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Biosurfactant-producing and oil-degrading *Bacillus subtilis* strains enhance oil recovery in laboratory sand-pack columns

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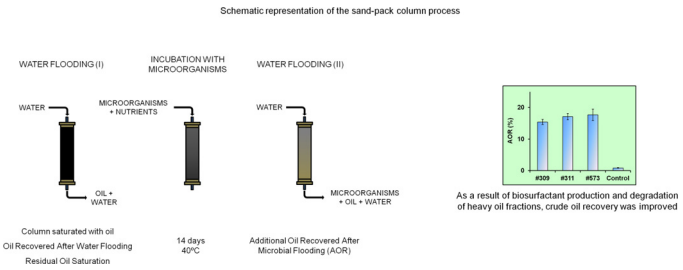
HIGHLIGHTS

- Sand-pack columns were designed to simulate the oil recovery operations.
- Indigenous *Bacillus subtilis* strains produced biosurfactants inside the columns.
- They also degraded the long-chain *n*-alkanes and reduced oil viscosity in porous media.
- Both processes led to an improvement in the oil recovery.
- Results obtained confirm the applicability of these isolates in MEOR.

GRAPHICAL ABSTRACT



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ABSTRACT

Microbial Enhanced Oil Recovery (MEOR) technology uses microorganisms and their metabolites to retrieve unrecoverable oil from mature reservoirs. *In situ* stimulation of biosurfactant-producing and oil-degrading microorganisms reduces the capillary forces retaining the oil inside the reservoir and decreases its viscosity, thus promoting oil flow and consequently production. In this work, a sand-pack column model was designed to simulate oil recovery operations and evaluate mobilization of residual oil by the selected microorganisms. Four different hydrocarbon mixtures and three *Bacillus subtilis* strains isolated from crude oil samples were used. Additional oil recoveries ranged from 6 to 24% depending on the hydrocarbon mixture and microorganism used. Biosurfactant production was observed with all the microorganisms and hydrocarbon mixtures studied. The oils recovered after incubation with *B. subtilis* isolates showed a reduction in the percentage of long-chain *n*-alkanes and lower viscosity when compared with the original oils. The results obtained suggest that stimulation of the selected *B. subtilis* strains *in situ* can contribute to mobilize entrapped oil in mature reservoirs.

1. 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 oil flow to the well head can be improved by injecting water into the wells. When the ratio of water to oil pumped out of the well becomes too high, 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, as well as the high interfacial tension between the hydrocarbon and aqueous phases that results in the high capillary forces that retain the oil in small pores within the reservoir rock [1,2]. Therefore, extracting the maximum amount of oil from reservoirs constitutes a major challenge to the oil industry. Recovery of entrapped oil usually involves the use of costly

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tertiary methods known as Chemical Enhanced Oil Recovery (CEOR). Several compounds are used for CEOR. Surfactants reduce interfacial tension between oil/water and oil/rock interfaces. Polymers are used to increase viscosity of water-flood. Acids, gases and solvents increase the permeability through the porous network and re-pressurize the oil reservoir. Since these compounds are petrochemicals, obtained from petroleum feedstock after refining and downstream processing, CEOR methods turned out to be economically unattractive as the finished products are used for the recovery of raw materials [2,3].

Microbial Enhanced Oil Recovery (MEOR) is an alternative tertiary oil recovery technology in which 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 [2,4]. 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 do not consume large amounts of energy as the thermal processes, nor depend on the price of crude oil as compared to many other chemical processes. Furthermore, microbial products are biodegradable and have low toxicity [1–3].

In situ biosurfactant production by microorganisms constitutes an effective mechanism to recover large amounts of the residual oil from mature oil fields [5–7]. 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, thus reducing surface and interfacial tensions [6]. Among them, lipopeptide biosurfactants produced by *Bacillus* species are capable of generating the low interfacial tension between the hydrocarbon and aqueous phases required to mobilize entrapped oil [8]. 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 [7,9]. *In situ* biosurfactant production at concentrations that allow the mobilization of significant amounts of residual oil has been demonstrated using selected microorganisms stimulated by the addition of proper nutrients into the wells [10,11].

Another important process in MEOR is the biodegradation of heavy oil fractions by microorganisms. In this process, heavy oil fractions are converted into lighter ones, reducing the viscosity of crude oil and improving its mobility through the reservoir, which increases oil recovery. In recent years, a wide variety of microorganisms able to degrade *n*-alkanes have been described, but only few reports presented the *Bacillus* species as oil degraders, usually under aerobic conditions [12–17]. The use of hydrocarbon degrading bacteria with the simultaneous ability to produce surface-active compounds is a good option for MEOR.

Laboratory studies on MEOR typically use sand-pack columns, which provide a suitable 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 can simulate the oil recovery operations usually conducted in reservoirs [9]. In this work, a sand-pack column model was used to study the effect of three different biosurfactant-producing and oil-degrading *Bacillus subtilis* strains, previously isolated from crude oil samples, on the mobilization of entrapped oil. Additionally, the degradation of oil hydrocarbons was evaluated in the oils recovered after the MEOR process.

