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PERFORMANCE OF A BIOSURFACTANT PRODUCED BY *Bacillus subtilis* LAMI005 ON THE FORMATION OF OIL / BIOSURFACTANT / WATER EMULSION: STUDY OF THE PHASE BEHAVIOUR OF EMULSIFIED SYSTEMS

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Abstract - In this study, the phase behaviour of emulsified systems (oil + biosurfactant + water) was analyzed. The biosurfactant was produced in a 4-L batch bioreactor by *Bacillus subtilis* LAMI005*,* using residual glycerine from biodiesel production as a carbon source. Fourier Transform Infrared Spectroscopy (FT-IR), Nuclear Magnetic Resonance (NMR), and High Performance Liquid Chromatography (HPLC) analyses demonstrated that the biosurfactant produced by *Bacillus subtilis* (LAMI005) consists of a lipopeptide similar to surfactin. The influences of temperature and the composition of oil + biosurfactant + water were determined by using phase diagrams. Three types of oil were used, namely: motor oil, hydrogenated naphthenic oil (NH140) and castor bean oil. The emulsified systems were analyzed using optical micrography. The results presented here indicated that the biosurfactant produced in this work presents a potential use as stabilizing agent for oil-in-water emulsions.
Keywords: Biosurfactants; Emulsions; Phase diagrams; Residual glycerol; *Bacillus subtilis*.

INTRODUCTION

Biosurfactants, one of the main classes of natural surfactants, are metabolic products of microbial origin (bacteria, fungi and yeasts) and exhibit surfactant properties, i.e., reduction of surface tension (Fiechter, 1992). Surfactin is produced by several strains of *Bacillus subtilis* (Nitschke and Pastore, 2002; Arima *et al*., 1968; Kowall *et al*., 1998). It is a cyclic peptide, with seven amino acids bound to a β-hydroxy fatty acid chain (which can contain from 13 to 15 carbon atoms). This structural variability allows for different homologous compounds and isomers to occur (Kowall *et al*., 1998; Lang, 2002; Kluge *et al*., 1988; Barros *et al*., 2007).

In general, emulsifiers contain a segment of the molecule that has amphipathic properties, forming a molecular film oriented according to its polarity, which reduces surface and interfacial tension between immiscible liquids. This characteristic is responsible for making mixtures of different polarities compatible, thus facilitating emulsion formation.

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Attwood and Florence (1983) define emulsions as heterogeneous systems of a liquid dispersed with another in the form of droplets that are generally larger than 0.1 μm in diameter. The occurrence of the following fundamental requirements is necessary in order for an emulsion to form: the coexistence of two immiscible liquids, sufficient agitation to allow one of the liquids to disperse, and an emulsifying agent (Arnold and Smith, 1992). Temperature is an important factor in stabilizing emulsions, since a higher temperature reduces the viscosity of oil and promotes a difference in density between the aqueous and oil phases. It also helps to increase the Brownian motion of droplets during the dispersal phase, and aids in the dissolution of emulsifying agents (Arnold and Smith, 1992). Another important factor in emulsion stabilization is the droplet size distribution, which has a direct impact on the viscosity of emulsions (Kokal, 2006). The destabilization of emulsions is governed by three main mechanisms, namely: creaming or sedimentation, flocculation, and coalescence. The first two phenomena are characterized by the effect of gravity, when the density of droplets and the density of the medium are unequal. Here, the emulsion separates into two distinct layers, with the droplets forming a cream or sediments, and leaving behind a liquid supernatant. The collision of particles can provoke flocculation, which can, in turn, lead to coalescence and the formation of larger globules. Flocculation is characterized by the separation of droplets. In the case of coalescence, the interfacial film must be interrupted, resulting in eventual rupture (Tadros, 2005).

In the literature, there are studies on the structure and properties of oil-in-water and water-in-oil emulsions using synthetic surfactants (Friberg *et al*., 2009; Binks and Rodrigues, 2009; Pichot *et al*., 2010). However, there is little research on the phase behaviour of oil-to-water systems emulsified by biosurfactants, notably with the use of surfactin. Therefore, phase diagrams of oil-in-water-in-biosurfactant (surfactin) were determined, varying the proportion of water/oil. For that, a microscopic technique has been used in order to determine the droplet size in the various systems studied.

MATERIALS AND METHODS

Microorganisms and Conditions of Maintenance

The strain *Bacillus subtilis* LAMI005, identified by the rRNA 16S sequence and deposited in the Genbank database with the following access number: FJ413046, was maintained in APGE medium (containing: peptone 5.0 g/L, glucose 5.0 g/L, yeast extract 2.5 g/L and Agar 15.0 g/L). The culture was kept on slants, refrigerated at 4 °C and replicated every 60 days.

