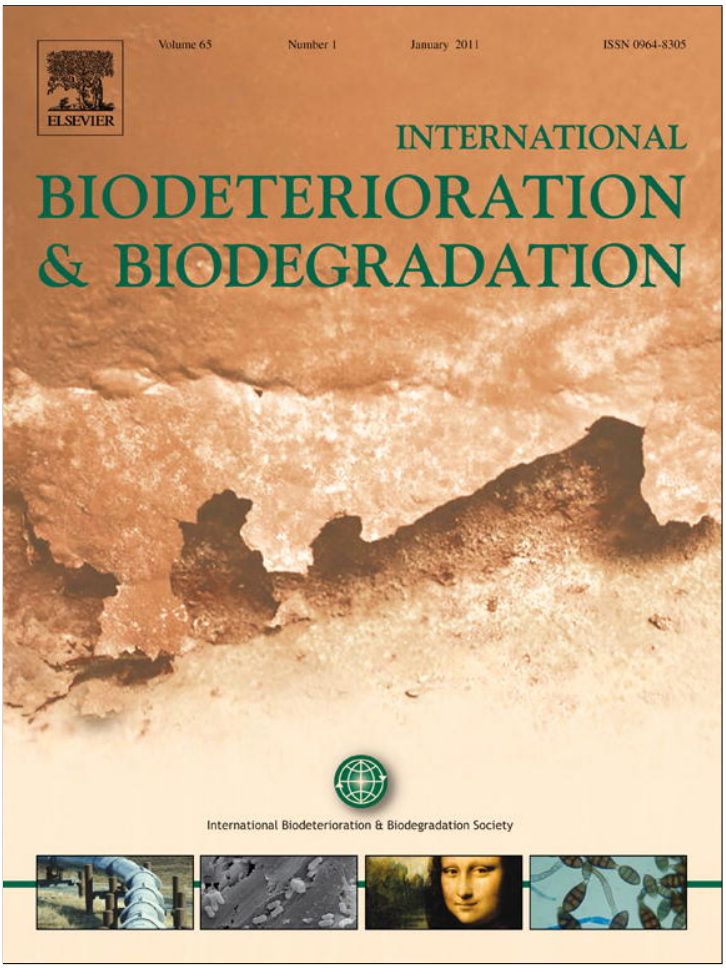

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Isolation and study of microorganisms from oil samples for application in Microbial Enhanced Oil Recovery

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ABSTRACT

Microbial Enhanced Oil Recovery (MEOR) is potentially useful to increment oil recovery from a reservoir beyond primary and secondary recovery operations using microorganisms and their metabolites. Stimulation of bacterial growth for biosurfactant production and degradation of heavy oil fractions by indigenous microorganisms can enhance the fluidity and reduce the capillary forces that retain the oil into the reservoir. MEOR offers major advantages over conventional EOR, namely low energy consumption and independence of the price of crude oil. In this work, the isolation and identification of microorganisms capable of producing biosurfactants and promote degradation of long-chain *n*-alkanes under conditions existent in oil reservoirs were addressed. Among the isolated microorganisms, five *Bacillus* strains were able to produce extracellular biosurfactants at 40 °C under anaerobic conditions in medium supplemented with hydrocarbons. Three isolates were selected as the higher biosurfactant producers. The obtained biosurfactants reduced the surface tension of water from 72 to 30 mN/m, exhibited emulsifying activity and were not affected by exposure to high temperatures (121 °C). These characteristics make them good candidates for use at conditions usually existing in oil reservoirs. Furthermore, it was here shown for the first time that *Bacillus* strains were able to degrade large alkyl chains and reduce the viscosity of hydrocarbon mixtures under anaerobic conditions. The results obtained show that the isolated microorganisms are promising candidates for the development of enhanced oil recovery processes.

1. Introduction

Oil is an essential source of energy and one of the main factors driving the economic development of the World. Its recovery comprises a primary phase, which produces oil and gas using the natural pressure drive of the reservoir; and a secondary phase, which involves stimulating the oil wells by the injection of fluids to improve the flow of oil and gas to the well-head (Lazar et al., 2007). While primary recovery produces 5–10% of the original oil in place, recovery efficiencies in the secondary stage range from 10% to 40% (Sen, 2008). Therefore, crude oil remaining in the reservoirs after conventional oil recovery operations makes up to two-thirds of the total oil reserves (Youssef et al., 2007, 2009; Suthar et al., 2008; Brown, 2010). The main factors responsible for the poor oil recovery from producing wells are the low permeability of some reservoirs, the high viscosity of the oil and the high interfacial tension between the hydrocarbon and aqueous phases, which result in high capillary forces that entrap oil in small pores within the reservoir (Sen, 2008; Brown, 2010). As the price of petroleum increases and petroleum reserves shrink, exploitation of oil resources in mature reservoirs is essential for meeting future energy demands. In the last years, more attention has been focused on tertiary recovery techniques, known as Chemical Enhanced Oil Recovery (CEOR), to mobilize entrapped oil (Sen, 2008; Youssef et al., 2009; Brown, 2010).

Chemical methods include the use of surfactants, polymers, acids, gases and solvents (Lazar et al., 2007). Surfactants reduce the interfacial tension between oil–water and oil–rock interfaces, decreasing the capillary forces that prevent oil moving through the rock pores; they also alter the wettability of the reservoir rock. Polymers are used to increase the viscosity of water in water-flooding operations, enhancing the effectiveness of the process. Furthermore, polymers plug oil-depleted zones, directing the water-flood to oil-rich channels to release residual oil. Acids are used to increase the permeability through the porous network. Gases and solvents decrease oil viscosity and promote its flow; gases also increase the pressure in the reservoir. It is generally accepted that about 30% of the oil contained in the reservoirs can be recovered using current CEOR technology (Bordoloi and Konwar,

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2008). However, these processes are environmentally hazardous, expensive, and leave undesirable residues which are difficult to dispose of, without adversely affecting the environment.

Microbial Enhanced Oil Recovery (MEOR) is an important tertiary oil recovery technology that represents a cost-effective and eco-friendly alternative to CEOR. In MEOR, selected microbial strains are used to synthesize compounds analogous to those used in CEOR processes to increase the recovery of oil from depleted and marginal reservoirs, thereby extending their life. MEOR is a less expensive process when compared with CEOR because microorganisms can synthesize useful products by fermenting low-cost substrates or raw materials. Furthermore, microbial products are biodegradable and have low toxicity (Lazar et al., 2007; Suthar et al., 2008; Banat et al., 2010).

