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Performance of a biosurfactant produced by a *Bacillus subtilis* strain isolated from crude oil samples as compared to commercial chemical surfactants

Deisi Altmajer Vaz[a,*], Eduardo J. Gudiña[b], Encarnación Jurado Alameda[a], José A. Teixeira[b], Lígia R. Rodrigues[b]

[a] *Chemical Engineering Department, Faculty of Sciences, University of Granada, 18071, Granada, Spain*
[b] *IBB – Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal*

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ABSTRACT

In this work, the biosurfactant produced by a *Bacillus subtilis* strain isolated from crude oil samples was characterized and its properties compared with commercially available chemical surfactants. The purified biosurfactant production yield ($Y_{P/X}$) was 0.20 g/g cell dry weight. The surface tension (29.0 mN/m) and critical micelle concentration (40 mg/l) were found to be similar to the values previously reported for surfactin. Temperature and pH stability of the biosurfactant were also evaluated. The biosurfactant was exposed to different temperatures (20, 37 and 46 °C) during two weeks and was found to be as stable as the commercial chemical surfactants Glucopone®215, Glucopone®650, Findet®1214N/23 and linear alkylbenzene sulfonates (LAS). Moreover, the biosurfactant subjected to 121 °C for 20 min did not exhibit a significant loss of surface activity. Contrary to the commercial chemical surfactants that were found to be stable over a wide range of pH (3.0–10.0), the biosurfactant was unstable precipitating at pH values below 5.0. The emulsification indexes showed that the biosurfactant possesses equal or superior capacity to form emulsions with n-hexadecane as compared to the commercial chemical surfactants. Moreover, the anti-adhesive activity of the biosurfactant and commercial chemical surfactants was evaluated. The biosurfactant showed some activity against *Staphylococcus aureus* and *Escherichia coli*. Nevertheless, no particular trend or special effect could be assigned to the use of commercial chemical surfactants as anti-adhesives. Results gathered in this work suggest that the biosurfactant recovered from *B. subtilis* EG1 constitutes an interesting alternative to the commercial chemical surfactants with potential use in several industries.

1. Introduction

Surfactants are amphiphilic compounds containing both hydrophobic and hydrophilic moieties, thus conferring them the ability to accumulate between fluid phases, such as oil/water or air/water, reducing the surface and interfacial tensions and forming emulsions [1]. Surfactants are classified according to their ionic properties in water (anionic, non-ionic, cationic and amphoteric) and are used in a great variety of applications for instance household detergents, food processing and industrial cleaners, cosmetics and personal care products, microbial enhanced oil recovery, remediation and bioremediation [2,3].

Traditionally, surfactants are produced by organic chemical reactions from petroleum feedstocks. Linear alkylbenzene sulfonates (LAS) are the most widely used anionic surfactants that find applications in detergents, pesticides, paints, polymers, textiles and paper industries, among others applications [4]. Fatty alcohol ethoxylates (FAE), such as Findet®1214N/23, constitute an important class of non-ionic surfactants used in several applications that include detergents, herbicides, pesticides, cosmetics, antistatic agents, and plasticisers, among others [5]. Goel [6] reported the use of FAE as an emulsifier agent in personal care, agricultural and pharmaceutical products. FAE correspond to the second largest group of surfactants after LAS and the leading group within the family of non-ionic ethoxylate surfactants [7]. Alkylpolyglucosides (APG), such as Glucopone®215 and Glucopone®650, are chemically produced from renewable resources such as fatty alcohols and sugars as the natural surfactants. APG have been used in cosmetics, household cleaning products, food and pharmaceutical products [8]. The growing awareness towards the use of renewable-based products and "green products" has stimulated the development of alternatives to these chemical surfactants. Natural oleochemical- or sugar-based surfactants (e.g. akylpolyglucosides) and microbial surfactants (biosurfactants) are examples of such environmental friendly options [9]. Comparing with chemical surfactants, these compounds have several advantages such as lower toxicity, higher

* Corresponding author. Tel.: +34 958241000x20164; fax: +34 958248992.
E-mail address: deisiav@ugr.es (D.A. Vaz).

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biodegradability and effectiveness at extreme temperatures or pH values [2,3].

Several biosurfactants have been reported to have a high surface activity together with a low critical micelle concentration (CMC), thus representing promising substitutes of their chemical counterparts [2]. Furthermore, these compounds are able to modify the bacterial surface hydrophobicity and consequently, microbial adhesion to solid surfaces [10]. However, currently only few biosurfactants are produced on an industrial scale [11]. In order to reduce their production costs and increase their competitiveness, various researchers have been working in the development of new and cheaper processes and/or in the use of low-cost raw materials [12–19].

Among the many classes of biosurfactants, lipopeptides are of great interest mainly due to their high surface activities and therapeutic potential [20]. Surfactin, the most well known lipopeptide produced by *Bacillus subtilis*, is a molecule that consists of a long-chain alpha-hydroxy fatty acid whose two functional groups close a short peptide chain of seven amino acid residues. There are three different types of surfactins (A, B and C), which are classified according to the differences in their amino acid sequences [20,21]. Furthermore, surfactin is considered the most powerful biosurfactant, presenting a surface tension of 27.9 mN/m [22] and an interfacial tension of the water/n-hexadecane system from 43 mN/m to values below 1 mN/m [20]. Therefore, it is expected that surfactin can exhibit a superior or at least similar performance to their chemical counterparts with all the above mentioned advantages of being a microbial surfactant.

