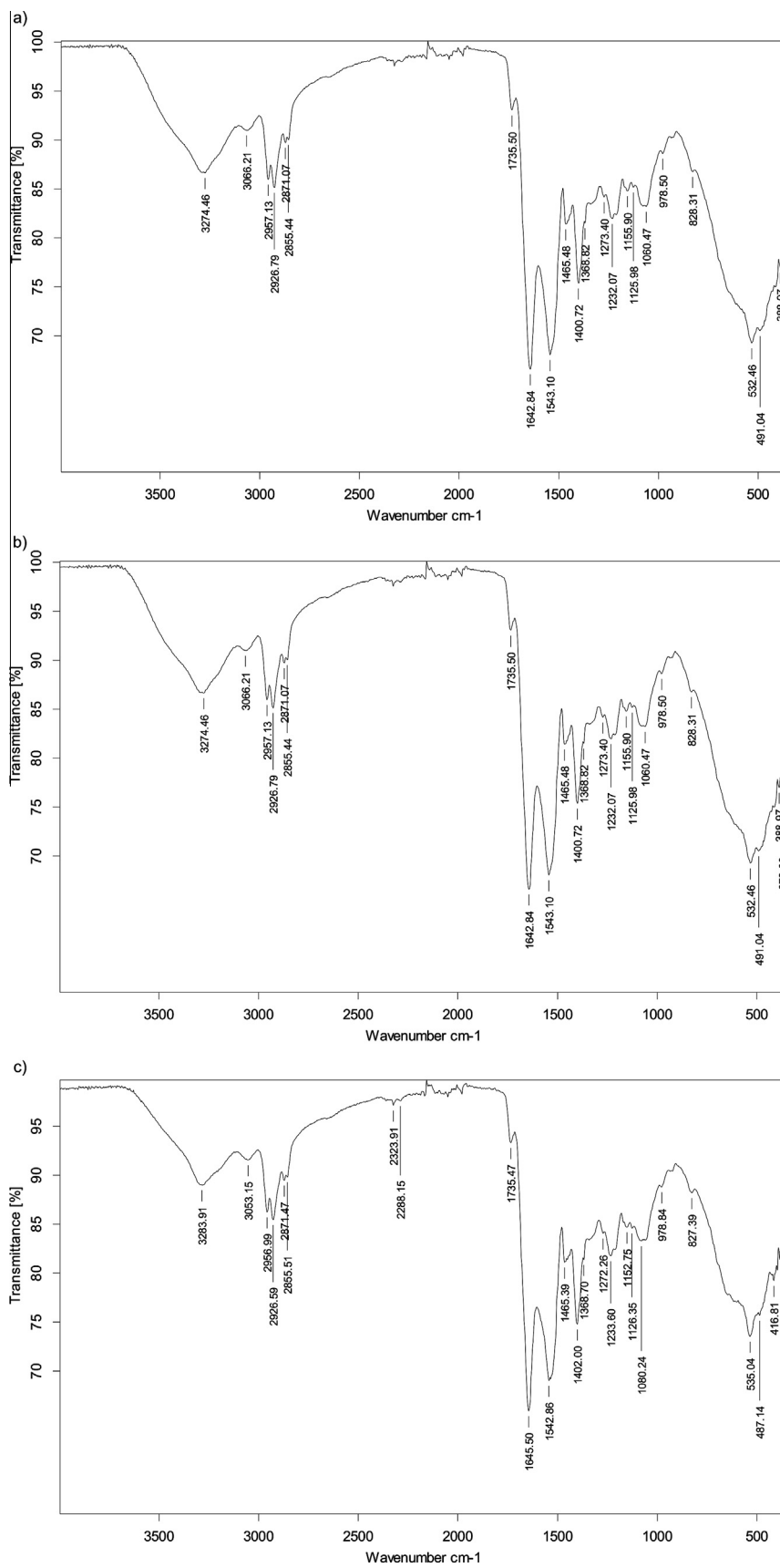


Fig. 1. FTIR-ATR spectra for biosurfactant extracts produced by isolates #309 (a), #311 (b) and #573 (c).