Table 1
Properties of the hydrocarbon mixtures: viscosity (η), density (ρ), API gravity (API) and *n*-alkanes range.

Hydrocarbon mixture	η (mPa s)	ρ (g cm^{-3})	API	*n*-alkanes range
Heating oil	1.34	0.76	–	C16–C30
Viscous paraffin	44.64	0.85	–	[a]
Arabian light oil	8.33	0.86	29.7°	C11–C24
Heavy crude oil	73.91	0.90	25.5°	C14–C32

Viscosity and density values were measured at 40 °C.
[a] Mainly composed of ramified hydrocarbons. *n*-alkane composition was not determined.

2. Materials and methods

2.1. Hydrocarbon mixtures

Four different hydrocarbon mixtures were used: heating oil, viscous paraffin, and two different types of crude oil, Arabian Light and heavy oil. Viscous paraffin was purchased from Merck (Merck, Darmstadt, Germany). Heating oil (Zibro^{TM}) was obtained from commercial sources. Heavy crude oil was obtained from a Brazilian oil field. Arabian Light oil was kindly provided by GALP (Portugal). The properties of each hydrocarbon mixture are presented in Table 1.

2.2. Microorganisms

Three *B. subtilis* strains (#309, #311 and #573) previously isolated from crude oil samples obtained from a Brazilian oil field at depths of 300–400 m [17] were used. These isolates produced extracellular biosurfactants with high surface and emulsifying activities at 40 °C under anaerobic conditions in medium supplemented with hydrocarbons. Furthermore, the isolates degraded the large alkyl chains of hydrocarbon mixtures under anaerobic conditions, reducing their viscosity. However, the three isolates exhibit some differences regarding the biosurfactants produced and their hydrocarbon degradation profiles [17,18]. These characteristics make them good candidates for application in the oil reservoir under study. The isolates were stored at −80 °C in LB medium supplemented with 20% (v/v) glycerol.

2.3. Sand-pack column assays

Sand-pack columns were designed to simulate the oil reservoir and used to evaluate the effect of microorganisms in enhanced oil recovery. Vertically oriented acrylic columns with a volume of 250 ml were uniformly packed with dry sand (previously sterilized). 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).

The experiments were carried out at 40 °C (this is the temperature of the oil reservoir under study, from which the microorganisms were isolated and where the field assays will be performed in the future) and different hydrocarbon mixtures were used, as described above. A schematic representation of this process is shown in Fig. 2. 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, in the same way the hydrocarbon mixture (previously sterilized) was injected into the column to replace water, until there was no more water coming out from the effluent. Original oil in place (OOIP, ml) was calculated as

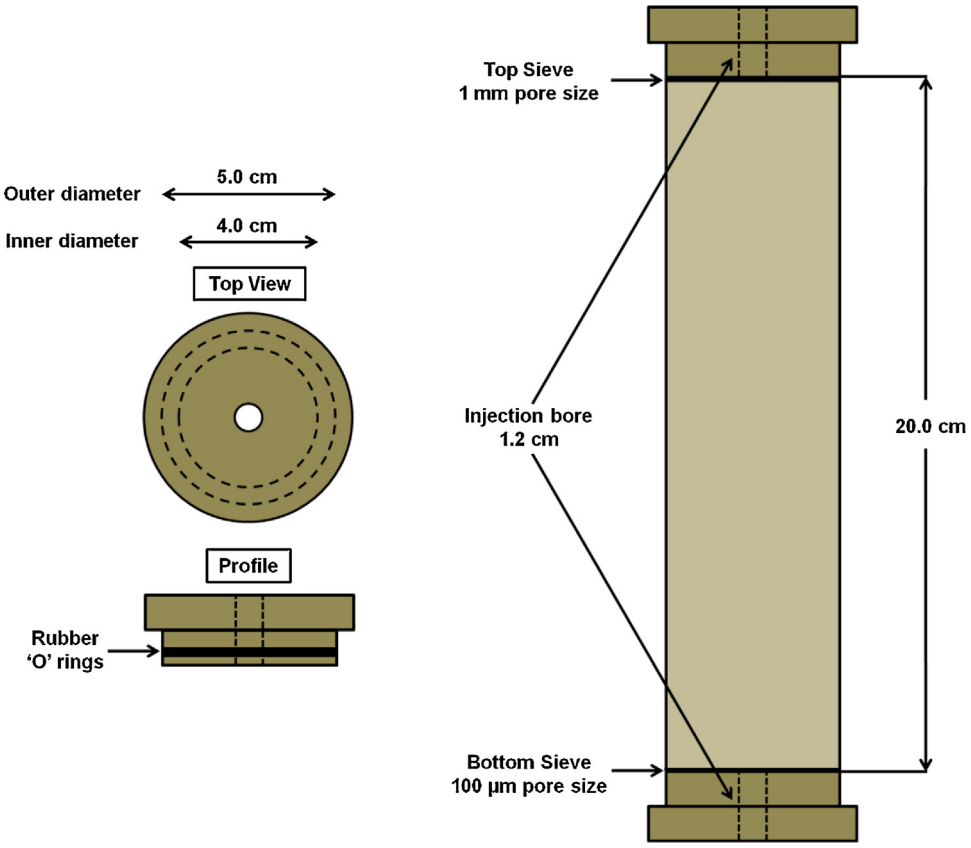

Fig. 1. Illustration of the sand-pack column model used to evaluate the mobilization of residual oil by microorganisms.
Source: Adapted from [9].

