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Biosurfactant producing microorganisms and its application to Enhance Oil Recovery at lab scale

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Abstract

Microbial Enhanced Oil Recovery (MEOR) is a tertiary oil recovery process where microorganisms and their metabolites are used to retrieve unrecoverable oil from mature reservoirs. Stimulation of biosurfactant production by indigenous microorganisms can reduce the capillary forces that retain the oil into the reservoir. The studied reservoir is characterized by alternated oil and water sand layers, with an average porosity of 25% and a permeability of 50 mD. It's a flat structure at 450 m depth, with an initial pressure of 32.4 bars and a temperature of 42.5ºC. The oil is paraffinic, with low viscosity, high pour point and a gravity of 25º API, with no gas dissolved. Due to these properties, the wells can't flow naturally and the production has to be lifted, making this reservoir a good candidate for MEOR application. This work addresses the isolation and identification of microorganisms capable of producing biosurfactants and degrading heavy oil fractions at the oil reservoir conditions. Five *Bacillus subtilis* strains isolated from oil samples were able to grow and produce extracellular biosurfactants at 40ºC under anaerobic conditions in medium supplemented with hydrocarbons. In addition, some of the isolates displayed a capacity to degrade, both in aerobic and anaerobic conditions, the large alkyl chains, and reduce the viscosity of hydrocarbon mixtures. A sand-pack column model was designed to simulate the oil recovery operations and evaluate the mobilization of residual oil by microorganisms. Additional oil recovery using *B. subtilis* isolates ranged from 19.8 to 35.0%, suggesting that stimulation of biosurfactant production by these strains *in situ* can contribute to mobilize entrapped oil. The novelty of this technique, compared to the conventional EOR methods, is the application of indigenous microorganisms to increase the oil recovery.

Introduction

The primary phase of oil recovery uses the natural stored energy of the reservoirs to produce oil and gas. As the reservoir pressure dissipates, the flow of oil to the well head can be improved by the injection of water into the wells. When the ratio of water to oil pumped out of the well becomes too great, the process is discontinued. However, after primary and secondary recovery operations, up to two-thirds of the original oil in place still remains in the reservoir. This is mainly due to the high viscosity of the residual oil, which limits its mobility, and the high interfacial tension between the hydrocarbon and the aqueous phases, which results in high capillary forces that retain the oil in small pores within the reservoir rock (Lazar et al. 2007; Sen 2008). A major challenge to the oil industry is extracting the maximum amount of oil from reservoirs. To recover entrapped oil, costly tertiary methods (including chemical and thermal process) are applied.

Microbial Enhanced Oil Recovery (MEOR) is an alternative tertiary oil recovery technology where microbial metabolites (biomass, biopolymers, gases, acids, solvents, enzymes and surface-active compounds) and activities (hydrocarbon metabolism, plugging) are used to improve the recovery of residual oil from depleted and marginal reservoirs, thereby extending their life (Brown 2010). This technology takes advantage of the ability of indigenous or injected microorganisms to synthesize useful products by fermenting inexpensive raw materials. MEOR processes offer major advantages over conventional EOR, namely they don't consume large amounts of energy as the thermal processes, nor depend on the price of crude oil as many chemical processes. Furthermore, microbial products are biodegradable and have low toxicity (Lazar et al. 2007; Sen 2008; Youssef et al. 2009).

Biosurfactant production by microorganisms *in situ* constitutes an effective mechanism to recover large amounts of the residual oil from mature oil fields (Almeida et al. 2004; Banat et al. 2010; Bordoloi and Konwar 2008). Biosurfactants are a heterogeneous group of surface-active molecules synthesized 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, reducing surface and interfacial tensions (Banat et al. 2010). Among them, lipopeptide biosurfactants produced by *Bacillus* species are capable of generating the low interfacial tension between the hydrocarbon and the aqueous phases required to mobilize entrapped oil (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 (Bordoloi and Konwar 2008; Suthar et al. 2008). Biosurfactant production *in situ* at concentrations that allow the mobilization of significant amounts of residual oil has been demonstrated using selected microorganisms stimulated by the addition of nutrients into the wells (Youssef et al. 2007).