Experimental Conditions for the Production of Biosurfactants

Culture Medium

The culture medium was prepared according to Móran *et al*. (2000), with slight modifications. The composition (in g/L) of the medium was: $(NH_4)_2SO_4$ (1.0); Na_2HPO_4. $7H_2O$ (7.2); KH_2PO_4 (3.0); NaCl (2.7); $MgSO_4$. $7H_2O$ (0.6); glycerol (20.0). The medium was sterilized in an autoclave at 110 °C for 10 minutes. After sterilization, 0.1% (v/v) of a micronutrient solution, previously sterilized by filtration (0.45 μm, Millipore, USA), was added. The composition of the micronutrient solution was also described by Móran *et al*. (2000), as follows (in g/L): $ZnSO_4.7H_2O$ (10.9); $FeSO_4.7H_2O$ (5.0); $MnSO_4.H2O$ (1.54); $CuSO_4.5H_2O$ (0.39); $Co(NO_3)_2.6H_2O$ (0.25); $NaB_4O_7.10H_2O$ (0.17); EDTA (2.50).

Propagation of the Inoculum

The microorganism was replicated in a Petri plate containing (in g/L): $(NH_4)_2SO_4$ (1.0); Na_2HPO_4. $7H_2O$ (7.2); KH_2PO_4 (3.0); NaCl (2.7); $MgSO_4$. $7H_2O$ (0.6); glycerol (20.0); Agar (15.0), and incubated at 30 °C for 48 hours. After this, three loopfuls of microorganisms were transferred to 500 mL Erlenmeyer flasks containing 300 mL of the culture medium to propagate the inoculum. The flasks were incubated in a rotary shaker (Tecnal – TE240, São Paulo, Brazil) at 180 rpm and at 30 °C for 24 - 48 hours.

Production of Biosurfactants in a Continuous Stirred Tank Bioreactor

The biosurfactant was produced in a 4-L bioreactor (Marconi) operating with 3 L of available volume, using the culture medium previously described. A concentration of 10% v/v of inoculum (0.82 g/L) was used and the fermentation was conducted at 30 °C, 200 rpm and with 1 L/min aeration. The samples were collected at pre-defined intervals to determine biomass and later centrifuged at 10.000 g for 20 minutes at 4 °C to remove the cells. The cell-free supernatant was then analyzed for surface tension, biosurfactant concentration and substrate consumption. The kinetics of biosurfactant production was

studied in terms of yield factors, specific substrate uptake rate (q_s) and specific product yield (q_p), according to Doran *et al*. (1995).

Extraction and Purification of the Produced Biosurfactant

The extraction of the biosurfactant from the culture medium was carried out according to Rocha *et al*. (2009). The cell-free broth was submitted to an acid precipitation procedure, at pH adjusted to 2.0 by the addition of HCl 3 M. The resulting solution was left to rest for 12 hours (overnight) at 4 °C, to allow the precipitation of surfactin, which was collected by centrifugation at 10.000 g for 20 minutes at 4 °C. The precipitate (crude biosurfactant) was re-suspended in 8 mL of distilled water at pH 8.0. Liquid-liquid extraction was carried out using equal volumes of sample and dichloromethane (Vetec, São Paulo, Brazil). It was agitated for 5 minutes and left to rest for 1 hour in a funnel to allow phase settling to occur. All of the phases of the solvent were collected in beckers and evaporated at room temperature. The product was re-suspended in 2 mL of methanol (Vetec, São Paulo, Brazil) for later analysis. After filtration by membrane (0.45 μm, Millipore, USA), a purified biosurfactant was finally produced.

Phase Behaviour of Emulsified Systems

The influence of oil and water, and temperature on the stability of the oil + biosurfactant + water emulsion was evaluated by means of pseudo-binary phase diagrams. A solution of biosurfactant in water at pH 8.0 (0.8 g/L), here named the aqueous-phase, was mixed with hydrophobic compounds (motor oil, castor bean oil and NH140 oil) in screw cap test tubes, in different proportions of aqueous solution (ranging from 10 to 50%). These systems were homogenized by using a vortex apparatus for 2 minutes. The screw cap test tubes were exposed to temperatures ranging from 30 to 85 °C in a thermostatic bath (Tecnal, model TE-184), and each 10 minutes, the phase behaviour was verified to better observe the phase transitions.

Analytical Methods

Cellular Concentration

Cellular growth was indirectly determined through turbidimetry, using a spectrophotometer (Genesys 20 series) at 600 nm, where biomass (g/L) was determined using a calibration curve of biomass against optical density (Giro *et al*., 2009).

Glycerol Concentration

Glycerol was determined by a colorimetric method in order to quantify triglycerides, using an enzymatic kit (Katal Biotecnológica Indústria e Comércio Ltda/ Minas Gerais). This method consisted of adding 2 mL of a colour reagent and 0.02 mL of the cell-free metabolite broth sample to a test tube, which was homogenized and placed in a thermostatic bath (Tecnal, model TE - 0541/1) at 37 °C for 10 minutes. Later, the absorbance of the mixture was determined at 500 nm in a spectrophotometer (Spectronic ® 20 Genesys).

Stability of the Emulsion

The stability of the emulsion was determined with a modification of the method proposed by Cooper and Goldenberg (1987). In this analysis, 2 mL of biosurfactant solution in water at pH 8.0 (0.8 g/L) was added to a test tube, containing 2 mL of a hydrophobic source. This mixture was agitated by vortex for 2 minutes and left to rest for 24 hours at 28 °C. After this period, the stability of the emulsion was ascertained visually. In this work, stability was evaluated against different hydrophobic sources, such as: castor oil, motor oil, soy bean oil, gasoline, diesel, hydrogenated naphthenic oils (NH 20 and NH 140), N-hexadecane and kerosene.