Biosurfactant MEOR represents one of the most promising methods to recover a substantial proportion of the residual oil from mature oil fields (Banat et al., 2010; Simpson et al., 2011). Biosurfactants are a heterogeneous group of surface-active molecules produced by microorganisms with both hydrophilic and hydrophobic domains, which allow them to partition at the interface between fluid phases with different degrees of polarity, such as oil–water or air–water interfaces. Due to these properties, biosurfactants reduce surface and interfacial tension and form stable emulsions where hydrocarbons can be solubilised in water, or water in hydrocarbons (Jenneman et al., 1983; Yakimov et al., 1995; Suthar et al., 2008; Banat et al., 2010). Biosurfactants produced by some microorganisms are capable of generating the low interfacial tension between the hydrocarbon and the aqueous phases required to mobilize entrapped oil (Yakimov et al., 1997; Youssef et al., 2007; Simpson et al., 2011). These compounds are good candidates for application in MEOR processes and can efficiently replace synthetic surfactants due to their specific activity, low toxicity, high biodegradability and effectiveness at extreme conditions of temperature, pressure, pH and salinity (Nitschke and Pastore, 2006; Abdel-Mawgoud et al., 2008; Bordoloi and Konwar, 2008; Pornsunthorntawee et al., 2008; Lotfabad et al., 2009).

There are two main strategies that can be adopted for the use of biosurfactants in enhanced oil recovery. Biosurfactants can be produced *ex situ* and subsequently injected into the reservoir; or they can be produced *in situ* by indigenous or injected microorganisms, stimulated by the addition of selected nutrients into the well. The first approach is expensive due to the capital required for bioreactor operation, product purification and introduction into oil-containing rocks. The second option is more favourable from an economic point of view, but requires the use of microorganisms capable of producing sufficient amounts of biosurfactant within the reservoir (Lazar et al., 2007; Banat et al., 2010).

Another mechanism of MEOR is the use of microorganisms to degrade heavy oil fractions. As a result the oil viscosity decreases and it becomes more fluid, lighter and more valuable. In some cases, combining multiple mechanisms by using consortia of microorganisms with different properties (ability to degrade heavy oil fractions and biosurfactant production) may be an effective strategy for enhanced oil recovery (Jinfeng et al., 2005). There are a number of microorganisms able to degrade hydrocarbons using them as carbon sources (Wentzel et al., 2007). During the last decade interesting results for the microbial *n*-alkane degradation have been reported (Grishchenkov et al., 2000; Li et al., 2002; Sabirova et al., 2006; Etoumi et al., 2008; Binazadeh et al., 2009). In particular, strains of *Gordonia amicalis* have shown ability to degrade large *n*-alkanes under aerobic and anaerobic conditions (Hao et al., 2008); *Pseudomonas fluorescens* are capable of degrading hydrocarbons with chain lengths between C12 and C32 (Banat, 1995); heavier *n*-alkanes (C36–C40) can be degraded by *Pseudomonas aeruginosa* strains (Hasanuzzaman et al., 2007); and a thermophilic *Bacillus* strain that degraded long-chain (C15–C36) but not short-chain (C8–C14) *n*-alkanes (Wang et al., 2006) has been reported. The use of biosurfactant-producing indigenous *Bacillus* strains to degrade the higher fractions of crude oil and enhance its flow characteristics has also been studied for a petroleum reservoir in the Daqing Oilfield (She et al., 2011).

The main drawback of MEOR is the difficulty of isolating microorganisms that can grow and produce the desired metabolic products under the environmental conditions that usually exist in oil reservoirs; many reservoirs are anaerobic and have high salinities and temperatures. Since few environmental parameters of the reservoirs can be manipulated, this also limits the number of reservoirs where microorganisms can be used for *in situ* treatment. *Bacillus subtilis* and *Bacillus licheniformis* strains have been isolated repeatedly from oil reservoirs, thus presumably are able to tolerate the reservoir conditions (Jenneman et al., 1983; Yakimov et al., 1995; Dastgheib et al., 2008; Ghojavand et al., 2008a; Youssef et al., 2009; Simpson et al., 2011).

In this work, several microorganisms were isolated from crude oil samples and screened for anaerobic growth, biosurfactant production and ability to degrade heavy oil fractions, in order to obtain suitable candidates for application in MEOR.

2. Materials and methods

2.1. Isolation of microorganisms from oil samples

Crude oil samples obtained from four wells (W-3, W-8, W-9 and W-11) of a Brazilian oil field were collected in sterile bottles. The temperature of the reservoir is about 40 °C. Samples were stored at room temperature until use. For isolation of microorganisms, two different methods were used. Direct isolation was performed by serially diluting crude oil samples, that were plated on agar solidified Bushnell–Hass medium and Raymond medium (containing 1% (v/v) *n*-hexadecane as the sole carbon source), as well as Luria–Bertani (LB) medium. Plates were incubated both in aerobic and anaerobic conditions at different temperatures. Enrichment cultures were prepared in 500 ml glass bottles containing 250 ml of mineral media (Bushnell–Hass medium and Raymond medium) supplemented with 1% (v/v) *n*-hexadecane as the sole carbon source. Crude oil samples (5 ml) were transferred to the bottles and incubated at 37, 42 and 50 °C for one month. To isolate bacterial strains, samples (200 μl) of the enrichment cultures were periodically spread on agar plates that were incubated at 37, 42 and 50 °C under aerobic and anaerobic conditions. After incubation, morphologically distinct colonies were re-isolated by transfer to fresh agar plates at least three times to obtain pure cultures. Isolate purity was verified through microscopic observation of Gram stained cultures. Pure cultures were stored at −80 °C in LB medium containing 20% (v/v) glycerol solution.

The composition of Raymond medium was (g/l): NaCl 50.0; Na_2HPO_4 3.0; NH_4NO_3 2.0; KH_2PO_4 2.0; yeast extract 0.5; $MgSO_4 \cdot 7H_2O$ 0.2; Na_2CO_3 0.1; $MnSO_4 \cdot 4H_2O$ 0.02; $CaCl_2$ 0.01; $FeSO_4$ 0.01; supplemented with 1% (v/v) *n*-hexadecane. The composition of Bushnell–Hass medium was (g/l): KH_2PO_4 1.0; K_2HPO_4 1.0; NH_4NO_3 1.0; $MgSO_4 \cdot 7H_2O$ 0.2; $FeCl_3$ 0.05; $CaCl_2$ 0.02; supplemented with 1% (v/v) *n*-hexadecane. The composition of LB medium was (g/l): NaCl 10.0; tryptone 10.0; yeast extract 5.0. All the media were adjusted to pH 7.0.

2.2. Screening of biosurfactant-producing strains

LB medium was used to study biosurfactant production by the different bacterial isolates. A single colony of each isolate was taken from the plate and transferred to 30 ml of LB liquid medium;

cultures were incubated at 40 °C and 120 rpm. Growth and biosurfactant production were examined under aerobic and anaerobic conditions. Anaerobic cultures were prepared removing oxygen by aseptically bubbling oxygen-free nitrogen into the flasks, which were sealed with rubber stoppers.