the volume of hydrocarbon retained in the column. Initial oil saturation (S_{oi}, %) and initial water saturation (S_{wi}, %) were calculated as follows:

$$S_{oi} = (\text{OOIP}/\text{PV}) \times 100 \quad (1)$$

$$S_{wi} = ((\text{PV} - \text{OOIP})/\text{PV}) \times 100 \quad (2)$$

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

$$S_{or} = ((\text{OOIP} - S_{orwf})/\text{OOIP}) \times 100 \quad (3)$$

Further, the residual oil was subjected to microbial recovery processes. The column was inoculated with 50 ml of the different microorganisms in Mineral Salt Solution supplemented with sucrose (MSS medium) diluted to an optical density (600 nm) of 0.2, sealed and incubated for 14 days at 40 °C. Control columns were inoculated only with MSS and incubated at the same conditions. This medium was selected in our previous work [17] as the most appropriate medium for biosurfactant production by these isolates. The MSS medium consisted of (g/l): NaCl 10.0; NH_4NO_3 2.0; Na_2HPO_4 5.0; KH_2PO_4 2.0; $MgSO_4 \cdot 7H_2O$ 0.2; sucrose 10.0. This medium contains nitrate (ammonium nitrate) which can be used as an alternative electron acceptor by the microorganisms whenever growing under anaerobic or oxygen limiting conditions, as demonstrated in our previous work. This is important because, although the conditions inside the sand-pack columns are not strictly anaerobic, the low amount of oxygen present should be quickly consumed by the microorganisms. After incubation, the column was flooded with water and the volume of hydrocarbon mixture recovered (oil recovered after microbial flooding (S_{ormf}, ml)) was measured volumetrically. Whenever required, the samples were centrifuged to break the emulsions formed. Additional Oil Recovery (*AOR*, %) was calculated as follows:

$$AOR(\%) = (S_{ormf}/(\text{OOIP} - S_{orwf})) \times 100 \quad (4)$$

All the experiments were performed in triplicate.

2.4. Hydrocarbon degradation

After conducting the sand-pack column assays with the different hydrocarbon mixtures and microorganisms, the *n*-alkanes degradation was evaluated. The oil recovered from the simulated MEOR experiments was diluted (20 mg/ml) in dichloromethane for gas chromatography (GC) analysis. GC analysis of each sample was performed using a CP 3800 Varian gas Chromatograph equipped with an on-column injector, FID detector, and DB-HT-SIMDIS capillary column (5 m × 0.53 mm i.d., 0.15 μm thickness) (Agilent J&W Scientific Inc., California, USA). Helium was used as the carrier gas at a constant flow rate of 18 ml/min. Injector and detector temperatures were 350 and 370 °C, respectively. For Arabian Light and heavy crude oil samples, the oven temperature was set at 40 °C during 5 min, raised to 350 °C at a rate of 5 °C/min, and at last kept at 370 °C during 15 min. For heating oil samples, the oven temperature was set at 40 °C during 8 min, raised to 300 °C at a rate of 5 °C/min, and at last kept at 300 °C during 15 min. The *n*-alkanes degradation