Biosurfactant Concentration

The concentration of biosurfactant was determined by High Performance Liquid Chromatography, coupled with a UV detector (Waters, model 2487) at 205 nm and equipped with a Symmetry C18 reversed phase column (150 x 4.6 mm, 5 μm, Waters, Ireland). The composition of the mobile phase was 20% of trifluoroacetic acid (3.8 mM) and 80% of acetonitrile. The flow rate was 1 mL/min at 30 °C and the volume of sample injections was 20 μL. The samples were quantified using a calibration curve prepared with standard surfactin (95% pure) from Sigma-Aldrich (Yeh *et al*., 2005). The concentration of biosurfactant was calculated from the chromatographic peaks, according to Wei and Chu (1998).

Spectroscopy in the Infrared Region (FT-IR)

Analysis of the surfactant chemical groups was carried out using FTIR (Fourier Transform Infrared Spectroscopy) spectra. The spectra were registered using a Shimadzu FTIR-8300 spectrometer from 400

and 4000 cm^{-1}, using potassium bromide (KBr) as a support.

Nuclear Magnetic Resonance (NMR) Spectroscopy

The biosurfactant samples, purified by extraction, were analyzed by ^1H NMR spectroscopy in a solution of deuterated methanol. The nuclear magnetic resonance spectra were obtained with a Brucker DRX-500 spectrometer operating at a frequency of 500 MHz for ^1H.

Optical Micrographs of the Emulsions

Optical micrographs were produced using an *Olympus CH31* microscope (Southall, UK) with an attached camera and objective lens of 200 X with a resolution of 1024 x 720. Different photographs were taken of each lamina for statistical analysis. *Image Pro 6.0* software (Media Cybernetics) was used to measure the droplet size distribution (DSD).

RESULTS AND DISCUSSION

Production of Biosurfactant by *Bacillus subtilis* LAMI005 in a Bioreactor

Figure 1 shows profiles of cellular growth (X), biosurfactant production and substrate consumption. From 1n(X) versus time (data not shown), it was observed that the culture reached the stationary phase after 44 hours of cultivation, with a maximum concentration of 3.47 g/L in 54 hours. It should be noted that the exponential phase lasted approximately 8 hours. Thus, a maximum specific cell growth rate ($\mu_{Xmáx}$) of 0.10 h^{-1} was found, see Table 1. When studying surfactin production by *Bacillus subtilis* MUV4 from glucose, other authors (Suwansukho *et al*., 2008) obtained the specific growth rate ($\mu_{Xmáx}$) of 0.14 h^{-1}, which is similar to the value achieved in this work. Observing the production of biosurfactant in Fig. 1, it can be seen that the concentration of biosurfactant increased during the microorganism's exponential phase. In addition, in comparing cellular growth with biosurfactant production using the specific growth rates μ_X and μ_P (data not shown), one can conclude that the production of biosurfactant occurred in association with cellular growth. The production of biosurfactant by *Bacillus subtilis* LAMI005 reached a maximum value of 263.64 mg/L in 30 hours of cultivation, while a decline in product formation after 72 hours of fermentation was observed. In a previous work (Sousa *et al*., 2012), *Bacillus subtilis* LAMI005 was cultivated in shake flasks and a higher concentration of biosurfactant (441.06 mg/L) was achieved after 72 h, using the same glycerin concentration. Due to the agitation rate in comparison with the air flow rate, the system is completely flooded and the oxygen transfer rate is certainly limited during the cultivation process. It is important to mention that, during this process, an important amount of foam is produced. Nevertheless, a by-pass arrangement can be used in order to deal with foam formation by means of a foam collector that guarantees the recirculation of the liquid culture (Giro *et al*., 2009). These authors (Giro *et al*., 2009) achieved a maximum concentration of biosurfactant of 232 mg/L in 48 hours, which remained constant until 72 hours, using a mineral medium supplemented with glucose and fructose. Compared with these results from the literature, the production of biosurfactant by *Bacillus subtilis* LAMI005 using glycerine was more efficient, since higher concentrations of biosurfactant were obtained. Kim *et al*. (1997) studied the production of biosurfactant by *Bacillus subtilis* C9 in a mineral and glucose medium and reported that the formation of product was associated with cellular growth. Neves *et al*. (2007) also observed the connection between the specific growth rate (μ_x), the rate of substrate consumption (μ_s) and the rate of biosurfactant formation (μ_p) during the cultivation of *Bacillus atrophaeus* ATCC 9372 in 2.5 g/L of glucose and 10.0 g/L of casein. Desai and Banat (1997) used *Bacillus sp.* IAF-343 to produce biodispersan and also considered the formation of the product to be associated with cellular growth.