The aim of the current work was to characterize and compare a biosurfactant produced by a *B. subitilis* strain isolated from crude oil samples with commercial chemical surfactants (LAS, Findet®1214N/23, Glucopone®215 and Glucopone®650) that are used for different purposes. The biosurfactant surface properties were determined, namely surface tension, stability at different temperature and pH conditions, emulsification index and anti-adhesive activity.

2. Materials and methods

2.1. Strain and culture conditions

A potential biosurfactant-producer microorganism was isolated from crude oil samples collected in a Brazilian oil field. The isolate was identified according to its 16S rRNA sequence as a *B. subtilis* EG1. The strain was stored at −80 °C in Luria-Bertani (LB) medium containing 15% (v/v) glycerol solution until use. The medium composition is: NaCl 10 g/l; tryptone: 10 g/l; yeast extract: 5 g/l; pH 7.0. Whenever required, frozen stocks were streaked on LB agar plates and incubated overnight at 37 °C for further culturing. Working stock cultures were stored at 4 °C for up to two weeks.

2.2. Growth curve

The *B. subtilis* EG1 was cultured in shake flasks without baffles containing 100 ml LB medium. To prepare pre-cultures, the strain from a frozen stock was streaked on a LB agar plate, which was incubated at 37 °C overnight. Afterwards, a single colony was taken from the plate and transferred into 50 ml of LB liquid medium, which was incubated at 37 °C and 120 rpm during 16 h. Next, 1 ml of this pre-culture was used to inoculate a second culture of LB medium (100 ml). The flask was incubated in ambient air at 250 rpm and 37 °C for 144 h. Samples were collected at different time intervals during the fermentation to determine biomass concentration. Bacterial growth was determined by measuring the optical density at 600 nm at different time intervals up to 144 h.

2.3. Biosurfactant production and isolation

Biosurfactant production by *B. subtilis* EG1 was carried out in shake flasks containing 500 ml of LB medium. Each flask was inoculated with 5 ml of a pre-culture prepared using the same culture medium and incubated overnight at 37 °C and 120 rpm. The flasks were then incubated for 144 h using the same operational conditions. At the end of the fermentation, cells were harvested by centrifugation (10,000 × *g*, 10 min, 10 °C) and cell dry weight was determined (48 h at 105 °C).

To recover the biosurfactant, the cell free supernatant was subjected to an acid precipitation as described elsewhere [23]. Briefly, the supernatant was adjusted to pH 2.0 with HCl 4 M and left overnight at 4 °C. Afterwards, the precipitate was collected by centrifugation (10,000 × *g*, 10 min, 4 °C) and washed twice with acidified water (pH 2.0). The precipitated biosurfactant (crude biosurfactant) was dissolved in 40 ml of demineralized water and the pH was adjusted to 7.0 using NaOH 1 M. Next, the biosurfactant solution was dialyzed against demineralized water at 4 °C in a Cellu-Sep© membrane (cut-off 6000–8000 Da, Membrane Filtration Products, Seguin, USA) for 48 h [20]. Finally, the so-called purified biosurfactant was freeze dried and stored at −20 °C for further use. The biosurfactant production was routinely evaluated through the determination of surface-activity. It is important to notice that since the surface tension was found to be constant after a given fermentation time, in the following experiments the biosurfactant was recovered at 48 h of fermentation.

2.4. Surface-activity determination

The surface tension of the culture broth supernatant and biosurfactant solution was measured according to the Ring method as previously described [24]. A KRUSS K6 Tensiometer (KRÜSS GmbH, Hamburg, Germany) equipped with a 1.9 cm De Noüy platinum ring was used. Surface tension values represent the average of three independent measurements performed at room temperature (20 °C).

2.5. Critical micelle concentration (CMC)

Critical micelle concentration is the concentration of an amphiphilic component in solution at which the formation of micelles is initiated. It is important for several biosurfactant applications to establish their CMC, as above this concentration no further effect is expected in the surface activity. The CMC was determined by plotting the surface tension as a function of the logarithm of biosurfactant concentration and was found at the point of intersection between the two lines that best fit through the pre- and post-CMC data. Concentrations ranging from 1 to 1667 mg/l of the purified biosurfactant recovered from *B. subtilis* EG1 were prepared in PBS (10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0) and the surface tension of each sample was determined by the Ring method at room temperature (20 °C) as described above. All measurements were done in triplicate.

2.6. Biosurfactant stability

The applicability of biosurfactants can be conditioned by pH and temperature; thus the stability of the purified biosurfactant and commercial chemical surfactants was determined by measuring their surface tension under different pH and temperatures. Purified biosurfactant solutions were prepared from the freeze-dried biosurfactant in PBS (1000 mg/l) and pH was adjusted as required using NaOH or HCl solutions. Commercial chemical surfactants were prepared in the same way. Three non-ionic commercial chemical surfactants: Glucopone®215 (Fluka, Sigma

Table 1
Main properties of the commercial chemical surfactants used in the current study according to the suppliers. DP: degree of polymerization; OE: number of ethylene oxide units; R: alkyl chain; CMC: critical micelle concentration at 37 °C.

Commercial name	Group	Chemical description	DP	OE	R	CMC* (mg/l)
LAS	Anionic	Linear alkylbenzene sulfonate	–	–	C10/13	1018
Findet®1214N/23	Non ionic	Fatty alcohol ethoxylate	–	11	C12/14	21
Glucopone®215	Non ionic	Alkylpolyglucoside	1.4	–	C8/12	241
Glucopone®650	Non ionic	Alkylpolyglucoside	1.3	–	C8/18	73

* [25].