Samples were taken at different time points 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 (10,000 × *g*, 20 min, 10 °C) and cell-free supernatants were used to measure surface tension and emulsifying activity. At the end of the fermentation, cells were harvested by centrifugation and cell dry weight (g/l) was determined (48 h at 105 °C).

2.3. Growth and biosurfactant production in mineral media

The bacterial strains were grown in 30 ml of different mineral media (medium E and Mineral Salt Solution (MSS) with and without *n*-hexadecane (1%, v/v)) at 40 °C. The composition of Medium E was (g/l): NaCl 50.0; K_2HPO_4 10.6; sucrose 10.0; KH_2PO_4 5.3; $(NH_4)_2SO_4$ 1.0; $MgSO_4 \cdot 7H_2O$ 0.25; supplemented with 1% (v/v) of trace salt solution. Trace salt solution contained (g/l): $MnSO_4 \cdot 4H_2O$ 3.0; EDTA 1.0; $FeSO_4 \cdot 7H_2O$ 0.1; $CaCl_2 \cdot 2H_2O$ 0.1; $CoCl_2 \cdot 6H_2O$ 0.1; $ZnSO_4 \cdot 7H_2O$ 0.1; $CuSO_4 \cdot 5H_2O$ 0.01; $AlK(SO_4)_2 \cdot 12H_2O$ 0.01; H_3BO_3 0.01; $Na_2MoO_4 \cdot 2H_2O$ 0.01. The Mineral Salt Solution consisted of (g/l): NaCl 10.0; sucrose 10.0; Na_2HPO_4 5.0; NH_4NO_3 2.0; KH_2PO_4 2.0; $MgSO_4 \cdot 7H_2O$ 0.2. Both media were adjusted to pH 7.0.

2.4. Biosurfactant production and isolation

Biosurfactant production was carried out in shake flasks containing 500 ml of LB medium. Each flask was inoculated with 5 ml of a pre-culture that was prepared using the same culture medium and incubated overnight at the optimum conditions established for each isolate. The flasks were then incubated at the same conditions until the stationary phase was reached. At the end of the fermentation, cells were harvested by centrifugation (10,000 × *g*, 20 min, 10 °C) and cell dry weight (g/l) was determined (48 h at 105 °C). To recover the biosurfactant, the cell-free supernatant was subjected to acid precipitation. Briefly, the supernatant was adjusted to pH 2.0 with HCl 6 M and left overnight at 4 °C. Afterwards, the precipitate was collected by centrifugation (10,000 × *g*, 20 min, 4 °C) and washed twice with acidified water (pH 2.0). The crude biosurfactant was dissolved in a minimal amount of demineralised water and the pH was adjusted to 7.0 using NaOH 1 M. Finally, the crude biosurfactant solution was freeze-dried and stored at −20 °C for further use.

2.5. Surface-activity determination

Surface tension measurements of culture broth supernatants and biosurfactant solutions were performed according to the Ring method described elsewhere (Gudiña et al., 2010). A KRÜSS K6 Tensiometer (KRÜSS GmbH, Hamburg, Germany) equipped with a 1.9 cm De Noüy platinum ring was used. To increase the accuracy of the surface tension measurements, an average of triplicates was determined. All the measurements were performed at room temperature (20 °C). Isolates which reduced the culture broth surface tension below 40 mN/m were considered as biosurfactant producers.

2.6. 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 or biosurfactant solution in glass test tubes. The tubes were mixed with vortex at high speed for 2 min and then 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). Emulsions formed by the different isolates were compared with those formed by a 1% (w/v) solution of the synthetic surfactant sodium dodecyl sulphate (SDS) in demineralised water, as proposed by Das et al. (1998). All emulsification indexes were performed in triplicate.

2.7. Critical micelle concentration (cmc)

Critical micelle concentration is the concentration of an amphiphilic component in solution at which the formation of micelles is initiated. Concentrations ranging from 0.001 to 5 g/l of the crude biosurfactants recovered from the different isolates 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 previously described. The *cmc* was determined by plotting the surface tension as a function of the logarithm of biosurfactant concentration and it was found at the point of intersection between the two lines that best fit through the pre- and post-*cmc* data. All measurements were done in triplicate.

2.8. Identification of the isolates

Bacterial isolates that displayed high biosurfactant production were selected and identified by partial 16S rRNA sequencing. The 16S rRNA gene was amplified by PCR using primers 341F and 907R. The resulting sequences were compared with sequences in the GenBank database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) using the nucleotide–nucleotide blast (BLASTn) network service, to determine their phylogenetic affiliations.

2.9. Stability studies

The effect of several environmental parameters on the activity of biosurfactants produced by the different isolates was determined. Stability studies were performed using the cell-free broth obtained by centrifuging the cultures at 10,000 × *g* for 20 min. In order to assess the effect of salinity on biosurfactant activity, the culture broth supernatants were supplemented with different NaCl concentrations (from 10 to 200 g/l) and the surface tension was measured as described above. To evaluate the stability of the biosurfactants to high temperatures, broth samples were incubated at 121 °C for 20 min and allowed to cool to room temperature. Surface tension and emulsification indexes were measured and compared to the corresponding values before heat treatment. The pH stability was studied by adjusting the cell-free broth to different pH values (2.0–13.0) using HCl or NaOH solutions, and then surface tension values were measured as described above. All the experiments were carried out in triplicate.

2.10. Growth at different temperatures and salinities

In addition to the lack of oxygen, temperature and salinity appear to be the most important environmental factors that influence bacterial growth in the reservoir. Therefore, media containing different NaCl concentrations (0–200 g/l) were used to grow the isolated bacteria and cultures were incubated at temperatures ranging from 40 °C to 60 °C for different time intervals.

2.11. Hydrocarbon degradation determination

The ability of the isolated microorganisms to degrade paraffinic mixtures under aerobic and anaerobic conditions was evaluated using two different experimental conditions. In the first experimental condition studied, YP medium supplemented with a paraffinic mixture A (containing *n*-alkanes between C14–C24) was used. In the second condition, Mineral Salt Medium (MSM) and a heavier paraffinic mixture B (containing *n*-alkanes between C20–C30) were used. The mixture A was prepared in order to make a preliminary assessment of the degradation ability of 5 different strains. The mixture B was prepared in order to evaluate the ability of 6 different strains to degrade heavier *n*-alkanes chains under anaerobic conditions, aiming at their potential application on Microbial Enhanced Oil Recovery.