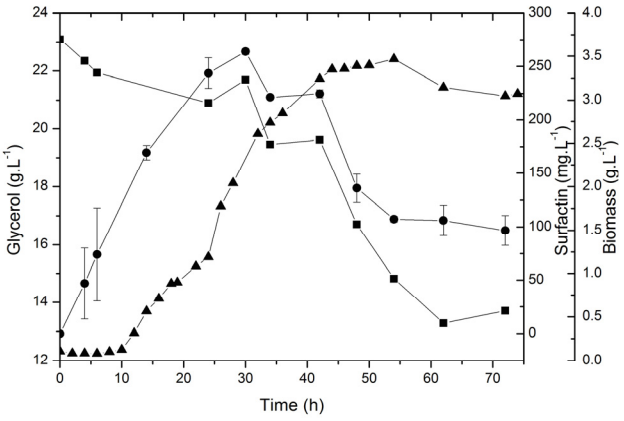

Figure 1: Profiles of cellular growth, production of biosurfactant, and substrate consumption in bioreactor tests using *Bacillus subtilis* LAMI005. (■) Substrate; (▲) Biomass; (●) Biosurfactant. Error bars represent the standard deviation.

Table 1: Product formation related to substrate consumption ($Y_{P/S}$) and dry cell biomass ($Y_{P/X}$), bacterial growth related to substrate consumption ($Y_{X/S}$), specific substrate utilization rate (q_s) and specific product yield (q_p) during growth of *Bacillus subtilis* LAMI005 in a 4-L batch bioreactor at 30 °C, 200 rpm and with 1 L.min^{-1} aeration.

Parameter	Value
$\mu_{Xmáx}$ (h^{-1})[a]	0.10
$Y_{P/S}$ (g g^{-1})[b]	0.11
$Y_{P/X}$ (g g^{-1})[b]	0.23
$Y_{X/S}$ (g g^{-1})[b]	0.49
q_s (g g^{-1} h)[c]	2.21
q_p (g g^{-1} h)[c]	0.16

[a] Calculated at the stationary phase; [b] Calculated at 24 h of fermentation; [c] Calculated at 6 h of fermentation

The cultivated strain consumed only 40% of the glycerol present in the culture medium, indicating that the carbon source does not appear to be a limiting substrate. A similar result was found by Davis *et al*. (1999) when they studied the production of surfactin by *Bacillus subtilis* ATCC 21332 in a bioreactor. These authors observed a large quantity of residual substrate when they used 30.0 g/L of glucose as the carbon source. They reported that production was not limited by the carbon source, since the production of surfactin grew until the end of the fermenting process, with a maximum concentration of 45.3 mg/L. Table 1 shows the product formation related to substrate consumption ($Y_{P/S}$) and cell biomass ($Y_{P/X}$), bacterial growth related to substrate consumption ($Y_{X/S}$), the specific substrate utilization rate (q_s) and specific product yield (q_p) during growth of *Bacillus subtilis* LAMI005. The kinetics of biosurfactant production by *Bacillus subtilis* LAMI005 showed a yield of biomass to substrate ($Y_{X/S}$) of 0.49, a yield of product to substrate ($Y_{P/S}$) of 0.11 and a yield of product to biomass ($Y_{P/X}$) of 0.23. Other authors (Suwansukho *et al*., 2008), studying surfactin production by *Bacillus subtilis* MUV4 from glucose, obtained similar values to $Y_{X/S}$, $Y_{P/S}$ and $Y_{P/X}$ of 0.713, 0.072 and 0.101, respectively. Vedaraman and Venkatesh (2011), who studied surfactin production by *Bacillus subtilis* MTCC 2423 from glucose and waste frying oils, determined the yield of biomass based on glucose (g biomass/g substrate) of 0.091, the yield of surfactin based on biomass (g surfactin/g biomass) of 0.23 and the yield of surfactin based on glucose (g of surfactin/g glucose) of 0.021. According to Davis *et al*. (1999), the kinetic parameters of surfactin production by *B. subtilis* ATCC 21332 may differ depending on the nutritional condition evaluated. They observed that $Y_{P/X}$, for instance, ranged from 0.0068 to 0.075 g/g.

Regarding the specific substrate utilization rate (q_S) and specific product yield (q_P), there are few data reported in the literature. For instance, Raza *et al*. (2007) studied the production of biosurfactant by a *Pseudomonas aeruginosa* mutant using vegetable oil refinery wastes and obtained q_S ranging from 1.20 to 1.26 g g^{-1} h and q_P ranging from 0.42 to 0.60 g g^{-1} h.

Structural Characteristics of the Biosurfactant Produced in the Bioreactor

Biosurfactant Concentration

Lipopeptides of *Bacillus* are classified into three families of cyclic compounds: iturin, surfactin, and fengycin. It is well known that these molecules can strongly affect the surface active properties of the solution (Raaijmakers *et al*., 2010). At this point our main goal was the use of different techniques (HPLC, FT-IR and NMR) to certify that *Bacillus subtilis* LAMI005 produced surfactin.

From HPLC analysis, it can be observed that the chromatogram of the biosurfactant sample produced by *Bacillus subtilis* LAMI005 showed similar retention peaks (A, B, C, D, E and F) to those observed with commercial surfactin (Sigma-Aldrich), see Fig. 2.