Aldrich, Spain), Glucopone®650 (Fluka, Sigma Aldrich, Spain) and Findet®1214N/23 (Kao Corporation, Tokyo, Japan), and an anionic commercial surfactant (LAS) from Kao Corporation were used. The main properties of the commercial chemical surfactants used are summarized in Table 1.

After preparing the surfactant solution at a given pH, ranging from 3.0 to 10.0, the surface tension was immediately measured as described above.

Furthermore, the stability of the surfactant solutions at different temperatures (20, 37 and 46 °C) was evaluated. For that purpose, two experiments were conducted. In the first one, surfactant solutions were subjected to different temperatures and the surface tension was measured at those temperatures. In the second experiment, the surfactant solutions were subjected to different temperatures over two weeks (336 h) after which surface tension was measured but at room temperature (20 °C).

Finally, the surfactant solutions stability at sterilizing conditions (121 °C, 20 min) was also evaluated. Solutions were stabilized at room temperature before surface tension measurement.

All surface tension measurements were performed in triplicate. The results were analyzed through a multiple comparison test based on the Tukey–Kramer criterion using the Statistics Toolbox of Matlab (Matlab version R2008a, The MathWorks Inc., U.S.).

2.7. Emulsification activity

Emulsification activity was determined by the addition of 2 ml of n-hexadecane to the same volume of purified biosurfactant or commercial chemical surfactant solutions in a test tube which was mixed vigorously with vortex for 2 min. The tubes were incubated at 25 °C and the emulsification index (EI) was determined after a given time (t) according to Eq. (1):

$$\mathrm{EI_t} = \left(\frac{H_e}{H_t}\right) \times 100 \quad (1)$$

where H_e and H_t are the height of emulsion and total height of the liquid in the tube, respectively. All emulsification indexes were performed in triplicate.

To study the emulsion stability the same protocol was used; the emulsification index (EI, %) was determined after 1 h (considered as the initial time, t_0); the tubes were incubated at 25 °C and the EI was measured periodically after 24 h and for two weeks; the emulsion stability (ES %) corresponds to the ratio between the EI at time t and t_0.

2.8. Anti-adhesion assays

The anti-adhesive activity of purified biosurfactant and commercial chemical surfactant solutions against two microbial strains, *Escherichia coli* and *Staphylococcus aureus*, was determined according to the procedure described by Rodrigues et al. [24] and Gudiña et al. [26]. Surfactant concentrations ranging from 400 to 3000 mg/l were studied.

3. Results and discussion

3.1. Kinetics of biomass growth and biosurfactant production

The relation between cell growth and surface-activity of the biosurfactant in time for *B. subtilis* EG1 was determined (Fig. 1). The biosurfactant production was monitored by surface tension measurement and emulsification activity (E_{24}) determination.

Biosurfactant production was found to be associated with cellular growth, as an increase in the biomass concentration lead to a decrease in the surface tension, especially in the first 4 h of the fermentation for which a pronounced decrease of surface tension was observed. In the case of a growth associated biosurfactant production there is a parallel relationship between the substrate consumption, growth and biosurfactant production [27]. Until 24 h, the surface tension continued dropping quickly reaching the lowest value (30.1 ± 0.2 mN/m) that remained nearly constant until the end of the fermentation (144 h), meaning that the CMC was most probably attained. Nevertheless, the E_{24} values continued increasing after 24 h and until the end of the fermentation, since although the CMC has probably been achieved, incremental amounts of biosurfactant result in a higher emulsifying activity. The highest value of E_{24} was obtained at the end of the fermentation (60.3 ± 0.4). Growth-associated biosurfactant production has been described for the production of biodispersan by *Acinetobacter calcoaceticus* [1]. In addition, biosurfactant production may occur, or be stimulated, by growing the microbial cells under growth-limiting conditions. *Pseudomonas aeruginosa* shows an overproduction of rhamnolipid when the culture reaches the stationary growth phase due to limitation of the nitrogen source [1]. Hence, the present observation that biosurfactant production by *B. subtilis* EG1 is maximal at the end of the fermentation is in accordance with the general notion on this point in the literature.

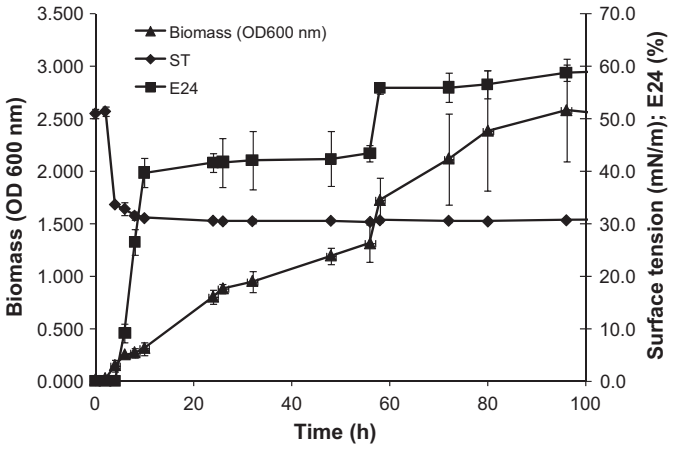

Fig. 1. Time course of growth and biosurfactant production by *B. subtilis* EG1 in LB medium.