The composition of YP medium was (g/l): peptone 20.0; yeast extract 10.0. The MSM contained (g/l): KH_2PO_4 4.7; KNO_3 4.0; $MgSO_4 \cdot 7H_2O$ 1.0; Na_2HPO_4 0.119; $FeSO_4 \cdot 7H_2O$ 0.015; $CaCl_2 \cdot 2H_2O$ 0.01; $MnCl_2 \cdot 4H_2O$ 0.01. MSM was used to evaluate the degradation of *n*-alkanes by the different isolates in medium without additional carbon sources, since YP medium contains peptone and yeast extract, which can be used as carbon sources by the microorganisms.

Flasks containing 50 ml of each culture media supplemented with 10% (w/v) of the respective sterile paraffinic mixture were prepared. The flasks were sealed with rubber stoppers. Anaerobic cultures were prepared removing oxygen by aseptically bubbling oxygen-free nitrogen into the flasks. The paraffinic mixture A was composed by a ratio of 3:1 of two synthetic liquid paraffins, RT20 and RT31, obtained from Rubitherm (Rubitherm GmbH, Berlin, Germany). Culture media were supplemented with 1% (w/v) of squalane (Sigma–Aldrich Co., St. Louis, USA), in order to evaluate the ability of the strains to degrade ramified hydrocarbons. The mixture B was prepared with 23% (w/w) of solid paraffin wax with a melting point of 50–52 °C (purchased from Sigma–Aldrich), dissolved in dodecane (99% of purity, also from Sigma–Aldrich). Cultures were performed at 40 °C and 150 rpm during 4 days under aerobic conditions, and during 15 days in anaerobic conditions. Control flasks were incubated at the same conditions without addition of microorganisms.

After the incubation time, the organic phase was separated and diluted (20 mg/ml) in dichloromethane for gas chromatography (GC) analysis. GC analysis of each sample was performed with CP 3800 Varian gas Chromatograph equipped with and on-column injection, FID detector, and DB-5HT capillary column (30 m × 0.32 mm i.d., 0.1 μm thickness) (J&W Scientific Inc., California, USA). Helium was used as gas carrier and a constant flow rate of 2 ml/min was set. Injector and detector temperatures were 350 and 370 °C, respectively. Oven temperature was set at 50 °C during 1 min, raised to 350 °C at the rate of 10 °C/min, and at last kept at 370 °C during 1 min. All the samples were analyzed in triplicate.

3. Results and discussion

3.1. Isolation of microorganisms from oil samples

When petroleum samples were spread on agar plates and incubated at different temperatures, no isolates were obtained, neither under aerobic nor anaerobic conditions. However, using the enrichment method, a total of fifty-eight bacterial strains were recovered. Results obtained are summarized in Table 1.

3.2. Screening of biosurfactant-producing strains

The different isolates were screened for extracellular biosurfactant production in LB medium under aerobic and anaerobic conditions at 40 °C. To evaluate biosurfactant production, both surface tension and emulsifying activity were taken into account. However, only those isolates that reduced surface tension were found to exhibit emulsifying activity (no bioemulsifier production was detected). Under aerobic conditions, thirty of the fifty-eight isolates reduced the culture medium surface tension below 40 mN/m, and therefore were considered biosurfactant producers. From these, only five isolates produced biosurfactants also under anaerobic conditions (Table 1). The most relevant results obtained are shown in Table 2.

Table 1
Number of isolates obtained from the different oil samples at different temperatures and results obtained regarding biosurfactant production from the screening process under aerobic and anaerobic conditions.

Sample	Number of isolates				Biosurfactant production	
	37 °C	42 °C	50 °C	Total	Aerobic	Anaerobic
W-3	3	3	14	20	8	2
W-8	3	11	7	21	12	0
W-9	2	3	3	8	2	0
W-11	2	1	6	9	8	3
Total	10	18	30	58	30	5

Biosurfactants produced by isolates #111 and #902 showed the highest emulsifying activity against *n*-hexadecane, similar to the one obtained with the synthetic surfactant SDS. However, surface tension values obtained with those isolates were not as low as the obtained with other isolates. Furthermore, although isolates #111 and #902 grew at anaerobic conditions, no surface tension reduction or emulsifying activity could be observed, which hampers their application in MEOR processes *in situ*, since most oil reservoirs are anaerobic. The other isolates described in Table 2 produced biosurfactants under both aerobic and anaerobic conditions. However, biomass and biosurfactant production were higher under aerobic conditions for all the isolates, which is in agreement with the results obtained by other authors (Yakimov et al., 1995, 1997; Dastgheib et al., 2008; Ghojavand et al., 2008a). Isolates #309, #311 and #573 showed the highest surface activity, reducing the surface tension of the culture broth from 51.3 mN/m to values around 30 mN/m, and emulsified *n*-hexadecane similarly to SDS. Isolates #309 and #311 were identified as the best biosurfactant producers under anaerobic conditions. Furthermore, emulsions formed by supernatants obtained from isolates #111, #309, #311, #573 and #902 remained stable for two weeks at 25 °C.

3.3. Identification of the isolates

The isolates described in Table 2 were identified according to the partial sequence obtained from their 16S rRNA genes. The sequences obtained were compared with those described in databases. The partial sequence of 16S rRNA genes from isolates #111 and #902 showed 100% similarity with *P. aeruginosa*. The partial sequence of 16S rRNA genes of the remaining isolates showed 100% similarity with *B. subtilis*. The sequences were deposited in the Gen Bank database under accession numbers JQ281104–JQ281110.

3.4. Study of biosurfactant production in mineral media

The isolates presenting the best results regarding biosurfactant production in LB medium were then evaluated under anaerobic conditions using Mineral Salt Solution (MSS), Mineral Salt Solution supplemented with 1% (v/v) *n*-hexadecane (MSSH) and medium E. The results obtained are gathered in Table 3. As can be seen from these results, the MSS (with and without *n*-hexadecane) offered better results than medium E for all the isolates. Addition of

Table 2
Surface tension values (mN/m), emulsification indexes (E_{24}, %) and biomass concentrations (g/l) obtained with the different isolates grown aerobically and anaerobically in LB medium at 40 °C. Surface tension values were determined six times at room temperature (20 °C). Emulsification indexes were calculated three times at 25 °C. Biomass concentration was calculated as dry weight (105 °C, 48 h). Results represent the average of three independent experiments ± standard deviation.