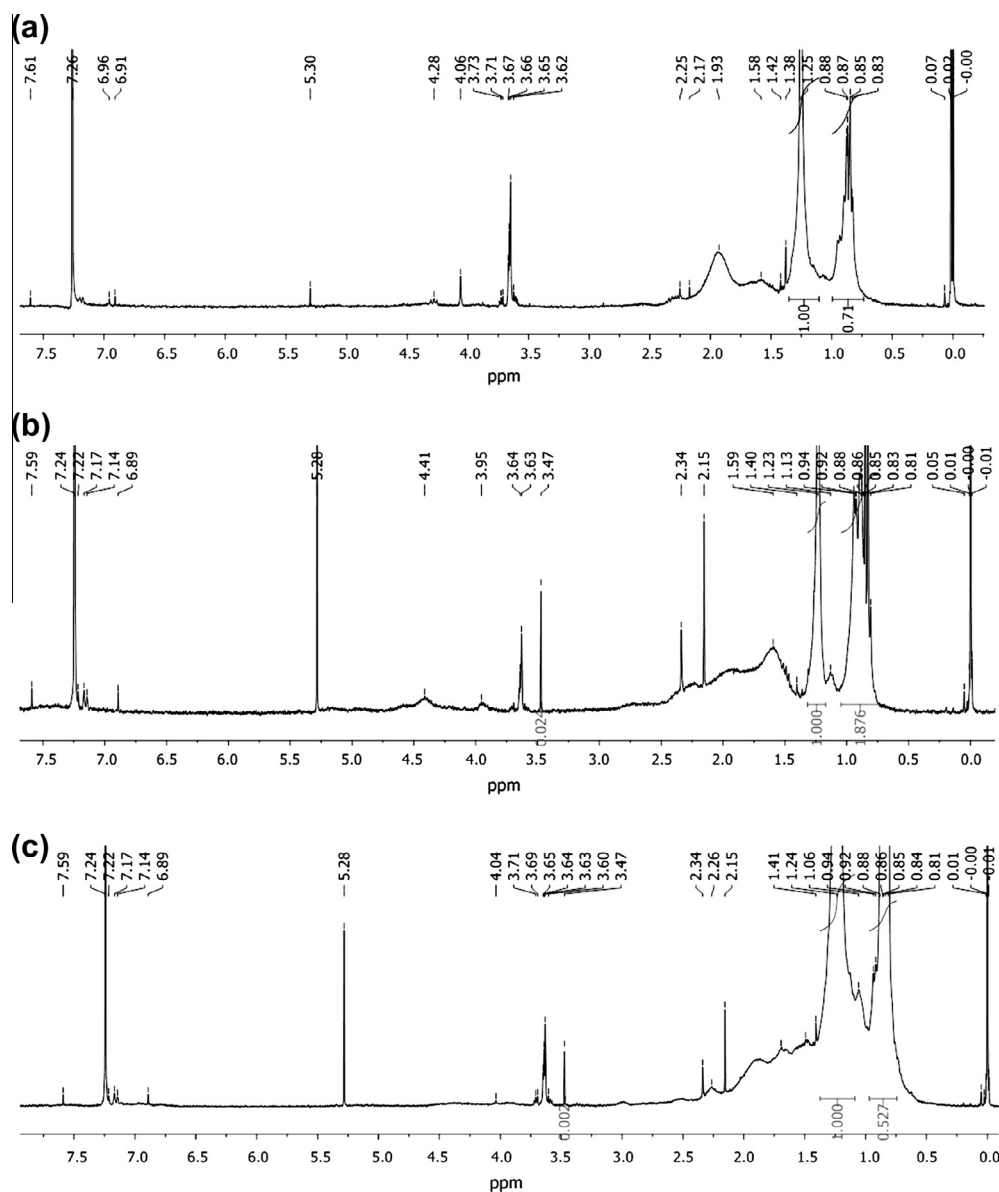


Fig. 2. <sup>1</sup>H NMR spectra for biosurfactant extracts produced by *B. subtilis* isolates #309 (a), #311 (b) and #573 (c).