Another important factor in MEOR is the degradation of heavy oil fractions to reduce oil viscosity and improve its mobility. In recent years, many studies have shown microorganisms capable to use heavy paraffinic oils as a carbon source (Wentzel et al. 2007). Despite the large number of species described as hydrocarbon degraders, only a few reports presented the ability of *Bacillus* strains to degrade the *n*-alkanes. A thermophilic *Bacillus* strain that had capacity to degrade long-chain rather that short-chain of *n*-alkanes was isolated by Wang et al. (2006) and recently, a petroleum reservoir in the Daqing Oilfiled was treated with *Bacillus* strains that degraded the higher fractions of crude oil and enhanced the flow characteristics (She et al. 2011). The efficiency of biodegradation of oil compounds is limited by poor mass transfer due to their high hydrophobicity, leading to low aqueous solubility. Surfactants and biosurfactants increase the aqueous solubility of hydrocarbons to enhance their bioavailability, leading to higher degradation rates. 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).

Laboratory studies on MEOR have typically used sand-pack columns, which provide a convenient bench-scale approach to evaluate oil recovery for several reasons: it is an economic model; a battery of columns can be set up simultaneously; and they simulate oil recovery operations of oil reservoirs (Suthar et al. 2008). In this study, a sand-pack column model was used to study the effect of biosurfactant-producing and oil-degrading microorganisms isolated from crude oil samples on the mobilization of entrapped oil.

Materials and Methods

Reservoir and fluid characteristics

The studied reservoir is characterized by alternated oil and water sand layers, with an average porosity of 25% and a permeability of 50 mD. The reservoir is a flat structure at 450 m depth, with no natural pressure support and with an initial pressure and temperature of 32.4 bars and 42.5ºC, respectively. The oil is paraffinic, with low viscosity, high pour point and a gravity of 25º API, with no gas dissolved. Due to these properties, the wells cannot flow naturally and the production has to be lifted. Based on these characteristics, the reservoir is a good candidate for MEOR application as a process to enhance the fluid flow and the ultimate recovery of the reservoir.

Isolation of microorganisms from oil samples

Crude oil samples obtained from four different wells of the studied reservoir were collected in sterile bottles. For the isolation of microorganisms enrichment cultures were prepared in 500 ml glass bottles containing 250 ml of 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 40ºC for one month. To isolate bacterial strains, samples (200 µl) of the enrichment cultures were periodically spread on LB agar plates that were incubated at 40ºC under aerobic and anaerobic conditions. After incubation, morphologically different colonies were re-isolated by transfer to fresh agar plates at least three times to obtain pure cultures. Isolates purity was verified through microscopic observation of Gram stained cultures. Pure cultures were stored at -80ºC in LB medium supplemented with 20% (v/v) glycerol.

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; $MgSO_4$ $7H_2O$ 0.2; Na_2CO_3 0.1; $MnSO_4$ $4H_2O$ 0.02; $CaCl_2$ 0.01; $FeSO_4$ 0.01; 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. Both media were adjusted to pH 7.0.

Screening of biosurfactant-producing strains in mineral media

The bacterial strains were grown in Mineral Salt Solution (MSS) with and without *n*-hexadecane (1%, v/v) at 40ºC. The MSS 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; $MgSO_4$ $7H_2O$ 0.2. The pH was adjusted to 7.0.

A single colony of each isolate was taken from the plate and transferred to 30 ml of MSS. Anaerobic cultures were prepared removing oxygen by aseptically bubbling oxygen-free nitrogen into the flasks, which were sealed with rubber stoppers. Cultures were incubated at 40ºC and 120 rpm. 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 (10000 × g, 20 min, 10ºC) and the cell-free supernatants were used to measure surface tension and emulsifying activity.

Surface-activity determination

Surface tension measurements of culture broth supernatants 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 (20ºC). Isolates which reduced the culture broth surface tension below 40 mN/m were considered biosurfactant producers.

Emulsifying activity determination

Emulsifying activity was determined by the addition of 2 ml of *n*-hexadecane to the same volume of cell-free culture broth supernatant in glass test tubes. The tubes were mixed with vortex at high speed for 2 min and then incubated at 25ºC for 24 hours. The stability of the emulsion was determined after 24 hours, 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. All emulsification indexes were performed in triplicate.