Infrared Spectroscopy (FT-IR)

Figure 3 shows the FT-IR spectra obtained from commercial surfactin (Sigma-Aldrich standard) compared with the biosurfactant produced by using *Bacillus subtilis* LAMI005. This analysis reveals that the biosurfactant produced presents the main characteristic groups of a surfactin molecule, indicating the presence of aliphatic hydrocarbon, as well as a peptide fraction. The most important absorption bands were assigned by comparison with spectra obtained from the literature (Joshi *et al*., 2008; Lin *et al*., 1994). In the spectrum shown in Fig. 3, seven main bands can be observed. The absorption band with a maximum of 3307 cm^{-1} corresponding to the N-H stretch can attributed to peptide residues. Another intense band with maxima of 2958 cm^{-1} and 2927 cm^{-1}, corresponding to the C–H (CH_3) and (CH_2) stretch, can be associated with the lipopeptide portion of the molecule. At 1726 cm^{-1}, a medium intensity band is observed that can be related to the absorption of C=O groups from lactonization. At 1651 cm^{-1}, a CO–N stretch points to the amide group. The bands at 1466 cm^{-1} and 1388 cm^{-1} indicate aliphatic chains (-CH_3,-CH_2). These results suggest that the biosurfactant produced by *Bacillus subtilis* LAMI005 in a medium containing glycerine is a cyclic lipopeptide, mainly surfactin.

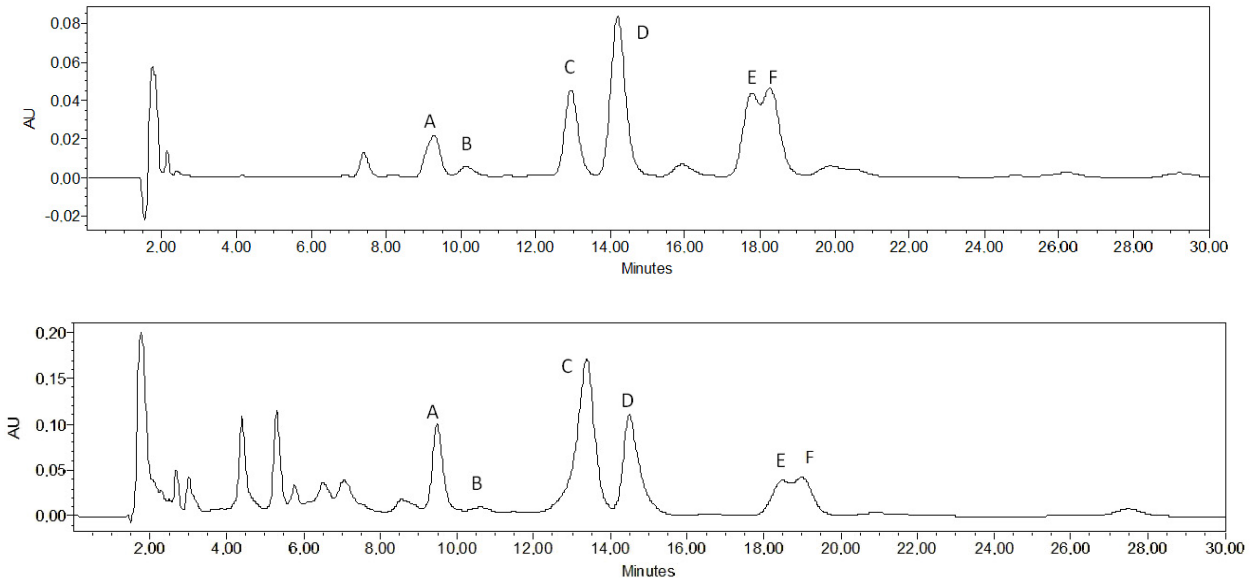

Figure 2: HPLC chromatogram of Sigma-Aldrich surfactin and of extracted biosurfactant sample, respectively. The final concentration of biosurfactant is equal to the sum of peaks A, B, C, D, E, F.

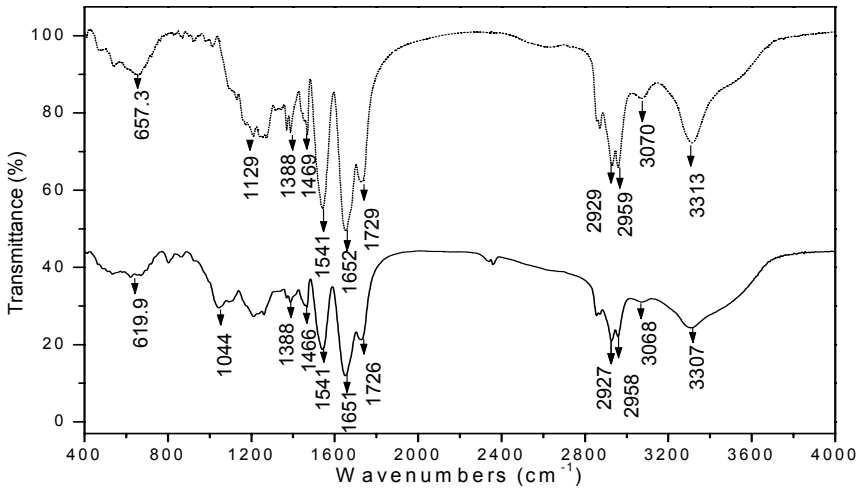

Figure 3: Infrared spectra of the Sigma-Aldrich standard surfactin molecule (dashed line) and of the extracted biosurfactant (line), respectively.