The integral relation of the two different regions of aliphatic chains (approximately 1.55–1.25 ppm and 0.80–0.90 ppm) can give an important correlation between the presence of *n*-surfactin and *iso*- or/and *anteiso*-surfactins. The results showed a higher presence of *iso* forms in the biosurfactants recovered from the supernatant of the isolate #311, and lower concentrations of those forms in the biosurfactant recovered from isolate #573. According to Youssef et al. [44], the surface activity of lipopeptides with structures analogous to surfactin increases when the ratio of *iso*- to *normal*-even-numbered fatty acids is increased. In our case, lower surface tension values ( $ST^{-2}$ ) and CMC were obtained for isolate #311 when compared with the isolate #573. However, for lichenysin, iturin and mycosubtilin an opposite behavior has been reported, and an increase in the percentage of branched-chain fatty acids was found to decrease the activity of the biosurfactant, whereas an increase in the percentage of straight-chains increased their surface activity [39]. This may be due to the different amino acid composition of those biosurfactants, which can result in different properties.

The <sup>1</sup>H NMR analysis of the different biosurfactant extracts produced by different isolates demonstrated the presence of different

surfactins mixtures, supporting the different surface activities and emulsifying properties previously discussed. Each surfactin mixture obtained with the *Bacillus* isolates herein studied was further characterized by mass spectrometry.

### 3.2.3. Mass spectrometry

The MALDI-TOF spectra of the biosurfactants obtained from each isolate (#309, #311 and #573) are shown in Fig. 3. The three mass spectra show that there are one well-resolved group of peaks at *m/z* values between 1000 and 1060. This group could be attributed to the different variants of surfactins, as previously described by Vater et al. [45]. Each surfactin mixture extract has peaks which can be attributed to the protonated forms, with sodium adducts. Each isolate spectrum displays three  $[M + Na]^+$  peaks at *m/z* = 1030.6, 1044.6 and 1058.7, that correspond to the heptapeptide moiety (L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu) characteristic of surfactins, linked to a C<sub>13</sub>, C<sub>14</sub> or C<sub>15</sub> hydroxy fatty acid chain, respectively (Fig. 4). The spectra here reported are very similar to those obtained by Vater et al. [45] and Leenders et al. [46]. In addition, it is possible to observe the presence of other surfactins in the extract mixtures, although in lower amounts. These results confirm