Strain	Aerobic			Anaerobic		
	ST (mN/m)	E_{24} (%)	[Biomass] (g/l)	ST (mN/m)	E_{24} (%)	[Biomass] (g/l)
#111	34.1 ± 0.6	56.5 ± 0.0	1.315 ± 0.346	53.7 ± 4.9	0.0 ± 0.0	0.363 ± 0.138
#191	31.0 ± 1.1	52.3 ± 2.4	1.364 ± 0.467	35.7 ± 1.5	2.6 ± 4.1	0.195 ± 0.050
#309	29.9 ± 0.1	51.6 ± 1.4	0.709 ± 0.252	31.0 ± 1.3	22.9 ± 11.7	0.218 ± 0.097
#311	30.1 ± 0.4	50.8 ± 0.0	0.365 ± 0.110	31.0 ± 1.3	22.7 ± 9.4	0.246 ± 0.041
#552	32.0 ± 0.2	46.1 ± 12.3	1.373 ± 0.379	35.4 ± 1.3	3.0 ± 3.7	0.207 ± 0.060
#573	30.5 ± 0.5	52.3 ± 1.5	1.686 ± 0.648	36.0 ± 2.4	2.2 ± 3.5	0.178 ± 0.041
#902	36.1 ± 0.6	56.5 ± 0.0	1.328 ± 0.011	56.0 ± 3.6	0.0 ± 0.0	0.413 ± 0.144
LB	51.3 ± 0.5	0.0 ± 0.0	–	51.3 ± 0.5	0.0 ± 0.0	–
SDS 1%	39.9 ± 0.4	55.0 ± 1.7	–	39.9 ± 0.4	55.0 ± 1.6	–

n-hexadecane to the culture broth was found to promote a higher biosurfactant production by some isolates and had no effect on the others. None of the isolates was able to grow or produce biosurfactants in MSS using *n*-hexadecane as the sole carbon source (*data not shown*). The lowest surface tension values (around 30 mN/m) were obtained by isolates #309, #311 and #573, with emulsifying indexes between 24% and 34%.

Several *P. aeruginosa* strains have been recovered from petroleum samples and oil contaminated places, and their biosurfactants exhibit good characteristics for application in MEOR, as demonstrated by simulations performed in sand-pack columns (Bordoloi and Konwar, 2008; Pornsunthorntawee et al., 2008; Lotfabad et al., 2009). However, those isolates were studied under aerobic conditions and the biosurfactants were produced *ex situ*. Therefore, although the authors suggested their possible use in MEOR, these strains are not appropriate for use in oil reservoirs. Most of the microorganisms that are potentially useful for application in MEOR *in situ* belong to the genus *Bacillus*. *Bacillus mojavensis* JF-2, a thermotolerant and halotolerant strain isolated from an oil field by Jenneman and collaborators (1983), grows and produces similar amounts of a lipopeptide biosurfactant under both aerobic and anaerobic conditions, reducing the surface tension of the medium below 30 mN/m, and additionally it is not inhibited by crude oil (Jenneman et al., 1983; Javaheri et al., 1985). Other strains isolated from oil fields and which applicability in MEOR has been demonstrated are *B. licheniformis* BAS50 (Yakimov et al., 1995, 1997), *B. licheniformis* ACO1 (Dastgheib et al., 2008) and *B. subtilis* PTCC1696 (Ghojavand et al., 2008a). Biosurfactants produced by those microorganisms recovered 22–60% of the residual oil entrapped in sand-packed columns (Yakimov et al., 1997; Dastgheib et al., 2008; Pornsunthorntawee et al., 2008; Suthar et al., 2008). Furthermore, Youssef et al. (2007) demonstrated that inoculation of oil wells with *Bacillus* strains and nutrients is possible, and that biosurfactants can be produced *in situ* at concentrations that are sufficient to mobilize significant amount of residual oil. Taking into account these data and the results obtained with isolates #309, #311 and #573 regarding biosurfactant production, they can be considered good candidates for application in MEOR.

3.5. Growth at different temperatures and salinities

Growth at several temperatures and salinities was evaluated for the different isolates in solid and liquid medium. *B. subtilis* isolates (#191, #309, #311, #552 and #573) were able to grow at temperatures between 40 and 55 °C in solid medium. At 45 °C they grew at NaCl concentrations up to 100 g/l. Isolates #309 and #311 grew at 50 °C in liquid medium, and biosurfactant production was similar to the obtained at 40 °C. However, isolates #191, #552 and #573 were unable to grow in liquid medium at temperatures higher than 45 °C. Similar results with several *Bacillus* strains have been reported by other authors (Jenneman et al., 1983; Javaheri et al., 1985; Yakimov et al., 1995, 1997; Dastgheib et al., 2008; Ghojavand et al., 2008a). For *P. aeruginosa* isolates #111 and #902 no growth was observed at temperatures higher than 42 °C neither in liquid or solid media.

3.6. Hydrocarbon degradation

Although the use of *Bacillus* strains for biosurfactant production has been widely described in the literature, their use to degrade *n*-alkanes has seldom been addressed. In order to evaluate the ability of the isolated *B. subtilis* strains to degrade *n*-alkanes, two different paraffinic mixtures (A and B) were used. The mixture A was incubated with isolates #191, #551, #571, #572 and #573 under aerobic and anaerobic conditions. The respective variations of relative weight fraction of each *n*-alkane present in mixture A compared with the control sample (incubated at the same conditions without microorganisms) are illustrated in Figs. 1 and 2.

The isolates studied were found to be able to grow in YP medium supplemented with paraffinic mixture A, both under

Table 3
Surface tension values (mN/m), emulsification indexes (E_{24}, %) and biomass concentrations (g/l) obtained with the different isolates grown in different media for 120 h at 40 °C and 120 rpm under anaerobic conditions. Surface tension values were determined six times at room temperature (20 °C). Emulsification indexes were calculated three times at 25 °C. Biomass concentration was calculated as dry weight (105 °C, 48 h). Results represent the average of three independent experiments ± standard deviation.

Strain	MSS			MSSH			Medium E		
	ST (mN/m)	E_{24} (%)	[Biomass] (g/l)	ST (mN/m)	E_{24} (%)	[Biomass] (g/l)	ST (mN/m)	E_{24} (%)	[Biomass] (g/l)
#191	41.7 ± 1.2	14.6 ± 1.9	0.194 ± 0.062	40.0 ± 2.0	13.3 ± 5.9	0.176 ± 0.055	50.5 ± 1.9	11.7 ± 2.1	0.188 ± 0.071
#309	31.4 ± 1.3	25.9 ± 4.2	0.221 ± 0.050	31.4 ± 0.5	24.6 ± 5.3	0.196 ± 0.068	32.2 ± 1.5	28.9 ± 2.7	0.193 ± 0.033
#311	30.5 ± 0.1	8.5 ± 4.0	0.245 ± 0.076	31.0 ± 0.6	34.2 ± 3.9	0.191 ± 0.042	36.4 ± 1.3	8.0 ± 2.8	0.175 ± 0.035
#552	48.6 ± 1.7	13.4 ± 1.6	0.263 ± 0.087	41.5 ± 1.9	14.1 ± 5.6	0.238 ± 0.008	56.3 ± 0.9	10.2 ± 4.8	0.160 ± 0.051
#573	31.5 ± 0.4	24.9 ± 2.3	0.284 ± 0.057	33.7 ± 1.4	19.4 ± 5.6	0.217 ± 0.073	43.2 ± 1.2	16.9 ± 7.5	0.230 ± 0.071
Control	66.4 ± 1.5	0.0 ± 0.0	–	63.3 ± 0.9	0.0 ± 0.0	–	65.6 ± 0.8	0.0 ± 0.0	–
SDS 1%	39.9 ± 0.4	55.0 ± 1.7	–	39.9 ± 0.4	55.0 ± 1.7	–	39.9 ± 0.4	55.0 ± 1.7	–