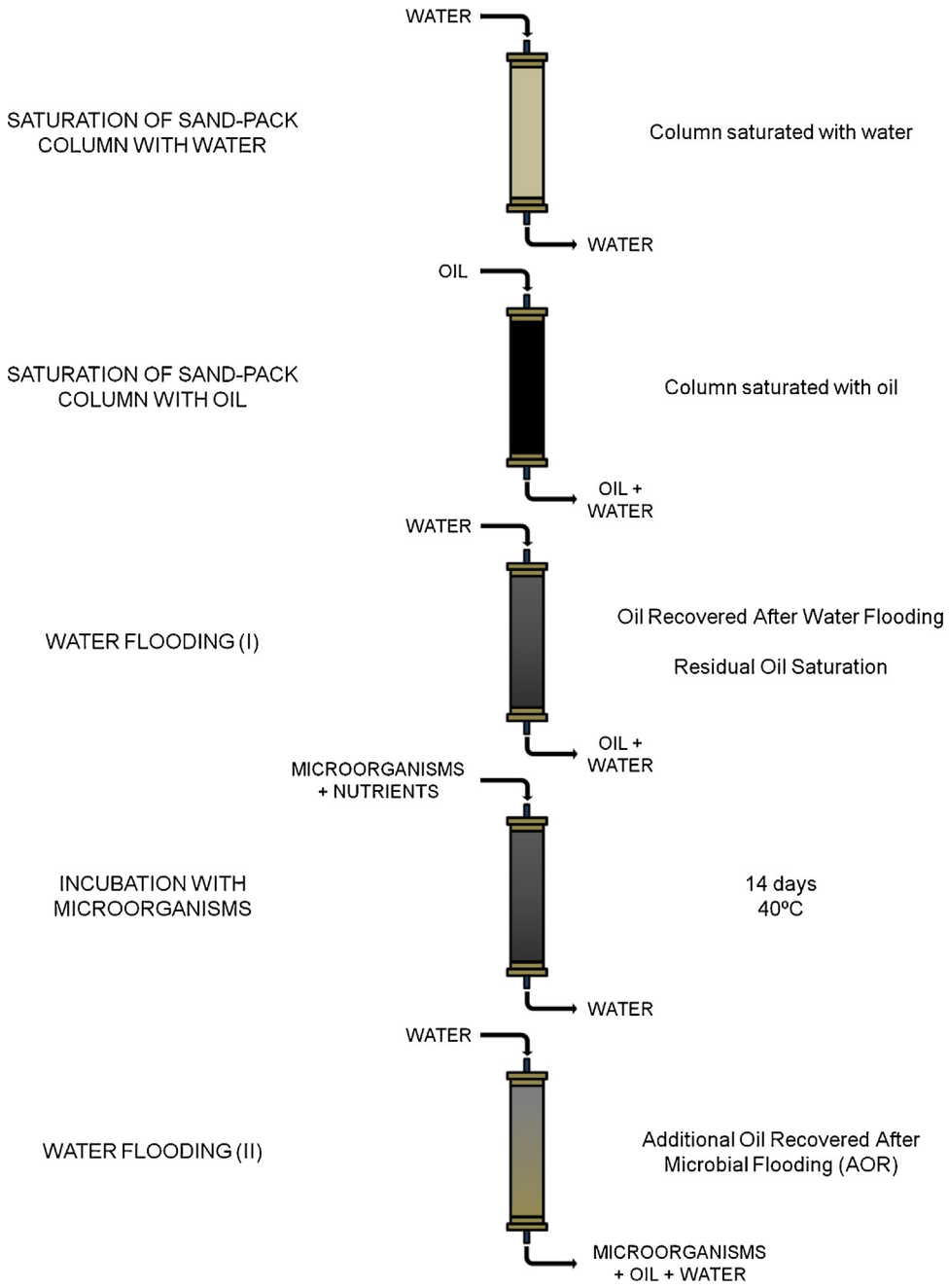

Fig. 2. Schematic representation of the sand-pack column process.

was evaluated by comparing the composition of hydrocarbons recovered after the treatment (with different microorganisms) with the hydrocarbons recovered in the abiotic control assays. All the samples were analysed in triplicate.

2.5. Viscosity

The dynamic viscosity (η) measurements were carried out using an automated Anton Paar (model SVM 3000) rotational Stabinger viscosimeter-densimeter at 50 °C and at atmospheric pressure (approximately 0.1 MPa). The viscosity of hydrocarbons recovered after the treatment with the different isolates was compared with the hydrocarbons recovered in the abiotic control columns. The relative uncertainty in dynamic viscosity measurements is ±0.35%.

2.6. Biosurfactant production in sand-pack columns

B. subtilis isolates used in this study have been previously found to produce biosurfactants both under aerobic and anaerobic conditions in the presence of different hydrocarbons in liquid medium [17]. In order to evaluate biosurfactant production in sand-pack columns, mini-sand-pack columns were prepared in 15 ml tubes. The tubes were uniformly packed with autoclaved dry sand. After that, the corresponding hydrocarbon mixture (previously sterilized) was introduced into the mini-column to attain the same percentage obtained in the sand-pack columns after the water flooding process. The remaining pore volume of the columns was saturated with a suspension of the corresponding microorganism in MSS medium (OD_{600nm} = 0.2). Afterwards, the columns were sealed and incubated at 40 °C for 14 days. Control columns were

Table 2
Summary of the results obtained in the MEOR sand-pack experiments using *B. subtilis* isolates #309, #311 and #573 and different hydrocarbon mixtures.