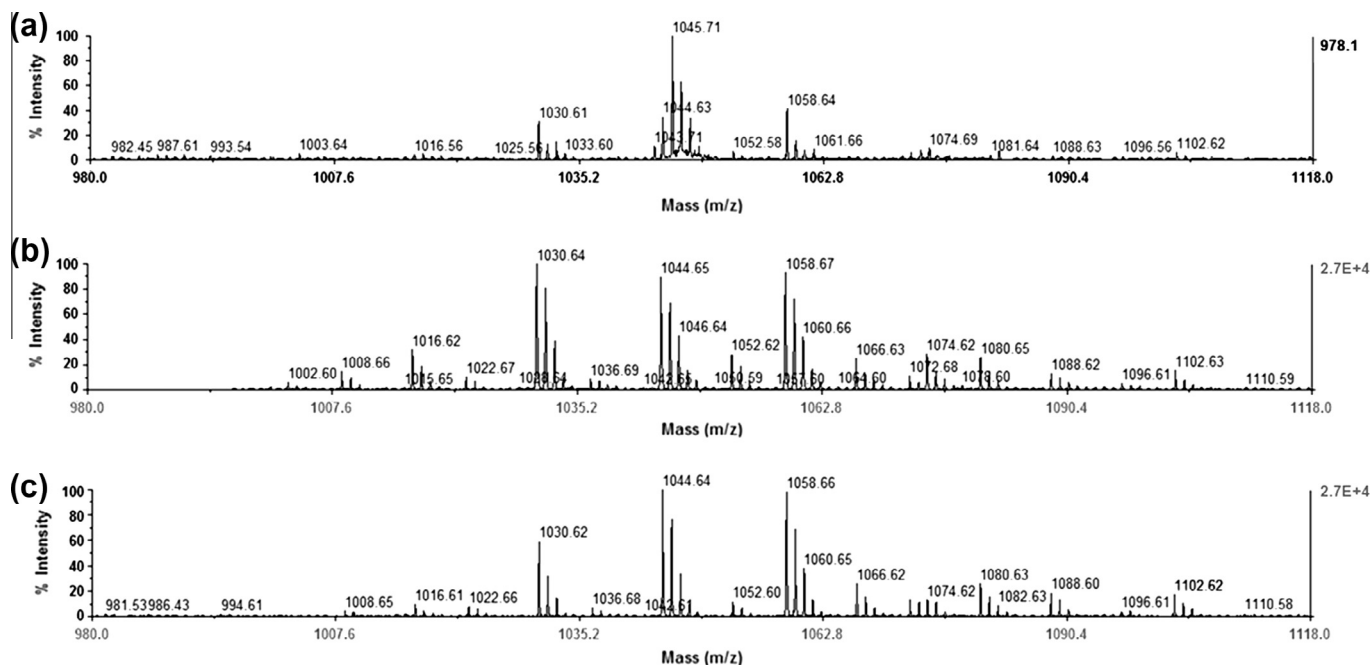


Fig. 3. MALDI-TOF spectra for surfactin-like extracts produced by *B. subtilis* isolates #309 (a), #311 (b) and #573 (c).

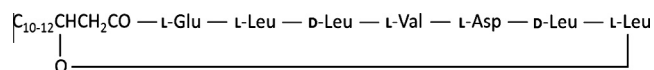


Fig. 4. Structure of surfactin.

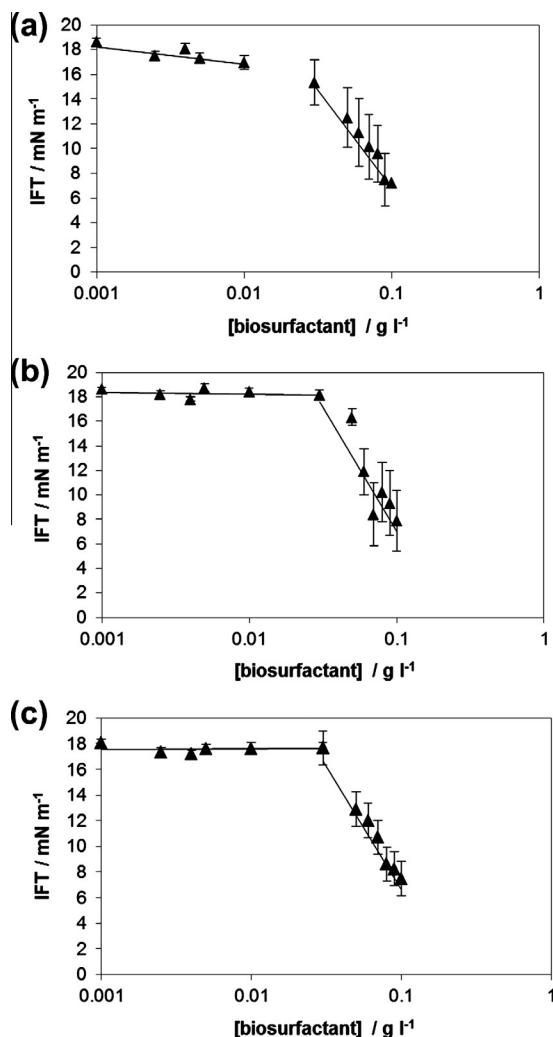
the data previously obtained by  $^1\text{H}$  NMR spectra, where it was clearly shown the presence of some mono-methyl ester surfactins, as well as the existence of different types of biosurfactants with different properties synthesized by the three isolates studied. Furthermore, comparing the three different spectra (Fig. 3) it was possible to found similarity between the mixtures obtained by isolate #311 and #573, in which approximate amounts of  $\text{C}_{13}$ -,  $\text{C}_{14}$ -, and  $\text{C}_{15}$ -surfactin are visible. On the other hand, the spectrum of the biosurfactant produced by the isolate #309 exhibited a more intense peak at 1045.7, corresponding to higher amounts of  $\text{C}_{14}$ -surfactin in the mixture. Youssef et al. [44] showed that *iso*- $\text{C}_{14}$  surfactin exhibits the optimum hydrophilic–lipophilic balance required for optimum surface activity. Also, Bacon et al. [47] reported that surfactins with  $\text{C}_{-14}$  acyl chains possess a higher surface activity. This could explain the higher efficiency of biosurfactants produced by isolate #309 when compared with the isolate #573.