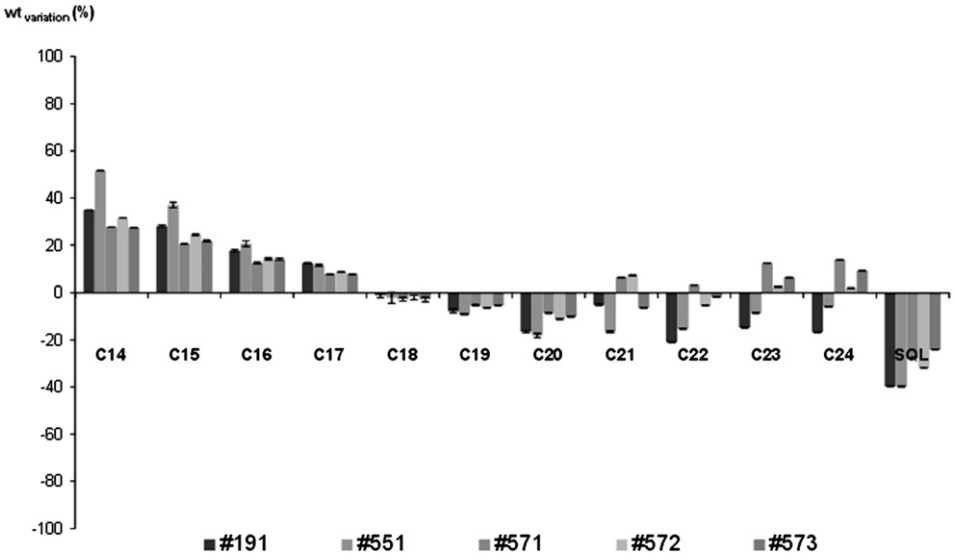

Fig. 1. Variation of relative weight fraction of *n*-alkanes present in paraffinic mixture A after incubation with bacterial isolates, for 4 days at 40 °C and aerobic conditions as compared to the control. (SQL: squalane).

aerobic and anaerobic conditions. The increase observed in the relative weight fraction of *n*-alkanes lower than C18 and the decrease observed in the percentage of *n*-alkanes between C18 and C20 after treatment with isolates #191, #551, #571, #572 and #573 indicate that those isolates degraded *n*-alkanes between C18 and C20 into lighter ones, both under aerobic and anaerobic conditions. In presence of oxygen, isolates #191 and #551 were able to degrade also *n*-alkanes higher than C20. Comparing the influence of oxygen on degradation of *n*-alkanes by the different isolates, significant differences were only observed for isolates #191 and #571. Isolate #191 showed a higher ability to degrade the long-chain *n*-alkanes (C18–C24) under aerobic conditions; however in absence of oxygen it was only able to degrade *n*-octadecane and *n*-nonadecane. On other hand, isolate #571 exhibited an opposite behaviour and better results were obtained under anaerobic conditions. For the remaining isolates, similar degradation values were obtained in both conditions. The experiments also showed that all the isolates were able to degrade ramified hydrocarbons (squalane) either with or without oxygen.

In view of the positive results obtained, the authors decided to study the anaerobic degradation in a mineral medium where the hydrocarbons were the only available carbon source. Furthermore, aiming at extending the range of *n*-alkanes studied a new mixture B containing *n*-alkanes up to C30 was used. It was observed that *B. subtilis* isolates #191, #309, #316, #551, #552 and #572 degraded the higher *n*-alkanes (>C27) under anaerobic conditions, and the percentage of *n*-alkanes with chains containing less than 25 carbons increased relatively to the control sample as shown in Fig. 3. The best results were obtained with isolates #191, #309 and #552.

The ability of a thermophilic *Bacillus* strain to degrade *n*-alkanes ranging from C15 to C36, but not those lower than C14

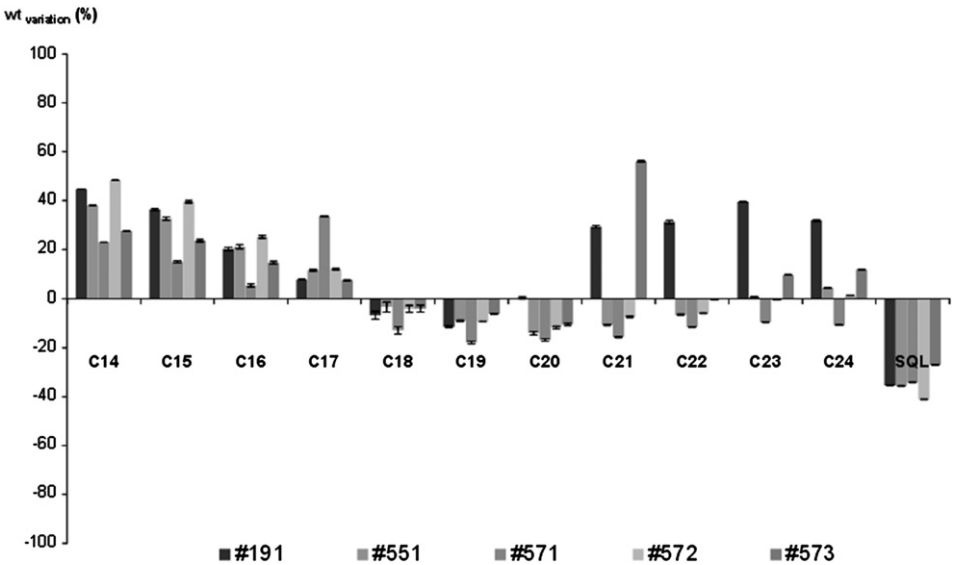

Fig. 2. Variation of relative weight fraction of *n*-alkanes present in paraffinic mixture A after incubation with bacterial isolates, for 15 days at 40 °C and anaerobic conditions as compared to the control.