Nuclear Magnetic Resonance (NMR) Spectroscopy

The ^{1}H-NMR spectrum of the biosurfactant isolated in this study is shown in Fig. 4. A comparative analysis between the standard sample of commercial surfactin from Sigma-Aldrich (Yeh *et al*., 2005) and other biosurfactants found in the literature (Lin *et al*., 1994; Liu *et al*., 2009) reveals that the main structural characteristics were detected in the isolated biosurfactant sample, meaning that the structure of the molecule produced is similar to that of the lipopeptide produced by *Bacillus subtilis*, in other words, surfactin. The ^{1}H-NMR spectrum (Fig. 4) confirms the presence of peaks corresponding to NH (δ 7 – 7.8 ppm) and to CH (δ 3.9 - 5.0 ppm), representing amino acids that are probably arranged in a cyclic structure. Also present are CH_2 groups of aliphatic chains (δ 1.25 – 1.55 ppm) and the CHO ester group (probably from lactonization) at δ 5.3 ppm. In addition, the δ 3.7 ppm peak may indicate the presence of monoesters.

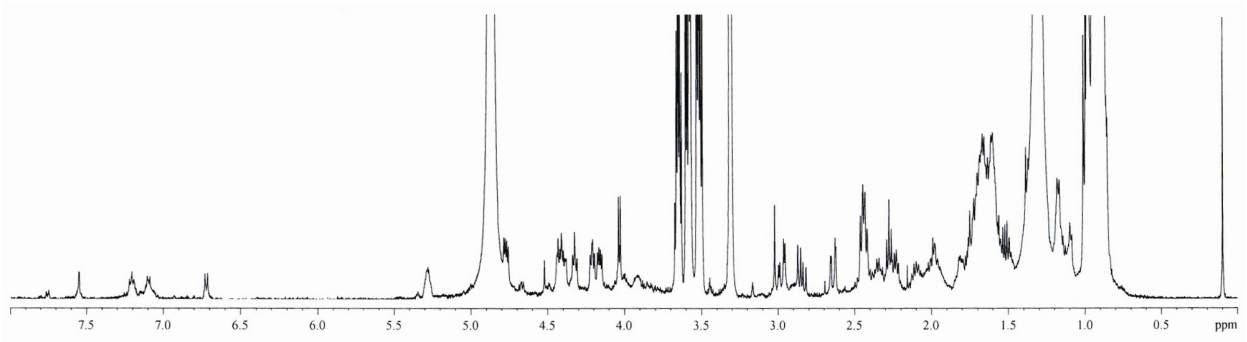

Figure 4: ^{1}H-NMR spectra of the extracted biosurfactant.

Behaviour of Phase Diagrams

Initially, emulsion stability of the biosurfactant produced by *Bacillus subtilis* LAMI005 against diverse hydrophobic sources was evaluated, using as reference the formation of an emulsion with stability during 24 hours at 28 °C. The best results were found by using motor oil, hydrogenated naphthenic oil (NH140) and castor bean oil. It is interesting to note that the biosurfactant presents an effective activity as an emulsifier agent for oils that show higher viscosity. Furthermore, it is interesting to mention that a shelf life analysis was performed for all oil + water + biosurfactant samples for at least two months. During this period the growth of microorganisms was not observed. This result can be attributed to the lipopeptides that present antimicrobial properties (Fernandes *et al*., 2007). This find can also be important for industrial applications, since, in the present case, a unique compound seems to have two important roles, as emulsifier, as well as antimicrobial agent.

Figure 5 shows the final result of the phase diagram analyses for (biosurfactant–motor oil), (biosurfactant–castor bean oil) and (biosurfactant–NH140 oil).

The studied systems were formed from an aqueous-phase (water at pH 8.0 and 0.8 g/L of biosurfactant) and an oil phase (motor oil, NH140 oil or castor bean oil). The diagram obtained for motor oil + biosurfactant + water system presents an aqueous-phase for a water cut above 30%. The diagram presents four specific regions, as follow: emulsion + oil (E + O), emulsion (E), emulsion + water + oil (E + W + O), and emulsion + water (E + W). The distribution of these regions depends on temperature and composition of surfactin. It is interesting to note that a sole emulsionated phase is formed at temperatures below 40 °C and surfactin composition until 0.3 v/v. For industrial applications, it could be interesting to work in this region, since phase separation is not observed. The castor oil + biosurfactant + water and NH140 oil + biosurfactant + water systems show similar results, with the presence of only two regions (E + W + O and E + W). In these cases, a unique emulsion phase region has not been observed. It can be seen that the separation region between E + W + O and E + W also depends on temperature and surfactin concentration, with an important dependence on temperature. The E + W + O region is larger for castor oil than for NH140 oil. For castor oil + biosurfactant + water the transition phase occurs at a temperature of 70 °C, except for 20% of surfactin. For NH140 oil + biosurfactant + water the temperature transition is reached at T = 80 °C, for surfactin concentration below 0.3 (above 0.4 the transition temperature decreases to 75 °C). This was also observed by Uddin *et al*. (2001) in a phase diagram for a water-in-surfactant binary system ($Si_{25}C_3EO_{15,8}$).