3.2. Purified biosurfactant yield and surface activity

A purified biosurfactant concentration of 150 mg/l was obtained corresponding to a yield per gram cell dry weight of 0.20 g/g. The comparison of the HPLC chromatograms obtained for the purified biosurfactant and the commercial surfactin (*data not shown*) suggests that the biosurfactant isolated in the current work corresponds to surfactin although less pure. Several authors have been developing strategies to optimize the production and recovery of purified surfactin using different carbon sources, media compositions, bioreactor design and downstream processes [28–30]. The aim of the current work was not to optimize the biosurfactant production but to compare its performance with commercial chemical surfactants. Nevertheless, the production yield obtained is in accordance with the ranges previously reported for surfactin in suboptimal conditions. Production yields of surfactin from glucose and other monosacharides have been reported in amounts ranging from 100 to 800 mg/l [22]. The use of foam fractionation techniques together with chemically defined media and optimized bioreactors lead to a surfactin concentration in the foam of 1670 mg/l [31].

Furthermore, the surface tension measured in the cell-free supernatants at the end of the fermentation was 30.3 ± 0.6 mN/m. The biosurfactant obtained after acid precipitation and resuspended in distilled water with pH adjusted to 7.0 presented a surface tension value of 33.7 mN/m. After dialysis, the so-called purified biosurfactant showed a surface tension value of 29.0 ± 0.7 mN/m. These findings confirm that no biosurfactant was lost during the recovery process since no significant changes in the surface tension could be observed. The surface tension values obtained for the crude and purified biosurfactant are comparable to those reported for surfactin by many other researchers [1,22,32–34].

3.3. Critical micelle concentration

In order to establish the CMC of the purified biosurfactant from *B. subtilis* EG1, the relationship between biosurfactant concentration and surface tension was determined. It is well known that important interfacial properties (such as detergency and solubilisation), are affected by the existence of micelles in solution [35]. Therefore, the CMC is widely used as index to evaluate the surface activity of a given surfactant. The freeze-dried biosurfactant was dissolved in PBS (pH 7.0) at different concentrations ranging from 1 to 1667 mg/l. A progressive decrease in surface tension was observed with the increase of biosurfactant concentration. For biosurfactant concentrations higher than 150 mg/l, the surface tension becomes stable, and there is no further significant reduction even at the highest concentrations tested. The surface tension *versus* biosurfactant concentration plot was used to determine the CMC as the point of interception between the two lines that best fit the decline and the constant plateau of surface tension. The CMC was found to be 40 mg/l with a minimum surface tension value of 29.0 ± 0.2 mN/m.

Comparing the biosurfactant CMC with the ones for the commercial chemical surfactants (Table 1) it was found that it is similar to Findet®1214N/23 (21 mg/l), slightly lower than Glucopone®650, and many times lower than Glucopone®215 and LAS [25]. These results suggest that the biosurfactant from *B. subtilis* EG1 is able to reduce the surface tension more effectively than the studied commercial chemical surfactants. From a practical point of view, it is important to distinguish between an effective and an efficient surfactant. Effectiveness is measured by the minimum value to which the surface tension can be reduced, whereas efficiency is measured by the surfactant concentration required to produce a significant reduction in the surface tension of water, namely the CMC [23]. Variations in CMC values were shown to be dependent on the nature of the solvent in which surfactin is dissolved, as well as the purity of the surfactin. For example, Abdel-Mawgoud et al. [23] demonstrated the high efficiency of a crude biosurfactant produced by *B. subtilis* BS5 based on the reduction of the surface tension of water from 70 to 36 mN/m. Other authors showed even lower values (27 mN/m) [36]. These differences may be attributed to lower surfactin purity in the first study mentioned. Actually, the authors reported a CMC value of 15.6 mg/l [23], whereas in the literature several examples suggest a value of 11 mg/l [33,36,37], although some other values (13, 22, and 17 mg/l) have also been stated [30].

Recently, Arutchelvi et al. [32] showed that crude surfactin produced by *B. subtilis* YB7 reduced the surface tension of water from 72 to 30 mN/m at a CMC of 40 mg/l. Therefore, the results obtained in the current work for the biosurfactant produced by a *B. subtilis* isolated from crude oil samples are in good agreement with the general notion in the literature.

3.4. Emulsification activity

As previously mentioned, several commercial applications have been described for surfactants and biosurfactants [11] based on their ability to stabilize emulsions. Table 2 compiles the emulsification index values for 24 h obtained for surfactant concentrations ranging from 100 to 1000 mg/l at 25 °C. Independently of the concentration, the higher E_{24} values were always found for Findet®1214N/23 and biosurfactant. Moreover, no emulsion was formed with Glucopone®215, and the surfactants Glucopone®650 and LAS presented similar E_{24} values.

For the lowest surfactant concentration studied (100 mg/l), only the fatty ethoxylated alcohol (Findet®1214N/23) and the biosurfactant were able to emulsify n-hexadecane. Additionally, among all the concentrations studied, the E_{24} values obtained for Findet®1214N/23 and biosurfactant are very similar. These results suggest that, under the current experimental conditions, the biosurfactant presents a similar performance as compared to that important commercial chemical surfactant. The higher E_{24} values obtained for Findet®1214N/23 are due to its hydrophilic–lipophilic balance number (HLB = 14.3, according to the supplier), which is in the usual range for oil/water emulsifiers [38]. Although there is no consensus on the HLB of surfactin, it is capable of lowering the surface tension of water to 27 mN/m, suggestive that it may also have an HLB near 10 [39,40].