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 the 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.

Hydrocarbon degradation determination

The ability of three isolated microorganisms to degrade paraffinic mixtures at anaerobic conditions was evaluated. A basal mineral salt medium (BMSM) supplemented with a paraffinic mixture containing *n*-alkanes between C20-C30 was used. The BMSM contained (g/l): KH_2PO_4 5.0; $NaNO_3$ 2.0; $MgSO_4$ $7H_2O$ 0.25; K_2HPO_4 $3H_2O$ 1.0; $(NH_4)_2SO_4$ 1.0; NaCl 5.0.

Flasks containing 50 ml of culture media supplemented with 0.1% or 1% (w/v) of sterile solid paraffin wax with a melting point of 50-52ºC (purchased from Sigma-Aldrich Co., St. Louis, USA) were sealed with rubber stoppers. Anaerobic cultures were prepared removing oxygen by aseptically bubbling oxygen-free nitrogen into the flasks. Cultures were incubated at 40ºC and 150 rpm during 15 days in anaerobic conditions along with control flasks incubated at the same conditions without addition of microorganisms.

At the end of the treatment the paraffinic mixture was quantified via the extraction of the residual solid paraffin in the culture media using an equal volume of dichloromethane, and the extract was dried and weighed accurately. The control was extracted via the same method and the percentage (w/w) of solid paraffin degradation estimated. The organic phase extracted was then diluted (20 mg/ml) in dichloromethane for gas chromatography (GC) analysis. GC analysis of each sample were 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 370ºC, respectively. Oven temperature was set at 50ºC during 1 min, raised to 350ºC at the rate of 10ºC/min, and at last kept at 370ºC during 1 min. All the samples were analyzed in triplicate.

Sand-pack column assays

Sand-pack columns were designed to simulate the oil reservoir and used to evaluate the effect of microorganisms in the oil recovery **(Fig.1)**. Vertically oriented acrylic columns with a volume of 250 ml were uniformly packed with acid washed dry sand. The columns were provided with a sieve and cap fixed at the bottom. After packing the sand tightly, a top sieve and cap were fixed. The caps on both the ends of the column were provided with holes for insertion of inlet and outlet tubes. Rubber 'O' rings surrounded the caps to hermetically seal the column.

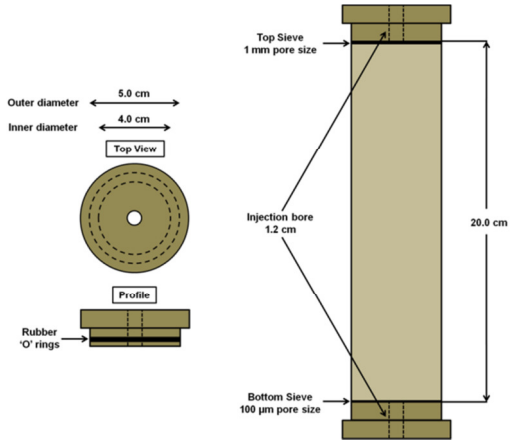

Fig.1 Illustration of the sand-pack column model used to evaluate the mobilization of residual oil by microorganisms. Adapted from Suthar et al. (2008).

The experiment was carried out at 40ºC. The column was first flooded with water at a constant flow rate of 3 ml/min; pore volume (PV, ml), defined as the empty volume of the model, was calculated by measuring the volume of water required to saturate the column. The porosity (%) of the column was calculated as the PV divided by the total volume of the column (250 ml). In the second step, liquid paraffin was injected into the column in the same way to replace water, until there was no more water coming out from the effluent. The volume of paraffin required to saturate the column was 200 ml. Original oil in place (OOIP, ml) was calculated as the volume of paraffin retained in the column. Initial oil saturation (S_{oi}, %) and initial water saturation (S_{wi}, %) were calculated as follows:

$$Soi\ (\%) = \frac{OOIP}{PV} \times 100 \quad (Eq.1)$$

$$Swi\ (\%) = \frac{PV - OOIP}{PV} \times 100 \quad (Eq.2)$$

The sand-pack column was incubated at 40ºC for 24 hours and afterwards flooded again with water to remove the excess of paraffin, until no more paraffin was observed in the effluent. The amount of paraffin recovered, so called oil recovered after water flooding (S_{orwf}, ml) was determined volumetrically. Residual oil saturation (S_{or}) was calculated as follows:

$$Sor\ (\%) = \frac{OOIP - Sorwf}{OOIP} \times 100 \quad (Eq.3)$$

The residual oil was subjected to microbial recovery processes. The column was inoculated with 50 ml of the different isolates in MSS (OD_{600nm}= 0.2), sealed and incubated for 14 days at 40ºC. After incubation, the column was flooded with water and the volume of paraffin recovered (oil recovered after microbial flooding (S_{ormf}, ml)) was measured. Additional Oil Recovery (AOR, %) was calculated as follows:

$$AOR\ (\%) = \frac{Sormf}{OOIP - Sorwf} \times 100 \quad (Eq.4)$$

Results and Discussion

Screening of biosurfactant-producing strains

A total of 58 isolates were obtained from crude oil samples. Biosurfactant production was evaluated in MSS under anaerobic conditions at 40ºC. Five of the isolates were found to produce biosurfactants under anaerobic conditions **(Table 1)**. The surface tension values were determined six times at room temperature (20ºC) and the Emulsification indexes were calculated three times at 25ºC (Results presented in **Table 1** represent the average of three independent experiments ± standard deviation). Furthermore, all isolates were identified as *B. subtilis* according to the partial sequence of their 16S rRNA genes. Biosurfactant production was not negatively affected by the presence of *n*-hexadecane (MSSH) in the culture broth. The best results regarding biosurfactant production were obtained with the isolates #309, #311 and #573. Those isolates reduced the surface tension of the culture broth to values around 30 mN/m and exhibited emulsifying indexes from 24 to 34%.

Table 1 Surface tension values (mN/m) and emulsifying indexes (E_{24}, %) obtained with the different isolates grown in MSS and MSSH for 120 hours at 40ºC and 120 rpm under anaerobic conditions.

	MSS		MSSH	
Strain	ST (mN/m)	E_{24} (%)	ST (mN/m)	E_{24} (%)
#191	41.7 ± 1.2	14.6 ± 1.9	40.0 ± 2.0	13.3 ± 5.9
#309	31.4 ± 1.3	25.9 ± 4.2	31.4 ± 0.5	24.6 ± 5.3
#311	30.5 ± 0.1	8.5 ± 4.0	31.0 ± 0.6	34.2 ± 3.9
#551	51.6± 1.5	7.1 ± 1.6	42.2 ± 2.4	13.6 ± 4.2
#573	31.5 ± 0.4	24.9 ± 2.3	33.7 ± 1.4	19.4 ± 5.6
Control	66.4 ± 1.5	0.0 ± 0.0	63.3 ± 0.9	0.0 ± 0.0
SDS 1%	39.9 ± 0.4	55.0 ± 1.7	39.9 ± 0.4	55.0 ± 1.7

Most of the microorganisms 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, 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 is not inhibited by crude oil (Javaheri et al. 1985; Jenneman et al. 1983). Other strains isolated from oil fields and which applicability in MEOR has been demonstrated are *Bacillus licheniformis* BAS50 (Yakimov et al. 1997; Yakimov et al. 1995), *B. licheniformis* ACO1 (Dastgheib et al. 2008) and *B. subtilis* PTCC1696 (Ghojavand et al. 2008). 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.

Hydrocarbon degradation

In order to evaluate the ability of the isolated *B. subtilis* strains to degrade the paraffinic oil fraction, a mixture of solid paraffin was incubated with isolates #309, #191 and #551 under anaerobic conditions. The respective anaerobic degradation (% w/w) of each paraffinic mixture is illustrated in **Fig.2** and compared with the control sample (incubated without microorganisms).