Oil recovery parameters	Hydrocarbon mixture			
	Control	#309	#311	#573
Heating oil				
OOIP (ml)	68.5 ± 4.9	68.2 ± 7.3	63.0 ± 1.7	69.0 ± 7.9
S_{oi} (%)	90.0 ± 5.7	76.5 ± 5.5	82.8 ± 0.4	90.6 ± 7.4
OOIP–S_{orwf} (ml)	25.0 ± 2.8	23.0 ± 8.1	31.7 ± 0.6	34.0 ± 5.3
S_{or} (%)	36.4 ± 1.6	34.9 ± 10.1	50.3 ± 2.3	49.4 ± 6.7
S_{ormf} (ml)	0.0 ± 0.0	5.0 ± 2.2	3.2 ± 0.3	2.3 ± 0.3
AOR (%)	0.0 ± 0.0	25.0 ± 0.8	10.3 ± 1.2	6.4 ± 0.6
Viscous paraffin				
OOIP (ml)	87.5 ± 3.5	86.5 ± 4.7	89.3 ± 3.8	87.0 ± 4.2
S_{oi} (%)	96.1 ± 2.4	89.1 ± 7.1	95.0 ± 0.7	94.0 ± 1.0
OOIP–S_{orwf} (ml)	44.0 ± 5.7	45.8 ± 5.1	47.3 ± 2.1	43.0 ± 4.2
S_{or} (%)	50.2 ± 4.5	52.9 ± 4.3	53.0 ± 0.3	49.4 ± 2.5
S_{ormf} (ml)	1.8 ± 0.4	11.8 ± 4.8	13.3 ± 4.5	8.5 ± 0.0
AOR (%)	4.0 ± 0.4	25.9 ± 10.6	27.9 ± 8.5	19.9 ± 1.9
Arabian light oil				
OOIP (ml)	80.0 ± 5.1	73.0 ± 3.6	80.5 ± 7.7	77.3 ± 5.9
S_{oi} (%)	69.0 ± 3.0	77.0 ± 3.4	79.8 ± 3.8	77.8 ± 3.7
OOIP–S_{orwf} (ml)	30.0 ± 5.7	24.3 ± 1.7	26.8 ± 2.4	24.0 ± 1.2
S_{or} (%)	37.7 ± 5.6	33.3 ± 2.8	33.3 ± 2.6	31.1 ± 1.1
S_{ormf} (ml)	1.3 ± 0.4	4.2 ± 1.5	5.3 ± 0.6	5.4 ± 0.8
AOR (%)	4.1 ± 2.2	17.1 ± 5.7	19.6 ± 1.2	22.4 ± 2.1
Heavy crude oil				
OOIP (ml)	91.8 ± 5.1	93.0 ± 1.4	91.0 ± 1.4	90.0 ± 2.8
S_{oi} (%)	98.9 ± 1.8	98.4 ± 2.2	95.8 ± 0.1	96.8 ± 1.4
OOIP–S_{orwf} (ml)	55.0 ± 5.0	52.0 ± 2.8	55.5 ± 0.7	54.0 ± 1.4
S_{or} (%)	59.7 ± 5.3	55.9 ± 2.2	61.0 ± 0.2	60.0 ± 0.3
S_{ormf} (ml)	0.5 ± 0.0	8.0 ± 0.0	9.5 ± 0.7	9.5 ± 0.7
AOR (%)	0.8 ± 0.1	15.4 ± 0.8	17.1 ± 1.0	17.7 ± 1.8

Results represent the average of three independent experiments ± standard deviation.

prepared in the same way but without addition of microorganisms. At the end of the incubation period, the content of each column was transferred to 50 ml test tubes. 5 ml of distilled water were added and the tubes were mixed using vortex for 2 min to extract the biosurfactants. The resulting mixture was centrifuged (9000 rpm, 30 min); the remaining hydrocarbon mixture was removed and the supernatant was filtered (0.2 μm) to remove residual hydrocarbon, sand and cells. The surface tension of the supernatants was determined according to the Ring method (as described by Gudiña et al. [17]) using a KRÜSS K6 Tensiometer (KRÜSS GmbH, Hamburg, Germany). These surface tension values were then used to calculate the concentration of biosurfactant using a calibration curve (surface tension *versus* biosurfactant concentration). The calibration curves were calculated for each strain using different concentrations of the corresponding crude biosurfactant (isolated as described by Gudiña et al. [17]) in distilled water below the critical micelle concentration (cmc). In this biosurfactant concentration range there is a linear relationship between biosurfactant concentration and surface tension. Therefore, whenever adequate, the supernatants obtained from the sand-pack columns were diluted to guarantee that biosurfactant concentrations were below the cmc.

3. Results and discussion

3.1. Sand-pack column assays

B. subtilis #309, #311 and #573 were used to perform the oil recovery assays with different hydrocarbon mixtures using sand-pack columns. The results obtained are shown in Table 2.

The pore volume of the columns was 96.1 ± 6.3 ml and the porosity 38.3 ± 2.4%. The OOIP values, the amount of hydrocarbon removed during the water flooding process (S_{orwf}) and the amount of entrapped hydrocarbon (OOIP-S_{orwf}) varied depending on the hydrocarbon mixture used (Table 2). These differences are most probably due to the different viscosities of the oils.

Table 3
Additional oil recoveries obtained with sand-pack column or core assays using different *Bacillus* isolates reported in the literature.

Microorganism	Substrate	AOR (%)	Reference
B. licheniformis XDS1, XDS2, *Bacillus cereus* XDS3	Crude oil	4.8–6.9	[16]
B. licheniformis BNP29	Crude oil	9.3–22.1	[19]
Bacillus brevis, *Bacillus polymyxa*, *B. licheniformis*	Paraffinic oil	18.0	[5]
B. licheniformis ACO1	Paraffin	21.7	[20]

When the different isolates were incubated for 14 days at 40 °C into the columns together with appropriate nutrients (MSS medium), an additional hydrocarbon recovery was observed for all cases as compared to the controls. However, different outcomes were obtained depending on the hydrocarbon mixture used (Table 2).