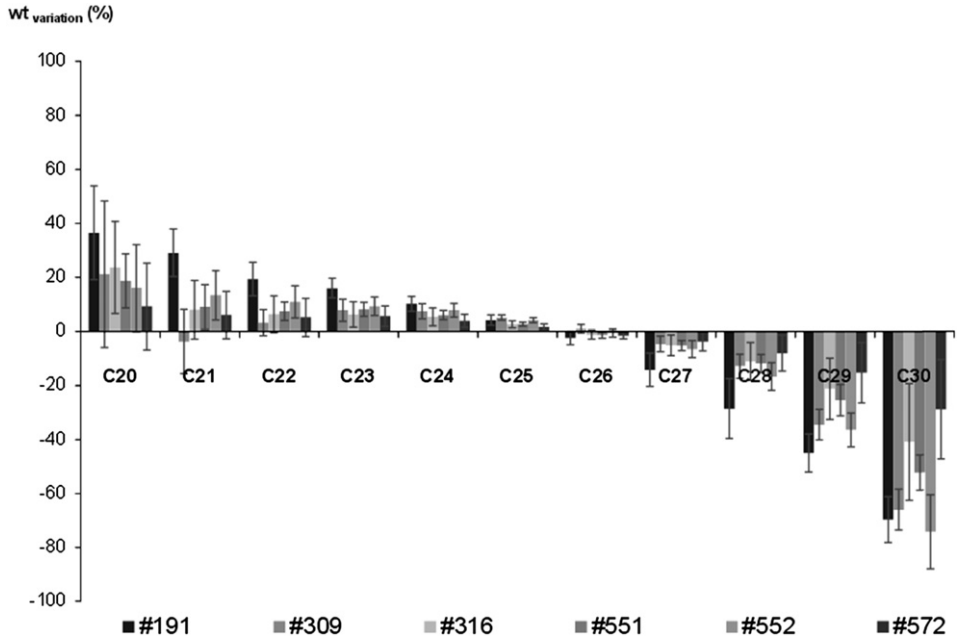

Fig. 3. Variation of relative weight fraction of *n*-alkanes present in paraffinic mixture B after incubation with bacterial isolates for 15 days, at 40 °C and anaerobic conditions as compared to the control.

was observed by Wang et al. (2006). She et al. (2011) observed the degradation of *n*-alkanes between C14 and C26 by three *Bacillus* strains isolated from an oil field. Moreover, Kato et al. (2001) isolated two *Bacillus thermoleovorans* strains from a petroleum reservoir which degraded *n*-alkanes between C12 and C30. Das and Mukherjee (2007) also observed that *B. subtilis* DM-04 preferentially degraded crude oil *n*-alkanes between C14 and C30. However, those assays were performed in aerobic conditions. *G. amicalis* LH3 utilizes a wide range of long-chain *n*-alkanes (C18–C36), although under aerobic conditions degraded preferentially *n*-alkanes between C18 and C24, whereas under anaerobic conditions degraded higher *n*-alkanes (C34–C36) (Hao et al., 2008). In MEOR processes, the degradation of long-chain *n*-alkanes under anaerobic conditions is a very important tool, since most of the oil reservoirs are anaerobic. Isolates #191, #309 and #552 showed the ability to degrade *n*-alkanes with chains higher than C27 into lighter ones under anaerobic conditions, which can be applied to improve the flow properties of crude oil. Furthermore, isolates #191 and #309 additionally showed biosurfactant production under anaerobic conditions, thus suggesting those isolates as attractive candidates for application in MEOR.

Table 4
Surface tension values (mN/m), emulsification indexes (E_{24}, %) and critical micelle concentrations (g/l), obtained with freeze-dried biosurfactants from the different isolates dissolved in PBS (pH 7.0). Samples were prepared with a concentration of 1 g/l for *B. subtilis* isolates and 5 g/l for *P. aeruginosa* isolates. Surface tension values were determined six times at room temperature (20 °C). Emulsification indexes were calculated three times at 25 °C. Results represent the average of three independent experiments ± standard deviation.

Isolate	ST (mN/m)	E_{24} (%)	cmc (g/l)
#111	35.5 ± 0.1	53.7 ± 0.0	2.3
#191	32.8 ± 0.2	34.8 ± 1.9	0.13
#309	30.4 ± 0.2	50.8 ± 3.6	0.02
#311	30.3 ± 0.3	51.3 ± 4.1	0.02
#552	32.0 ± 0.3	31.1 ± 2.0	0.10
#573	31.2 ± 0.2	43.3 ± 1.6	0.03
#902	37.9 ± 0.1	52.3 ± 0.0	2.3

3.7. Critical micelle concentration (cmc)

Freeze-dried biosurfactants obtained from the different isolates were dissolved in PBS (pH 7.0) at a concentration of 1 g/l for *B. subtilis* isolates, and 5 g/l for *P. aeruginosa* strains, and surface tension, as well as emulsifying activity were determined. As can be seen from Table 4, the lowest surface tension values were obtained with isolates #309 and #311; those isolates also showed the highest emulsifying activities, except for the isolates #111 and #902 (but in this case, biosurfactant concentration was five times higher). To obtain the *cmc*, the original concentrations were serially diluted with PBS buffer and the surface tension was determined. The effective concentration of biosurfactants from *B. subtilis* isolates was found to be much lower than that of biosurfactants from *P. aeruginosa* strains, with *cmc* values 20–100 times lower. The lowest *cmc* values (0.02 g/l) corresponded to isolates #309 and #311, with a minimum surface tension value of 30 mN/m.

Lipopeptide biosurfactants produced by *Bacillus* strains include, among others, surfactin and lichenysin, which reduce surface tension to values around 26–36 mN/m, with *cmc* values from 0.01 to 0.04 g/l, and emulsification indexes of about 60% (Javaheri et al., 1985; Yakimov et al., 1995; Nitschke and Pastore, 2006; Abdel-Mawgoud et al., 2008; Ghojavand et al., 2008b;

Table 5
Effect of NaCl concentration on surface activity. Surface tension values (mN/m) obtained with culture broth supernatants at different NaCl concentrations (10–200 g/l). Surface tension values were determined six times at room temperature (20 °C). Results are expressed as means ± standard deviations of three independent measurements.