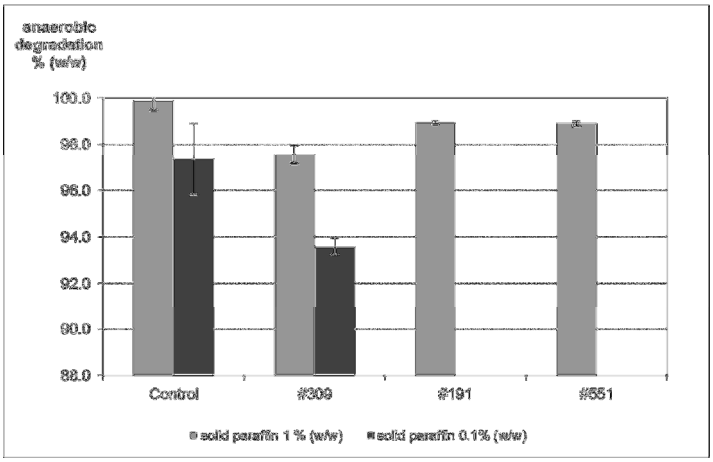

Fig.2 Values of anaerobic degradation of 0.1% and 1% (w/w) solid paraffin by bacterial isolates after 15 days of incubation at 40ºC and 150 rpm.

The isolates studied were able to grow in BMSM medium supplemented with solid paraffinic wax, under anaerobic conditions. After the treatment with #191 and #551 bacterial isolates, the percentage of paraffin degraded was of about 1% (w/w). The highest degradation rates observed (circa 2.5%) were obtained for the isolate #309. Thus, the isolate #309 was selected to further study its ability to degrade a mixture with a lower concentration of solid paraffin wax (0.1% (w/w)). The anaerobic degradation (% (w/w)) for this system and the respective control sample are also presented in Figure 2.

The results show that in presence of lower concentrations of paraffin, the strain #309 presents a higher capacity for anaerobic degradation, and degrades approximately 6% (w/w) of solid paraffin. These results are in good agreement with those reported by Etoumi (Etoumi 2007), which showed that microbial degradation of paraffinic hydrocarbon of crude oil was higher for lower concentrations of crude oil.

The paraffin extracted after the treatment with isolate #309 was analyzed by GC and the relative degradation of each *n*-alkane was measured. The respective variations of relative weight fraction of each *n*-alkane present in the paraffin mixture are illustrated in **Fig.3**.

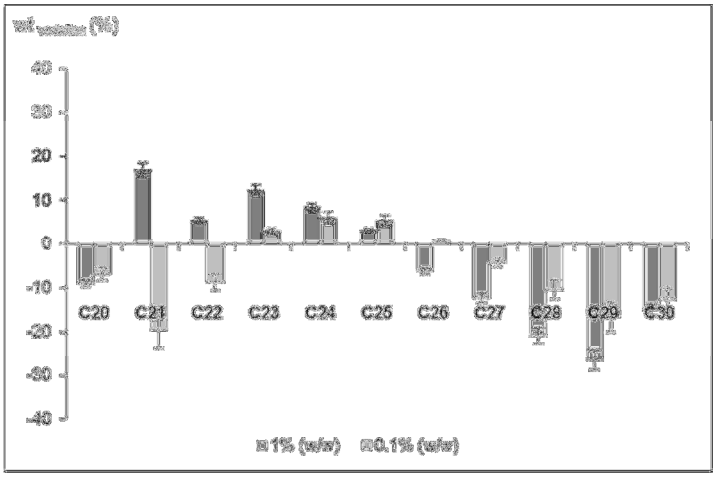

Fig.3 Variation of relative weight fraction of *n*-alkanes present in solid paraffinic mixture after incubation with isolate #309, for 15 days at 40ºC and anaerobic conditions as compared to the control (error bars show the average error).

For both paraffin concentrations the isolate #309 led to a decrease in the alkanes higher than C26, while for the lower paraffin concentration (0.1% (w/w) a decrease in the lower alkanes was also observed suggesting a degradation of the whole range of alkanes above C20 at these conditions. The aerobic degradation of *n*-alkanes has been also reported by other authors (Das and Mukherjee 2007; Etoumi 2007; Kato et al. 2001; She et al. 2011; Wang et al. 2006), however anaerobic studies using *B. subtilis* strains have not been described. One exception is our recent work (Gudiña et al. 2012) in which it was studied the anaerobic abilities of different *B. subtilis* isolates to degrade *n*-alkanes. In that work the same isolate (#309) showed identical *n*-alkanes degradation abilities to higher concentrations of solid paraffin.