The isolate #309 was the most effective when heating oil was used as hydrocarbon (25% of additional oil recovered (AOR) after incubation with microorganisms). In the experiments conducted with viscous paraffin, the highest AOR values (26–28%) were obtained with isolates #309 and #311. Regarding the experiments performed with crude oil, using Arabian Light, the highest recovery was obtained with the isolate #573 (22%), whereas with heavy crude oil similar oil recoveries were obtained with the three isolates (15–17%).

As shown in Table 3, enhanced oil recovery has been demonstrated by several authors with different hydrocarbon mixtures in sand-pack columns or cores after growing *in situ* different *Bacillus* strains which have been reported to produce surface-active compounds. The AOR values reported were between 5 and 22%. In some cases, the strains produced also extracellular polymers, which can contribute to enhance oil recovery as they increase the efficiency of the water-flooding operation [19].

3.2. Evaluation of hydrocarbon degradation

In order to evaluate the degradation of the different hydrocarbon mixtures after being recovered from the sand-pack column assays performed with *B. subtilis* isolates #309, #311 and #573, the oils recovered were analysed by GC and the relative degradation of each *n*-alkane was measured and compared with the oils recovered in the abiotic control columns (abiotic controls were subjected to the same incubation period and flood processes, to avoid the possible effect of the loss of hydrocarbons which may be retained in the system during the process). The oil recovered from the viscous paraffin experiments could not be analysed by GC, since this hydrocarbon mixture was mainly composed of ramified hydrocarbons. The respective variations of relative weight fraction of each *n*-alkane present in the different oil samples as compared with the control assays are illustrated in Fig. 3.

As observed by the GC analysis, the three hydrocarbon mixtures recovered after the treatment with the strains #309, #311 and #573 were degraded. It was also observed that all the *B. subtilis* strains preferentially degraded the higher *n*-alkane fractions of each hydrocarbon mixture. Due to the low amount of hydrocarbons recovered in the control assays, the degradation data obtained may not be representative of all the oil entrapped in the columns. However, the degradations herein obtained were consistent with previous experiments conducted with these isolates in liquid medium [17].

Analysing the results obtained with heating oil, it was found that all the isolates degraded the higher *n*-alkanes (C25–C30),

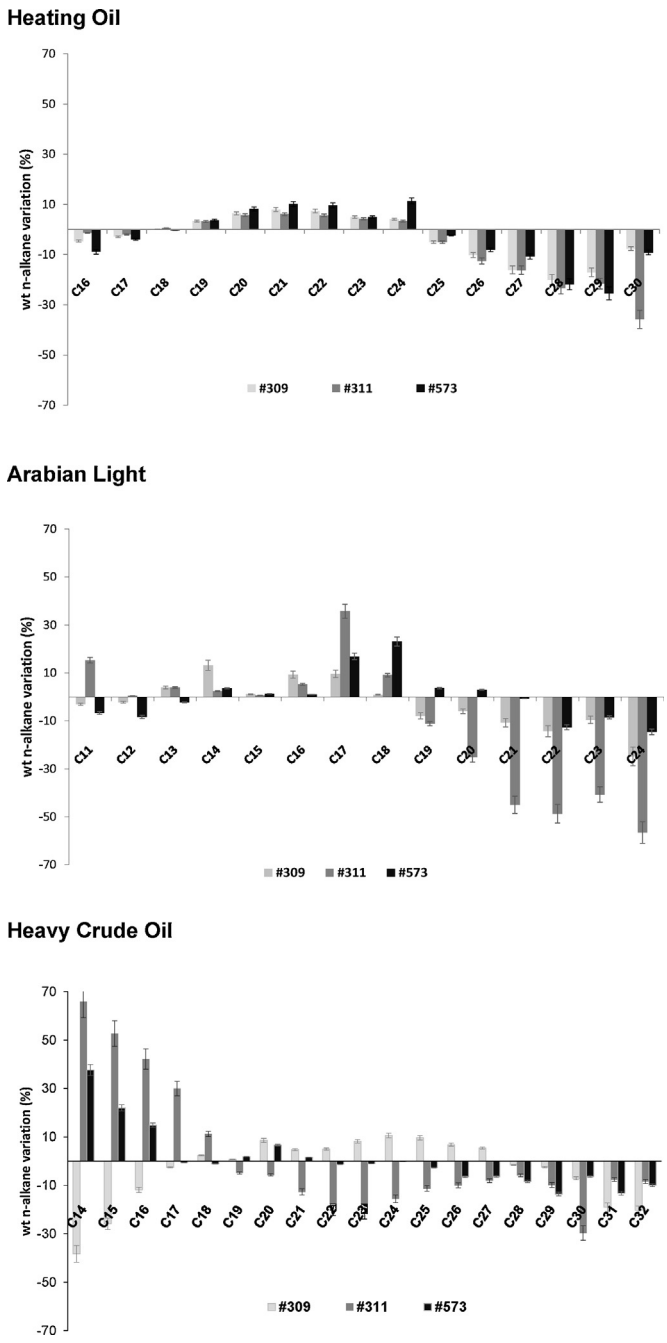

Fig. 3. Variation of relative weight fraction of *n*-alkanes present in the different oil samples (Heating oil, Arabian Light Oil and Heavy Crude Oil) after *B. subtilis* isolates #309, #311 and #573 incubation for 14 days at 40 °C in sand-pack columns. Results are compared to control columns and error bars represent the average error of three independent assays.

causing an increase in *n*-alkanes in the C19–C24 range with respect to the original oil. The isolate #311 showed the highest degradations. The Arabian Light Oil was composed of *n*-alkanes up to C24. In this case, the oil recovered after the sand-pack column assays showed a reduction in *n*-alkanes higher than C19 for isolates #309 and #311; and higher than C21 for isolate #573. As in the case of heating oil, the highest degradation was obtained with the isolate #311. The GC analysis also showed an increase in the percentages of C17 and C18 *n*-alkanes when compared with the controls, perhaps due to the increase of the isoprenoids (pristane and phytane) compositions.