Furthermore, higher values of E_{24} were found for the biosurfactant comparing to LAS and both alkylpolyglucosides. Altmajer [41] found experimental HLB values of 12.6 and 12.8 for Glucopon®215 and Glucopon®650 (both from Henkel), respectively. Moreover, the Sauter diameter obtained in emulsions formed with Findet®1214N/23 and a mixture of fatty acids was smaller than those obtained with Glucopon®215 and Glucopon®650, suggesting that Findet®1214N/23 is a better emulsifier than both alkylpolyglucosides.

Ghojavand et al. [34] studied the emulsification index of a biosurfactant synthesized by a member of the *B. subtilis* group (PTCC 1696) which was isolated from an Iranian oil field with several typical hydrocarbons, n-hexane, n-heptane, cyclohexane and n-nonane. The maximum emulsification activity was 64.4% with cyclohexane, and the biosurfactant was found to be able to emulsify oil, which is essential for enhanced oil recovery. The emulsification index of a biosurfactant produced by *B. subtilis* was also determined by Makkar and Cameotra [42], using motor oil and culture broth. According to the authors, the E_{24} was found to be 33.3, thus demonstrating its capacity to emulsify oil. Additionally, Abdel-Mawgoud et al. [23] evaluated the E_{24} of surfactin against different oil phases. The surfactin containing crude cell free supernatants showed high emulsification indexes with n-hexadecane, kerosene and diesel. The E_{24} values reported for n-hexadecane were 60% which is in

Table 2
Emulsification indexes (EI,%) and emulsion stability (ES,%) obtained for the biosurfactant and commercial chemical surfactants dissolved in PBS (pH 7.0) at 100, 500 and 1000 mg/l. Emulsification indexes were determined at different time points (1, 24, 168 and 336 h). Measurements were performed at 25 °C. Results are expressed as the means ± standard deviations of three independent experiments.

Time (h)	Concentration (mg/l)	EI (%)				ES (%)			
		LAS	Findet®1214N/23	Glucopone®650	Biosurfactant	LAS	Findet®1214N/23	Glucopone®650	Biosurfactant
1	100	–	41.5 ± 1.2	–	37.8 ± 1.7	–	–	–	–
	500	43.0 ± 0.6	60.9 ± 0.4	44.9 ± 0.7	60.6 ± 0.0	–	–	–	–
	1000	52.7 ± 1.8	62.8 ± 0.0	56.5 ± 1.4	61.6 ± 1.3	–	–	–	–
24	100	–	41.3 ± 5.4	–	36.6 ± 1.9	–	93.0 ± 0.8	–	96.8 ± 0.5
	500	41.0 ± 1.4	60.6 ± 1.7	44.1 ± 0.1	59.0 ± 1.4	95.3 ± 1.7	97.6 ± 0.6	98.2 ± 1.5	97.4 ± 2.2
	1000	51.7 ± 2.4	61.8 ± 0.1	55.8 ± 1.3	59.4 ± 0.8	97.9 ± 1.0	98.4 ± 0.0	98.7 ± 0.1	96.5 ± 0.7
168	100	–	41.3 ± 5.4	–	25.6 ± 3.3	–	96.2 ± 5.4	–	67.5 ± 5.5
	500	36.7 ± 4.7	59.1 ± 0.4	41.2 ± 0.1	44.0 ± 1.4	85.2 ± 9.7	96.2 ± 1.5	91.7 ± 1.4	72.6 ± 2.3
	1000	51.7 ± 0.1	57.4 ± 2.1	56.7 ± 0.1	52.5 ± 2.1	97.9 ± 1.0	91.3 ± 3.2	98.5 ± 0.5	85.3 ± 5.3
336	100	–	41.3 ± 5.4	–	24.9 ± 2.7	–	96.2 ± 5.4	–	65.7 ± 4.2
	500	36.7 ± 4.7	59.1 ± 0.4	41.2 ± 0.1	43.0 ± 1.4	85.2 ± 9.7	96.2 ± 1.5	91.7 ± 1.4	70.9 ± 2.3
	1000	51.7 ± 2.4	57.4 ± 2.1	55.0 ± 2.4	51.5 ± 2.1	97.9 ± 1.0	91.3 ± 3.2	97.4 ± 6.7	83.7 ± 5.3

good agreement with the values obtained in the current work. Similarly, other authors using a biosurfactant from *Lactobacillus pentosus* obtained EI between 38% and 42% with gasoline, and 14% for the commercial surfactin [19]. Similar values were reported for kerosene and their emulsions were found to be stable for 72 h. These findings show that surfactin even in a crude form possesses a high ability to stabilize emulsions, thus representing a potential candidate to be used in bioremediation of hydrocarbon-contaminated sites or in the petroleum industry.

According to Rosenberg and Ron [39], the low molecular mass bioemulsifiers are generally glycolipids (e.g. trehalose lipids, sophorolipids and rhamnolipids) or lipopeptides (e.g. surfactin, gramicidin S and polymyxin). Bioemulsifiers have important advantages over traditional surfactants, which should allow them to become prominent in industrial and environmental applications. Their potential commercial applications include bioremediation of oil-polluted soil and water, enhanced oil recovery, replacement of chlorinated solvents used in cleaning-up oil-contaminated pipes, vessels and machinery, use in the detergent industry, formulations of herbicides and pesticides and formation of stable oil-in-water emulsions for the food and cosmetic industries.