Microscopic measurements were carried out to observe the aspect and size of emulsion particles in phase transition regions for all oil + water + biosurfactant systems. Droplet size was measured immediately after the emulsification process. The droplet size distribution is shown in Fig. 6.

Using digital micrographic analysis with a relative frequency of 0.6 (60%), it can be observed that, at a temperature of 40 °C, in the motor oil + water + biosurfactant emulsion containing 20% biosurfactant solution, the diameter of the drops was around 1μm. Meanwhile, in the emulsion containing 50% of aqueous-phase for this system, an increase in temperature to 55 °C resulted in a lower relative frequency of drops of the same diameter. Although droplet size analyses have been performed only for freshly-formed emulsions, it can be suggested that the predominant process for the formation of non-emulsified oil in the points selected in Fig. 5 A is probably sedimentation. For the emulsion of 50% at 55 °C, its phase transition can probably be explained by coalescence. These inferences are due to the diameter ranges with higher frequency. For smaller-sized

droplets, it is more difficult for coalescence to occur when compared to sedimentation, as the droplets are too small and uniform (Kokal, 2006). In Fig. 6 B, a micrograph of the emulsion produced by the castor bean oil + water + biosurfactant system at 75 °C reveals more dispersed particles that have larger droplet diameters between 1 and 3 µm, with a frequency of 0.2 (20%). For NH140 oil + water + biosurfactant, droplets dispersed in a continuous medium could be observed with a diameter of between 1 and 3 µm at a frequency of 0.4 (Figures 5 C and 6 D). Nevertheless, it should be noted that in order to determine the dominat destabilization mechanism it would be necessary to carry out a kinetic analysis of droplet size.

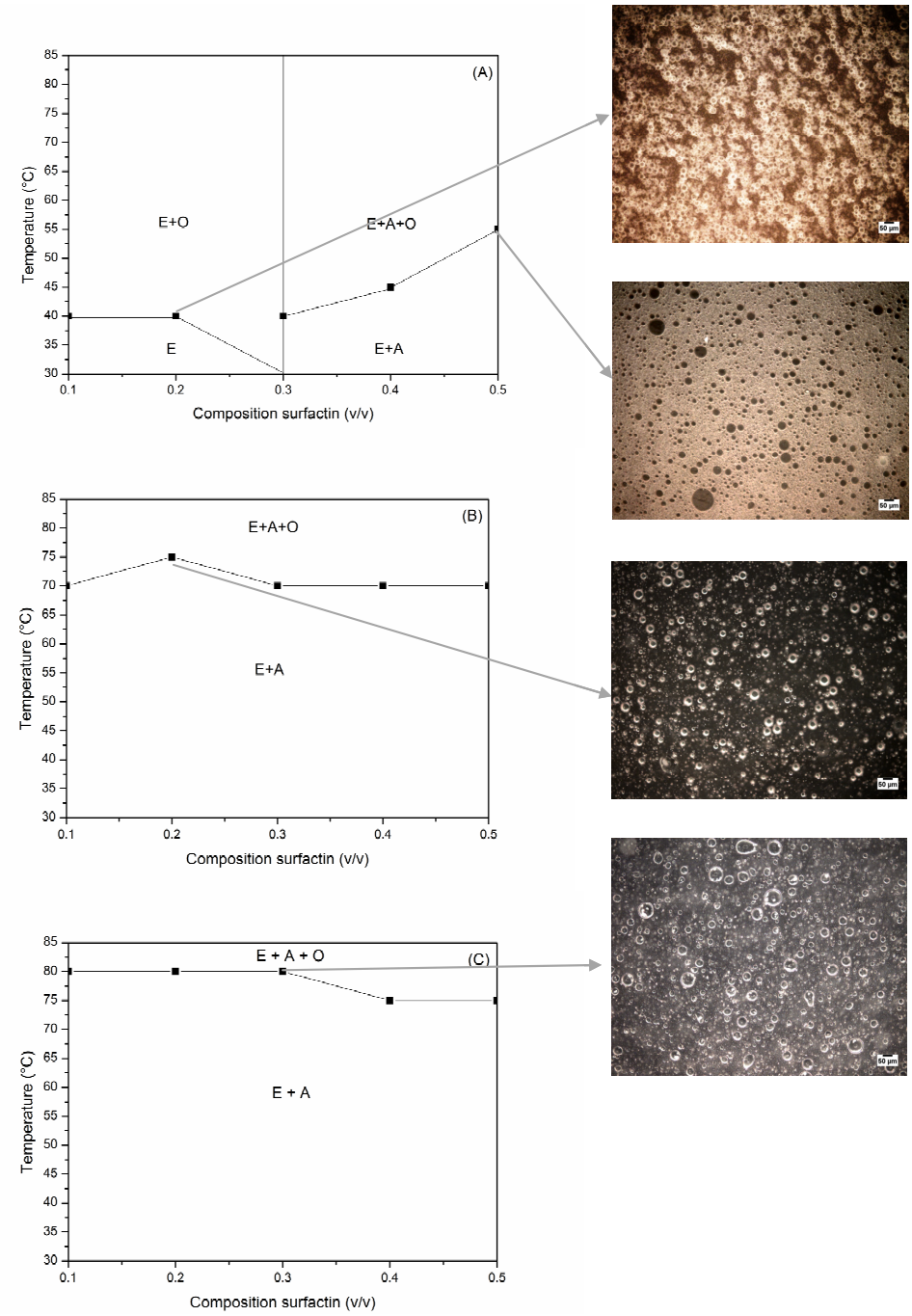

Figure 5: Phase diagrams: effect of temperature and of dispersal of biosurfactant solution with different hydrophobic oil sources and the representative micrographs for the points indicated. (A) motor oil; (B) castor bean oil; (C) NH140 oil. Legend: E – emulsion phase; A – aqueous-phase; O – oil phase.