The study of *n*-alkanes variation of the heavy crude oil recovered showed that all the strains were able to degrade the hydrocarbon chains higher than C27. Among them, the isolate #311 exhibited the highest degradation with an increase in the relative weight fraction of *n*-alkanes lower than C19, and a decrease in the percentage of *n*-alkanes higher than C18. These results indicate that this isolate degraded the higher *n*-alkanes into lighter ones during the sand-pack column assays. Similarly, the isolate #573 showed the ability to degrade the long-chain *n*-alkanes (>C25), thus increasing the percentage of *n*-alkanes with chains containing 20 carbons or less than 17 carbons, when compared with the control sample. On the other hand, the isolate #309 displayed a different oil-degradation profile, degrading the higher *n*-alkanes (>C28) as well as the lower ones (chains lower than 17 carbons).

Several microorganisms able to degrade heavy oil hydrocarbons have been described [21], but only a few reports refer *Bacillus* species as oil degraders. *Bacillus thermoleovorans* strains with the ability of degrading *n*-alkanes up to C23 at 70 °C were isolated by Kato et al. [12]. A thermophilic *Bacillus* strain that degraded long-chain (C15–C36) rather than short-chain *n*-alkanes was isolated by Wang et al. [14]. *Bacillus* strains isolated from a Brazilian oil reservoir were found to degrade *n*-alkanes of Arabian Light and Marlin oil [13]. Das and Mukherjee [15] reported that *B. subtilis* DM-04 preferentially degraded crude oil *n*-alkanes between C14 and C30. She et al. [16] studied three *Bacillus* strains isolated from an oil reservoir in the Daqing Oilfield (China) which degraded *n*-alkanes with chain lengths between C14 and C26. However, all these assays were performed under aerobic conditions. Recently, our group showed the ability of the three *B. subtilis* strains used in the current study to preferentially degrade the large alkyl chains of different paraffinic mixtures under anaerobic conditions [17]. The most common pathway for *n*-alkanes degradation under aerobic conditions is via terminal oxidation, in which *n*-alkanes are first oxidized to the corresponding primary alcohol, which is further oxidized by alcohol and aldehyde dehydrogenases. Although the conditions existent in the sand-pack columns are not strictly anaerobic, the oxygen present must be quickly consumed by the microorganisms, so it is still not clear how the *n*-alkanes degradation can occur under oxygen limiting conditions. Several authors have reported in the last years the degradation of *n*-alkanes under anaerobic conditions using other microorganisms, even strict anaerobic bacteria [3,4,22–26]. However, the pathways involved in this process have not yet been elucidated, although many researchers have suggested that nitrate can work as an alternative electron acceptor for hydrocarbon degradation under anaerobic conditions [24,26].

The results herein obtained using different hydrocarbon mixtures (including heavy crude oil) were similar to the ones previously reported in liquid medium [17], thus suggesting that these isolates can be used to reduce the percentage of long-chain *n*-alkanes of crude oil also in porous media (sand-pack column). The comparison between different hydrocarbons showed that even changing their *n*-alkanes composition, the different isolates maintained the ability to degrade preferentially the heavier oil fractions. Therefore, these results are very interesting in view of their application in MEOR processes in reservoirs containing heavy and paraffinic oils.

As a result of the degradation of high *n*-alkanes present in the crude oil into lighter ones, a reduction in oil viscosity is expected, with the subsequent improvement in the flow properties. The viscosity of the heavy crude oil recovered after the sand-pack column assays was measured, and the respective variation relatively to the control samples (oil recovered from the abiotic control columns) is presented in Table 4.

The results presented in Table 4 show that the viscosity of heavy crude oil samples recovered after the sand-pack column assays was reduced when compared with the control samples. The viscosity of

Table 4
Relative viscosities variation ($\Delta\eta$) (at 50 °C) of heavy crude oil recovered from sand-pack column assays using *B. subtilis* isolates #309, #311 and #573 relatively to the control (the associated errors are lower than 6%).

	Bacillus subtilis isolates		
	#309	#311	#573
$\Delta\eta$/(%)	−21.4	−28.7	−32.0
η of the heavy crude oil (control) at 50 °C/(mPa s)	37.6		

the crude oil decreased more than 20% in all the cases after treatment with microorganisms, being the isolate #573 the one that promoted the highest viscosity reduction (32%).