In the current work, the stability of the emulsions formed was also monitored each 24 h over a two week period (336 h). The experimental data (Table 2) showed that the emulsification indexes remained almost constant and that the emulsions were very stable with ES values ranging from 85% to 99% in most cases.

From the results gathered in the current work, and except for Glucopone®215, it was shown that the commercial chemical surfactants were able to form stable emulsions with hexadecane, confirming its good properties as emulsifying agents. Biosurfactant and Findet®1214N/23 were found to be especially useful for producing stable oil/water emulsions, that can be required in applications such as remediation of soils, household and industrial cleaning systems, among others. The alkylpolyglucosides, recognized as safe (GRAS), can be used as emulsifying agents in food manufacturing. Moreover, the results showed that LAS can be used as an efficient emulsifying agent, yielding E_{24} values similar to those of Glucopone®650, which is in agreement with the work reported by Jurado et al. [43].

3.5. Biosurfactant stability

The applicability of biosurfactants in several fields depends on their stability at different temperatures and pH values.

3.5.1. Temperature effect

Usually the surface activity of a given surfactant is determined at 20–25 °C, however there are many applications (e.g. detergents) for which the surfactants are used at higher temperatures. Therefore, the surface tension of the biosurfactant and commercial chemical surfactants was determined at three different temperatures (Table 3). Independently of the temperature, the biosurfactant surface tension (1000 mg/l) was found to be similar to those obtained for the commercial chemical surfactants, except for Findet®1214N/23 for which the surface tension values were slightly higher. Hence, it can be assumed that the biosurfactant surface activity is comparable to the commercial products. The results were analyzed by a two-way ANOVA followed by a multiple comparison test. The differences observed between the surface tension values at 37 and 46 °C were found to be non significant (p-value >0.01) for all the surfactants studied. Nevertheless, the differences observed between the surface tension at 20 °C and 37 or 46 °C were significant for all cases.

An insignificant impact on the surface tension was observed for the commercial chemical surfactants in the temperature range studied. The temperature effect on the surfactants

Table 3
Surface tension values (mN/m) measured at 20, 37 and 46 °C for the biosurfactant recovered from *Bacillus subtilis* EG1 and the commercial chemical surfactants dissolved in PBS (pH 7.0) with a concentration of 1000 mg/l. Results are expressed as means ± standard deviations of three independent experiments.

Temperature (°C)	Surface tension (mN/m)				
	LAS	Findet®1214N/23	Glucopone®215	Glucopone®650	Biosurfactant
20	31.4 ± 0.3	37.9 ± 0.1	31.8 ± 0.1	32.0 ± 0.2	30.9 ± 0.1
37	31.1 ± 0.1	36.6 ± 0.3	30.8 ± 0.2	31.8 ± 0.2	30.2 ± 0.1
46	30.9 ± 0.1	36.4 ± 0.1	30.7 ± 0.3	31.2 ± 0.1	29.9 ± 0.1

stability was more pronounced for Findet®1214N/23 for which a 4% decrease in the surface tension was measured for increasing temperatures. Decreases of 3.5%, 3.2%, 2.6% and 1.5% were measured for Glucopone®650, biosurfactant, Glucopone®215 and LAS, respectively. Nevertheless, these surface tension variations were considered negligible and all the surfactants were found to be stable to the temperature changes evaluated.

The effect of temperature on the surface activity of chemical surfactants has been reported in literature [35,44–46]. Globally, Findet®1214N/23 presented slightly higher surface tension values at increasing temperatures. This is due to the strong dependence on temperature of the phase behaviour presented by fatty alcohol ethoxylates. Frequently, when cloud point temperatures are reached, a second phase appears as the result of the loss of solubility of the surfactant in water [45]. In contrast, alkylpolyglucosides show a distinct behaviour from fatty alcohol ethoxylates or anionic surfactants. The increase of temperature leads to a decrease in the free energy of the air/liquid boundary, thus to a reduction in the surface tension values. Moreover, for concentrations above the CMC, the alkylpolyglucosides are not affected by temperature, probably due to the loss of a large amount of heat during the destruction of the hydrogen bonds that are formed between the water molecules and the free hydroxyl group in the glucose head [35]. The anionic surfactants are more water-soluble than non-ionic ones and they are not known to show the clouding phenomenon in aqueous solutions [45].

Additionally, the effect of exposing the biosurfactant and commercial chemical surfactants to different temperatures over time was studied. Surfactant solutions dissolved in PBS at a concentration of 1000 mg/l were incubated at 20, 37 and 46 °C. The surface tension was measured at room temperature at time zero and after two weeks (336 h). The results are summarized in Table 4. As can be seen, independently of the temperature, the commercial chemical surfactants and biosurfactant surface activities remain unaltered after 336 h exposure to the different temperatures studied. Furthermore, the stability of the different surfactants after incubation for 20 min at 121 °C was studied (Table 4). Biosurfactant and commercial chemical surfactants exhibited high thermal stability since only small variations in the surface activity could be observed. These results are in good agreement with the study from Portilla et al. [19] that reported that the biosurfactants produced by *L. pentosus* were stable after 24 h of exposure to 10, 25 and 40 °C. Further, Ghojavand et al. [34] investigated the stability a biosurfactant from a *B. subtilis* strain at 100 °C at different exposure times. The authors reported that for exposure times inferior to 120 min, no interference on the surface activity could be found, thus corroborating the results obtained in the current work. Also, Abdel-Mawgoud et al. [23] verified the stability of the biosurfactant from *B. subtilis* BS5 at 121 °C. The authors found that there was no reduction in the surface activity to any degree upon exposure to 100 °C for upto 1 h. Furthermore, autoclaving the biosurfactant (1000 mg/l) at 121 °C for 10 min did not reduced the surface activity and it even resulted in a slight decrease in the surface tension. This increase in the activity may be attributed to the heat-dependent coagulation and precipitation of substances such as proteins contaminating the surfactin solution; such substances might have been co-extracted with surfactin during the extraction steps. This coagulation was demonstrated by the presence of white coagulum at the bottom of the test tubes containing the surfactin solution after autoclaving [23]. Additionally, several biosurfactant concentrations (10, 25, 50 and 100 mg/l) under the CMC were tested (*data not shown*) and the surface tension values before and after the exposure to 121 °C for 20 min were found to be similar, thus suggesting that no degradation occurred.