### 3.3. Interfacial water–oil tension properties

To use these biosurfactants in MEOR processes it is important to first understand their interfacial properties in liquid–liquid equilibrium. Although several researchers have studied the surface activities at air/water [28,31–33,48,49] and the oil/water/rock systems wettability (by the contact angle measurement) [50,51], only few reports present information on the properties of lipopeptides at the liquid/liquid interfaces [52,53]. Therefore, to overcome this scarcity, the interfacial tension of a water/oil system was studied for each crude biosurfactant at different concentrations (Fig. 5). The analysis of the interfacial tension values showed that the biosurfactants produced by the isolates #311 and #573 exhibit a similar ability to decrease the IFT of the Arabian light crude oil/water system. These two biosurfactant mixtures only showed surfactant activities above their CMC (0.02 and 0.03  $\text{g l}^{-1}$ , respectively, as

determined in our previous work [28]), while in the case of the biosurfactant produced by the isolate #309 it occurred a slight decrease of the IFT before the CMC was reached (0.02  $\text{g l}^{-1}$  [28]), being this decrease more significant afterwards. This may be due to the higher percentage of  $\text{C}_{14}$ -surfactin in the mixture of biosurfactants produced by this isolate, as discussed previously, whereas the similar interfacial-activities observed with the biosurfactants produced by #311 and #573 are in accordance with their surfactin composition, earlier characterized by  $^1\text{H}$  NMR and mass spectrometry. The three biosurfactant mixtures were found to reduce the IFT values up to 5  $\text{mN m}^{-1}$ . However, higher IFT decreases were expected based on reports from the literature [52]. Yet, the methodology herein used presents some limitations regarding the drop formation, since for biosurfactant concentrations above 0.1  $\text{g l}^{-1}$  the drop is unstable originating its immediate release, thus for higher biosurfactant concentrations it was not possible to determine the IFT values. Regarding the chemical surfactants Enordet and Petrostep, their CMCs were found to be 1  $\text{g l}^{-1}$ . For a concentration of 0.1  $\text{g l}^{-1}$  (the highest biosurfactant concentration tested) the IFT values were similar to those obtained with demineralized water (about 21  $\text{mN m}^{-1}$ ), whereas for a concentration of 1  $\text{g l}^{-1}$ , Enordet and Petrostep reduced the IFT to 6.0 and 10.5  $\text{mN m}^{-1}$ , respectively. The results obtained show that biosurfactants studied in this work are more efficient than chemical surfactants. Al-Bahry et al. [54] observed that surfactins produced by a *B. subtilis* strain grown on molasses reduced the IFT between water and *n*-hexadecane from 23 to 5  $\text{mN m}^{-1}$  at a concentration of 2.2  $\text{g l}^{-1}$ . However, when higher concentrations of biosurfactant were used, the IFT was reduced up to 1  $\text{mN m}^{-1}$ . Deleu et al. [52] reported the reduction of the dodecane/water IFT to values around 2  $\text{mN m}^{-1}$  using surfactin produced by *B. subtilis*, being this reduction higher than the observed with other biosurfactants, such as iturin A (14.94  $\text{mN m}^{-1}$ ) and fengycin (11.63  $\text{mN m}^{-1}$ ). Urum and Pekdemir [53] studied the ability of several biosurfactants to decrease the IFT of *Ekofisk* crude oil and reported that biosurfactants have a higher potential than the chemical surfactant SDS (7.0  $\text{mN m}^{-1}$ ), as for example lecithin (5.0  $\text{mN m}^{-1}$ ), rhamnolipid (4.5  $\text{mN m}^{-1}$ ), saponin (6.0  $\text{mN m}^{-1}$ ) and tannin (4.5  $\text{mN m}^{-1}$ ). In summary, the reduction of IFT observed indicates the ability of the surfactin mixtures produced by the three *B. subtilis* isolates herein studied to