The reduction in oil viscosity is in close agreement with the AOR values obtained for each microorganism, as a direct relationship was observed between the increase in oil recovered and the decrease of oil viscosity. These results were expected since the reduction in oil viscosity enhances the oil fluidity in the reservoir.

3.3. Biosurfactant production in sand-pack columns

The results obtained regarding biosurfactant production by the different microbial isolates in sand-pack columns are shown in Table 5. As it can be seen from those results, all the isolates produced biosurfactants in sand-pack columns with the different hydrocarbons tested. In the control columns, no biosurfactant production was observed. For all the cases evaluated, the lowest biosurfactant production was obtained with the isolate #573, whereas isolates #309 and #311 produced similar amounts of biosurfactant. Furthermore, for all isolates the lowest biosurfactant production was obtained when heating oil was used as hydrocarbon mixture.

Biosurfactants, which reduce the interfacial tension between water–oil–sand and emulsify hydrocarbons, contributed to the enhanced oil recoveries observed. As reported by several authors, low biosurfactant concentrations (close to the cmc) are enough to mobilize entrapped oil [10,27,28], although their effect depends on the system studied. Even biosurfactant concentrations lower than the cmc can contribute to mobilize entrapped oil [29]. The cmc values determined for the crude biosurfactants produced by isolates #309, #311 and #573 were 20, 20 and 30 mg/l, respectively [17]. Taking into account these values, for isolate #573 the amount of biosurfactant produced was lower than the cmc in all the cases (except for heavy crude oil). Likewise, biosurfactants production by isolates #309 and #311 with heating oil was below their cmc. However, with the other hydrocarbon mixtures, the concentration of biosurfactant produced was approximately four times the cmc. According to these results, it can be suggested that for all hydrocarbon mixtures evaluated, the biosurfactants produced contributed to enhance the oil recovery.

As previously mentioned, sand-pack columns provide a suitable approach to study the ability of injected microorganisms to increase oil recovery in reservoirs, because these models allow the simulation of oil recovery operations in oil fields. After the water flooding process, residual oil is trapped in the pores of the reservoir rocks. Biosurfactants produced by microorganisms reduce the interfacial tension at the oil–water–rock interface, thus reducing the capillary forces that prevent oil from moving through rock pores.

Table 5
Biosurfactant concentrations (mg/l) obtained in the mini-sand-pack column assays performed with the different isolates and hydrocarbon mixtures. Results represent the average of three independent experiments ±standard deviation.

Hydrocarbon	Biosurfactant concentration (mg/l)		
	#309	#311	#573
Heating oil	20.1 ± 3.3	22.0 ± 1.6	8.2 ± 1.2
Viscous paraffin	86.7 ± 1.8	85.2 ± 3.6	23.1 ± 2.7
Arabian light oil	82.2 ± 3.9	72.6 ± 2.9	24.7 ± 1.8
Heavy crude oil	85.0 ± 2.2	82.4 ± 3.1	30.7 ± 2.2

The increases in oil recovery observed in sand-pack columns can be due to several factors, and it is not straightforward to assign a percentage of recovery to a specific one. *B. subtilis* isolates used in this study produce biosurfactants which reduce surface tension and emulsify hydrocarbons, decreasing the interfacial tension at the oil–water interface. In sand-pack column assays performed with heating oil and viscous paraffin, the lowest recoveries were obtained with isolate #573. This isolate was also found to exhibit the lowest biosurfactant production with both hydrocarbons. However, with the other hydrocarbon mixtures studied, a direct relationship between biosurfactant production and the amount of oil recovered is not evident. Furthermore, the microorganisms herein used have the ability of degrading long-chain *n*-alkanes, reducing oil viscosity, which can contribute to enhance the mobilization of the hydrocarbon into the column. In sand-pack column assays performed with heavy crude oil, the highest recoveries were obtained with isolate #573, which was found to produce less biosurfactants than the other two isolates. However, this was the isolate that caused the highest reduction in crude oil viscosity. Indeed, as previously mentioned there are other factors that can contribute to the increase in oil recovery, such as the production of gases by the microbial isolates, increasing the pressure into the column; or the plugging of high permeability channels due to the accumulation of biomass, which redirects water to oil rich zones, thus increasing oil production.

In field assays, inoculation of mature oil wells with *Bacillus* strains resulted in biosurfactant production (20–90 mg/l) and in an increase in oil production in the inoculated wells, as well as in a decrease in the water:oil ratio. These figures were maintained for a period of 40–60 days following the treatment, therefore constituting a cost-effective process [10,11].

4. Conclusions

B. subtilis isolates were evaluated for their oil-degrading and biosurfactant-production capabilities in sand-pack columns. Different hydrocarbon mixtures were studied and the isolates were able to increase the oil recovery in all cases, 6–25% for heating oil, 16–24% for viscous paraffin, 13–18% for Arabian Light oil and 15–17% for heavy crude oil. *In situ* treatment of heavy and paraffinic oils with *B. subtilis* can thus contribute to improve their fluidity and to reduce interfacial tension, increasing the additional oil recoveries. These isolates are good candidates for use in MEOR process and may be useful to recover residual oil from mature reservoirs.

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