3.5.2. pH effect

In order to study the pH effect on biosurfactant and commercial chemical surfactants stability, the surface tension of several surfactant samples prepared with a concentration of 1000 mg/l at different pH values (3–10) was determined. As can be seen in Fig. 2, the minimum surface tension of the biosurfactant was obtained at pH values between 5.5 and 6.0 (29.9 ± 0.2 mN/m). The surface activity of the biosurfactant remained relatively stable to pH changes between 5.0 and 10.0, showing higher stability at alkaline than acidic conditions. At pH 10.0, the surface tension value is only one unit higher than at pH 7.0, whereas at pH 5.0 it is nine units higher. Additionally, for pH values lower than 5.0, the samples become turbid due to partial precipitation of the biosurfactant. These results are in accordance with the ones reported by Abdel-Mawgoud et al. [23], in which surfactin was found to be soluble in aqueous solutions at pH values higher than 5.0. Also, the authors demonstrated that the optimum solubility for surfactin is at pH 8.0–8.5. This may be attributed to the acidic nature of surfactin. The molecule contains two carboxylic groups (glutamic acid residue and aspartic acid residue) that confer it its anionic nature. Surfactin showed nearly no surface activity at pH 2.0–4.0, and maximum activity at pH 6.0 [34]. Likewise, other authors [19] showed that the surface activity of biosurfactants from *L. pentosus* are not negatively affected by pH values between 7.5 and 10.5, although their stability is challenged by acid pH values.

The surfactin stability over a wide range of pH and temperatures, together with its excellent surface and emulsifying activities, make it a potential candidate to be used in bioremediation of

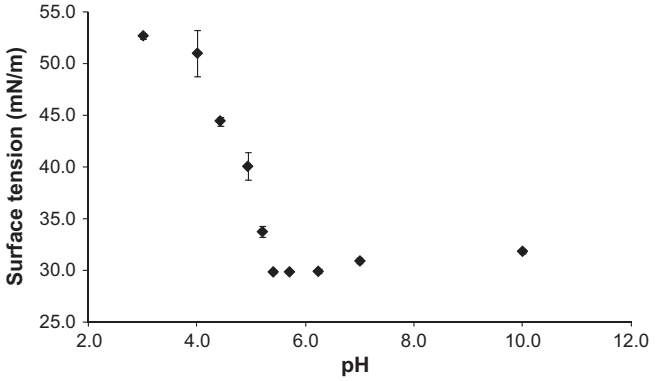

Fig. 2. Effect of pH on the surface tension of the biosurfactant recovered from *B. subtilis* EG1. Samples were prepared with a concentration of 1000 mg/l at different pH values and measurements were done at room temperature (20 °C). Results represent the average of three independent measurements and error bars represent standard deviations.

Table 4
Surface tension values (mN/m) measured at room temperature for the biosurfactant recovered from *Bacillus subtilis* EG1 and the commercial chemical surfactants dissolved in PBS (pH 7.0) with a concentration of 1000 mg/l when exposed for 336 h to different temperatures (20, 37 and 46 °C), and immediately after exposure to 121 °C for 20 min (0.33 h). Results are expressed as means ± standard deviations of three independent experiments.

Temperature (°C)	Incubation time (h)	Surface tension (mN/m)			
		LAS	Findet®1214N/23	Glucopone®650	Biosurfactant
20	0	31.4 ± 0.3	37.9 ± 0.1	32.0 ± 0.2	30.9 ± 0.1
	336	31.3 ± 0.1	38.2 ± 0.1	32.1 ± 0.1	30.8 ± 0.1
37	0	31.4 ± 0.3	37.9 ± 0.1	32.0 ± 0.5	30.9 ± 0.1
	336	31.3 ± 0.1	38.1 ± 0.1	32.1 ± 0.1	30.7 ± 0.1
46	0	31.4 ± 0.3	37.9 ± 0.1	32.0 ± 0.5	30.9 ± 0.1
	336	31.2 ± 0.1	38.0 ± 0.1	32.1 ± 0.1	30.8 ± 0.1
121	0.33	31.4 ± 0.3	38.1 ± 0.1	32.0 ± 0.1	29.8 ± 0.2

Table 5
Surface tension values (mN/m) measured at room temperature for the commercial chemical surfactants with a concentration of 1000 mg/l at different pH values. Results are expressed as means ± standard deviations of three independent experiments.

pH	Surface tension (mN/m)			
	LAS	Findet®1214N/23	Glucopone®215	Glucopone®650
3.0	32.1 ± 0.2	31.2 ± 0.2	31.9 ± 0.1	37.1 ± 0.1
7.0	31.2 ± 0.1	31.5 ± 0.5	31.8 ± 0.2	37.2 ± 0.1
10.0	32.0 ± 0.0	30.6 ± 0.4	31.9 ± 0.1	37.4 ± 0.2

contaminated sites and in the petroleum industry (microbial enhanced oil recovery (MEOR)) where drastic conditions commonly prevail [34].