**Fig. 5.** Interfacial tension values ( $\text{mN m}^{-1}$ ) versus logarithm of biosurfactant concentration ( $\text{g l}^{-1}$ ) for each biosurfactant extract from *B. subtilis* isolates #309 (a), #311 (b) and #573 (c).

**Table 3**

Percentages of oil recovered by the different biosurfactants and chemical surfactants at a concentration of  $1 \text{ g l}^{-1}$ . Results represent the average of three independent experiments  $\pm$  standard deviation.

Biosurfactant/chemical surfactant	% of oil recovered
<i>B. subtilis</i> #309	$22.1 \pm 2.4$
<i>B. subtilis</i> #311	$19.0 \pm 1.6$
<i>B. subtilis</i> #573	$18.8 \pm 1.1$
Enordet	$11.7 \pm 1.9$
Petrostep	$8.7 \pm 1.3$
Control	$0.0 \pm 0.0$

remove oil from reservoir. As the IFT between crude oil and water is reduced, the capillary force between the oil and the reservoir rock is also reduced. Therefore, this reduction leads to an increased mobilization of the oil in the reservoir which is of great interest to enhance oil recovery in mature reservoirs.

#### 3.4. Application of chemical surfactants and biosurfactants in removal of crude oil from sand

The performance of biosurfactants and chemical surfactants in oil recovery was studied using crude oil contaminated sand. As can be seen from the results obtained (Table 3), the different

biosurfactants at a concentration of  $1 \text{ g l}^{-1}$  recovered between 19% and 22% of oil, whereas the recoveries obtained with the chemical surfactants at the same concentration were between 9% and 12%. From the results obtained it can be concluded that these biosurfactants are more effective in oil recovery when compared with the chemical surfactants Enordet and Petrostep.

#### 4. Conclusion

In the current work, the biosurfactant production by three *B. subtilis* strains was optimized using different carbon and nitrogen sources. The strains were found to produce biosurfactants with better surface-activities and emulsification properties when sucrose was used as carbon source. Crude biosurfactant mixtures were found to be mainly constituted of surfactins with an heptapeptide moiety (L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu), linked to a C<sub>13</sub>, C<sub>14</sub> or C<sub>15</sub> hydroxy fatty acid chain. Although several similarities were found among the biosurfactant mixtures produced by the different isolates, it was evident the presence of different variants of surfactins that could be implicated in the distinct emulsification and surface-active properties found. These biosurfactant mixtures were able to decrease the interfacial tension of Arabian light crude oil/water system more efficiently than chemical surfactants, and showed better results in oil recovery, thus suggesting their interest for use in MEOR processes.

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