Isolate	NaCl (g/l)				
	10	50	100	150	200
	ST (mN/m)				
#191	30.8 ± 0.2	28.5 ± 0.1	29.4 ± 0.4	38.0 ± 4.1	49.5 ± 2.3
#309	29.6 ± 0.2	27.0 ± 0.0	27.8 ± 0.1	31.5 ± 1.4	37.4 ± 0.5
#311	29.8 ± 0.1	27.0 ± 0.0	27.9 ± 0.2	31.2 ± 0.2	38.8 ± 0.8
#552	31.4 ± 0.1	29.0 ± 0.1	30.6 ± 0.3	45.0 ± 3.9	51.5 ± 0.7
#573	30.6 ± 0.1	28.1 ± 0.1	30.1 ± 0.3	42.4 ± 4.4	47.7 ± 0.8

Table 6
Effect of pH on surface activity. Surface tension values (mN/m) obtained with culture broth supernatants at different pH values. Surface tension values were determined six times at room temperature (20 °C). Results are expressed as the means ± standard deviations of three independent experiments.

Isolate	pH					
	2.0	3.0	4.0	5.0	6.0	7.0
			ST (mN/m)			
#191	56.2 ± 2.3	49.7 ± 3.4	38.9 ± 1.6	30.8 ± 0.1	29.0 ± 0.1	30.9 ± 0.3
#309	60.0 ± 2.0	54.6 ± 1.8	43.8 ± 4.0	31.8 ± 0.1	27.5 ± 0.1	29.8 ± 0.1
#311	62.5 ± 1.2	63.4 ± 2.2	46.5 ± 5.3	31.2 ± 0.3	27.7 ± 0.1	29.8 ± 0.1
#552	60.1 ± 1.8	55.7 ± 2.5	47.3 ± 2.6	33.0 ± 0.6	29.1 ± 0.2	31.7 ± 0.2
#573	61.7 ± 1.2	56.4 ± 2.4	41.8 ± 2.7	30.3 ± 0.1	28.6 ± 0.2	30.8 ± 0.2
	8.0	9.0	10.0	11.0	12.0	13.0
#191	30.8 ± 0.3	31.0 ± 0.3	31.2 ± 0.2	31.3 ± 0.2	32.6 ± 0.1	35.2 ± 0.3
#309	29.9 ± 0.1	30.1 ± 0.1	30.1 ± 0.2	30.2 ± 0.1	33.0 ± 0.1	34.8 ± 0.1
#311	29.9 ± 0.1	30.4 ± 0.1	30.4 ± 0.2	30.4 ± 0.1	33.5 ± 0.1	34.4 ± 0.1
#552	31.3 ± 0.3	31.8 ± 0.2	32.0 ± 0.1	32.2 ± 0.2	34.2 ± 0.2	34.9 ± 0.2
#573	30.9 ± 0.1	31.0 ± 0.2	31.2 ± 0.1	31.3 ± 0.2	34.1 ± 0.4	35.0 ± 0.4

Pornsunthorntawee et al., 2008; Vaz et al., 2012). In sand-pack column assays performed with different *Bacillus* strains, Suthar and co-workers (2008) observed that the combination of surface and emulsifying activity resulted in higher oil recovery when compared with strains possessing merely surface activity, since emulsifiers stabilize oil–water emulsions, which facilitates oil displacement (Suthar et al., 2008).

3.8. Effect of temperature, pH and salinity on biosurfactant stability

In order to assess the effect of salinity on biosurfactant activity, culture broth supernatants were supplemented with different NaCl concentrations, ranging from 10 to 200 g/l, and surface tension was measured. Data obtained are presented in Table 5. The maximum surface tension reduction was reached with a NaCl concentration of 50 g/l for all the isolates, and at 100 g/l the surface tension values were still lower than the obtained at 10 g/l, similar to the results described by other authors (Yakimov et al., 1995; Nitschke and Pastore, 2006; Abdel-Mawgoud et al., 2008; Dastgheib et al., 2008; Ghojavand et al., 2008b). NaCl concentrations above 100 g/l resulted in higher surface tension values.

The pH of biosurfactant-containing culture media was changed from 2.0 to 13.0 and surface tension values were measured (Table 6). Biosurfactant activity was retained at a pH range of 5.0–11.0 with a minimum deviation in surface tension for all the isolates, showing higher stability at alkaline than acidic conditions, and the minimum surface tension values were obtained at pH 6.0 for all the isolates, as previously described (Nitschke and Pastore, 2006; Abdel-Mawgoud et al., 2008; Ghojavand et al., 2008b; Vaz et al., 2012). Below pH 5.0, surface tension increased due to the biosurfactant precipitation.

Table 7
Surface tension values (mN/m) and emulsification indexes (E_{24}, %) obtained with culture broth supernatants from the different isolates before and after exposure to 121 °C for 20 min. Surface tension values were determined six times at room temperature (20 °C). Emulsification indexes were calculated three times at 25 °C. Results are expressed as means ± standard deviations of values from triplicate experiments.

	Before		After	
	ST (mN/m)	E_{24} (%)	ST (mN/m)	E_{24} (%)
#191	31.8 ± 0.1	50.8 ± 0.0	32.6 ± 0.1	42.4 ± 0.0
#309	29.9 ± 0.1	50.8 ± 0.0	30.0 ± 0.1	53.7 ± 0.0
#311	29.8 ± 0.0	50.8 ± 0.0	30.1 ± 0.1	53.7 ± 0.0
#552	31.9 ± 0.2	46.6 ± 1.9	32.8 ± 0.2	43.8 ± 2.0
#573	30.6 ± 0.3	52.3 ± 1.9	31.1 ± 0.1	52.3 ± 1.9

Usually, lipopeptide biosurfactants retain their surface-active properties after incubation at 100 °C for 2 h (Ghojavand et al., 2008b), and their stability when exposed to 121 °C has been described by several authors (Nitschke and Pastore, 2006; Abdel-Mawgoud et al., 2008; Dastgheib et al., 2008; Vaz et al., 2012). To study the stability of biosurfactants at high temperatures, culture broth supernatants obtained at the end of the fermentation were incubated at 121 °C for 20 min. Surface tension and emulsification activity were measured before and after heating. As can be seen from Table 7, the temperature increased had no negative effect in surface activity.

4. Conclusion

In the current study, *B. subtilis* strains exhibiting desirable properties for application in MEOR were isolated from crude oil samples. Some isolates grew under anaerobic conditions in medium with NaCl concentrations up to 100 g/l and temperatures up to 45–50 °C and were not inhibited by the presence of hydrocarbons. The biosurfactants produced by those isolates have some properties, including thermo- and salt-tolerance; ability to reduce the surface tension of water to 30 mN/m; ability to emulsify hydrocarbons and critical micelle concentrations of 0.02–0.03 g/l. Some *B. subtilis* isolates were also capable of degrading the heavier *n*-alkanes in different paraffinic mixtures, both under aerobic or anaerobic conditions at 40 °C. And some isolates (#191 and #309) exhibited simultaneously the ability for biosurfactant production and hydrocarbon degradation, making them promising candidates for MEOR. Their usefulness for MEOR applications will be further evaluated using laboratory scale sand-pack columns in the future.

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