The stability of the commercial chemical surfactants at different pH values was also determined (Table 5). The statistical analysis of the results suggested that pH has no significant effect on the surface activity for Glucopone®650 and LAS. For Glucopone®215 and Findet®1214N/23, the surface tension differences obtained at pH 3.0 and 7.0 were found to be not statistically significant. However, significant differences could be found at pH 10.0 and 7.0 (p-value <0.01). Furthermore, pH was found significantly affect the surface tension of the biosurfactant solutions (Fig. 2).

The commercial chemical surfactants were found to be more stable to pH changes as compared to the biosurfactant over the whole range of pH values studied. The instability of the biosurfactant to acidic pH is probably due to the presence of proteinaceous contaminants. Nevertheless, it is important to notice that above pH 5.0 the biosurfactant showed a superior surface activity as compared to the commercial chemical surfactants, thus representing a viable alternative for a number of applications.

3.6. Anti-adhesive activity

The biosurfactant from *B. subtilis* EG1 was found to possess some anti-adhesive activity against *S. aureus* and *E. coli* (Table 6). The highest anti-adhesive percentage was obtained for *S. aureus* (28.6%) for a concentration of 3000 mg/l. On the contrary, a low activity was observed for *E. coli* (10.3%) at the same concentration. Involvement of biosurfactants in microbial adhesion and desorption has been widely described [10]. The prior adsorption of biosurfactants to solid surfaces might constitute an effective strategy to reduce microbial adhesion and preventing colonization by pathogenic microorganisms in several industries [3,24,26].

Similar studies on the anti-adhesive properties of surfactin on polystyrene surfaces were performed by Zeraik and Nitschke [47]. The authors demonstrated that *S. aureus* attachment to polystyrene could be reduced 42.2% by conditioning the surface with surfactin. Furthermore, Rivardo et al. [48] reported a decrease of 97% and 90% on the *E. coli* CFT073 and *S. aureus* ATCC 29213 biofilm formation respectively, due to the presence of surfactin. Results gathered in the current work regarding the anti-adhesive activity of the biosurfactant produced by *B. subtilis* EG1 were much less pronounced probably due to a lower purity of the biosurfactant used as compared with the above mentioned studies.

To the author's knowledge, this is the first report on the potential anti-adhesive activity of the commercial chemical surfactants against *S. aureus* and *E. coli*. Except for Glucopone®650, residual inhibition of *E. coli* was observed for all the surfactants. Regarding *S. aureus*, all the surfactants greatly promoted its adhesion in a concentration dependent manner with the exception of Glucopone®215. These commercial chemical surfactants present very different chemical structures and features that might condition the way they adsorb to the polystyrene surfaces, and therefore the functional groups exposed at the surface that will interact with bacteria. No particular trend or special

Table 6
Anti-adhesive properties of the biosurfactant recovered from *Bacillus subtilis* EG1 and commercial chemical surfactants. Negative controls were set at 0% to indicate the absence of biosurfactant. Positive percentages indicate the reductions in microbial adhesion when compared to the control, and negative percentages indicate increased microbial adhesion. Experiments were performed in triplicate and correspond within 15%.

Surfactant	Microorganism	Surfactant concentration (mg/l)			
		400	800	1500	3000
LAS	*S. aureus*	−36.4	−41.0	−50.5	−55.3
	E. coli	5.9	5.4	5.6	5.3
Findet®1214N/23	*S. aureus*	−6.2	−18.8	−18.6	−19.3
	E. coli	2.7	4.9	3.9	9.6
Glucopone®215	*S. aureus*	13.1	11.6	10.9	13.2
	E. coli	2.7	7.3	8.8	6.1
Glucopone®650	*S. aureus*	−2.3	−7.9	−30.4	−34.5
	E. coli	16.1	22.6	18.1	19.6
Biosurfactant	*S. aureus*	9.6	18.4	22.9	28.6
	E. coli	9.5	10.8	10.4	10.3

effect could be assigned to the use of these surfactants as anti-adhesives.

4. Conclusions

In this work, the biosurfactant produced by a *B. subtilis* strain isolated from crude oil samples was characterized and compared to commercial chemical surfactants. The minimum surface tension and the critical micelle concentration were similar to the values previously reported for biosurfactants isolated from other *Bacillus* sp., such as surfactin. Furthermore, similarly to the commercial chemical surfactants, the biosurfactant was found to be highly stable to temperature changes, even when exposed to high temperatures over two weeks. Nevertheless, the biosurfactant was found to be stable only at pH values above 5.0 contrarily to their chemical counterparts. This was probably due to the presence of proteinaceous contaminants present in the biosurfactant mixture, and could eventually be optimized through further purification steps. Interestingly, this biosurfactant was found to hold equal or superior capacity to form emulsions with n-hexadecane as compared with the chemical surfactants. Although the biosurfactants from *Bacillus* sp. have been reported by several authors to possess a great anti-adhesive activity, the same was not observed in the current work most probably due to the low purity of the biosurfactant. Moreover, the commercial chemical surfactants evaluated also did not reveal any anti-adhesive effect. In summary, the biosurfactant from *B. subtilis* EG1 presents interesting features, and therefore constitutes an alternative with potential use in several industries.

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