Sand-pack columns assays

B. subtilis #309, #311 and #573 were selected to perform the oil recovery assays using sand-pack columns. The results obtained are shown in **Table 2**, (results represent the average of three independent experiments ± standard deviation).

Table 2 Oil recovery in sand-pack columns using isolates #309, #311 and #573.

	#309	#311	#573	Control
PV (ml)	92.5 ± 3.5	94.0 ± 3.5	92.5 ± 3.5	91.0 ± 1.4
Porosity (%)	37.0 ± 1.4	37.6 ± 1.4	37.0 ± 1.4	36.4 ± 0.6
OOIP (ml)	88.0 ± 2.8	89.3 ± 3.8	87.0 ± 4.2	87.5 ± 3.5
S_{oi} (%)	95.1 ± 0.6	95.0 ± 0.7	94.0 ± 1.0	96.1 ± 2.4
S_{wi} (%)	4.9 ± 0.6	5.0 ± 0.7	6.0 ± 1.0	3.9 ± 2.4
S_{orwf} (ml)	43.0 ± 0.2	42.0 ± 1.7	44.0 ± 0.0	43.5 ± 2.1
OOIP-S_{orwf} (ml)	45.0 ± 7.1	47.3 ± 2.1	43.0 ± 4.2	44.0 ± 5.7
S_{or} (%)	51.0 ± 6.4	53.0 ± 0.3	49.4 ± 2.5	50.2 ± 4.5
S_{ormf} (ml)	15.8 ± 2.5	12.3 ± 1.1	8.5 ± 0.0	1.8 ± 0.4
AOR (%)	35.0 ± 0.0	23.1 ± 1.2	19.9 ± 1.9	4.0 ± 0.4

As can be seen from **Table 2**, the pore volume was in the range of 91-94 ml, and the OOIP was between 87.0 and 89.3 ml. Water flooding was able to recover about 42.0-44.0 ml of the OOIP, leaving about 50% of paraffin unrecovered in the column. When the different isolates were introduced in the column with the appropriate nutrients (MSS) and incubated for 14 days at 40ºC, additional paraffin was recovered. The best result was obtained with the isolate #309, which recovered 35% of the entrapped paraffin; isolates #311 and #573 recovered 23 and 20% of paraffin, respectively. In the control columns, which were incubated in the same conditions but without microorganisms, the amount of paraffin recovered was about 4%, so it can be suggested that the remaining paraffin was recovered due the action of microorganisms. **Table 3** compiles the recovery efficiencies obtained by other authors in sand-pack column assays in situ using different *Bacillus* strains isolated from oil reservoirs.

Table 3 Results of additional oil recovery from sand-pack column assays using different *Bacillus* isolates.

Microorganism	Substrate	AOR (%)	Reference
B. licheniformis XDS1 *B. licheniformis* XDS2 *Bacillus cereus* XDS3	Crude oil	4.8-6.9	(She et al. 2011)
B. licheniformis BNP29	Crude oil	9.3-22.1	(Yakimov et al. 1997)
Bacillus brevis *Bacillus polymyxa* *B. licheniformis*	Paraffinic oil	18.0	(Almeida et al. 2004)
B. licheniformis ACO1	Paraffin	22.0	(Dastgheib et al. 2008)

As can be seen, large additional oil recoveries were obtained with all the isolates and these were particularly important with isolate #309 which were higher than the reported in other studies. The results gathered in the current work show that the isolated microorganisms are promising candidates for application in MEOR processes.

Conclusions

In the current work some *B. subtilis* strains were isolated from crude oil samples from the target reservoir. Five isolates were able to grow and produce extracellular biosurfactants at 40ºC under anaerobic conditions in a culture medium supplemented with hydrocarbons. Those isolates reduced the surface tension of the culture broth to 30 mN/m and were able to emulsify hydrocarbons. The isolate #309 was also capable of degrading a solid paraffinic mixture, mainly the heavier *n*-alkanes under anaerobic conditions. The simulation of the oil recovery operations using the sand-pack column showed that three isolates (#309, #311 and #573) recovered between 19.8 and 35% of the entrapped oil. These results suggest that stimulation of biosurfactant production and anaerobic degradation of heavy oil fractions *in situ* can contribute to mobilize the entrapped oil and improve the oil fluidity.

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