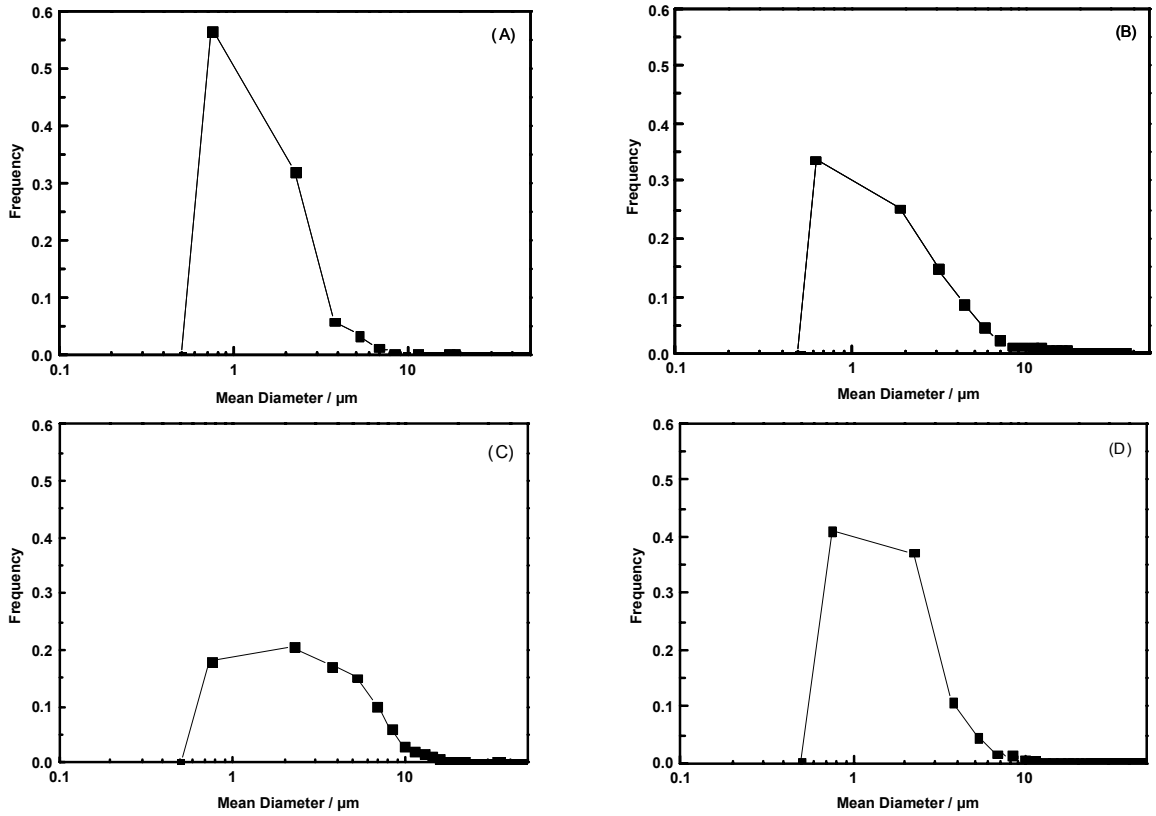

Figure 6: Droplet size distributions (DSD). (A) 20% (v/v) of aqueous phase in motor oil emulsion at 40 °C. (B) 50% (v/v) aqueous phase in motor oil emulsion at 55 °C. (C) 20% (v/v) aqueous phase in castor beans oil emulsion at 75 °C. (D) 30% (v/v) aqueous phase in NH 140 oil emulsion at 82 °C.

CONCLUSION

With respect to the experiments conducted in the 4-L bioreactor, an expressive production of biosurfactant (263.64 mg/L) was observed when compared to the data reported in the literature. Analyses carried out with High Performance Liquid Chromatography (HPLC) showed that surfactin was produced. FTIR and NMR spectra indicated the presence of aliphatic and carbonyl groups, amide bands and –OH/ –NH groups, characteristic of surfactin molecules. It can be concluded that the surfactant produced by *Bacillus subtilis* is a cyclic lipopeptide that is comparable to the commercially available surfactin. These results show a potential application of biosurfactant produced in this work in several industrial sectors, such as bioremediation, waste treatment, and microbial activity. Phase diagrams were determined for the biosurfactant-motor oil, biosurfactant-castor bean oil, and biosurfactant-NH140 systems, which were observed using microscopic images of the formed emulsions.

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