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ORIGINAL PAPER

Efficiency of the EPS emulsifier produced by *Ochrobactrum anthropi* in different hydrocarbon bioremediation assays

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Abstract *Ochrobactrum anthropi* strain AD2 was isolated from the waste water treatment plant of an oil refinery and was identified by analysis of the sequence of the gene encoding 16S rDNA. This bacterium produced exopolysaccharides in glucose nutrient broth media supplemented with various hydrocarbons (*n*-octane, mineral light and heavy oils and crude oils). The exopolysaccharide AD2 (EPS emulsifier) synthesized showed a wide range of emulsifying activity but none of them had surfactant activity. Yield production varied from 0.47 to 0.94 g of EPS l^{-1} depending on the hydrocarbon added. In the same way, chemical composition and emulsification activity of EPS emulsifier varied with the culture conditions. Efficiency of the EPS emulsifier as biostimulating agent was assayed in soil microcosms and experimental biopiles. The AD2 biopolymer was added alone or combined with commercial products frequently used in oil bioremediation such as inorganic NPK fertilizer and oleophilic fertilizer (S200 C). Also, its efficiency was tested in mixture with activated sludge from an oil refinery. In soil microcosms supplemented with S200 C + EPS emulsifier as combined treatment, indigenous microbial populations as well as hydrocarbon degradation was enhanced when compared with microcosms treated with NPK fertilizer or EPS emulsifier alone. In the same way EPS emulsifier stimulated the bioremediation effect of S200 C product, increasing the number of bacteria and decreasing the amount of hydrocarbon remained. Finally, similar effects were obtained in biopile assays amended with EPS emulsifier plus activated sludge. Our results suggest that the bioemulsifier EPS emulsifier has interesting properties for its application in environment polluted with oil hydrocarbon compounds and may be useful for bioremediation purposes.

Keywords *Ochrobactrum anthropi* · Bioemulsifier · Biodegradation · Hydrocarbon

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Introduction

Microbial biosurfactants and bioemulsifiers are produced by a wide variety of diverse microorganisms and they have different chemical structures and properties [6, 28]. In general, bacteria make low molecular weight molecules that efficiently reduce surface and interfacial tensions such as glycolipids [18, 35] and lipopeptide [22]; [32], and high molecular weight polymers that are efficient emulsifiers such as emulsan [45], alasan [30] or biodispersan [36]. Within the first group, it is worth mentioning the rhamnolipids produced by *Pseudomonas* species, composed of two molecules of rhamnose and two molecules of 3 hydroxyacids. Also remarkable are the trehalolipids produced by several species of *Rhodococcus*, *Arthrobacter* and *Mycobacterium* composed of trehalose, nonhydroxylated fatty acid and mycolic acids and the sophorolipids produced by *Candida* and *Torulopsis*, in which sophorose is combined with long chain hydroxyacid [11, 37]. The high molecular weight biopolymers containing polysaccharides, polysaccharides attached to lipids; or polysaccharides attached to

proteins have been described as efficient emulsifying agents of numerous hydrocarbon compounds [4, 10, 20, 28, 33, 40].

Regardless of the different chemical composition and applications that bioemulsifiers show, the main field of research nowadays is focused on the mass production of these compounds on an industrial scale [17]. Many research [25, 42] papers have been published showing improved methods for rhamnolipids production. Recently, the relationships between several exploratory variables and one or more response variable [Response Surface methodology (RSM)] have been used to identify optimal C and N source in order to optimize rhamnolipid production from a *P. aeruginosa* S2 strain [13] and bioemulsifier production by *Candida lipolytica* [2].

The diversity of these compounds results in a broad spectrum of potential applications, being valuables in industries as diverse as agriculture, food industry, leather, textile or paper industries, cosmetic and pharmaceutical industries, etc. However, the largest possible market for biosurfactants and bioemulsifiers could be the oil industry, both for petroleum production and for bioremediation of oil contaminated sites [21, 34].

Biodegradation is one of the primary mechanisms for elimination of petroleum and other hydrocarbon pollutants from the environment [26]. It is considered an environmentally acceptable way of eliminating oils and fuel because the majority of hydrocarbons in crude oils and refined products are biodegradable, and hydrocarbons degrading microbes are ubiquitous [23].

Petroleum hydrocarbon compounds bind to soil components and are difficult to remove and degrade. Bioemulsifiers can emulsify hydrocarbons enhancing their water solubility and increasing the displacement of oily substances from soil particles and thus making them more available to microorganisms [6]. It has been demonstrated that emulsifying agents enhance the degradation process due to better solubilization of hydrocarbon prior to microbial degradation [15]. Addition of compounds able to emulsify hydrocarbons into microscopic droplets increases the surface area exposed to bacteria, this produces a readily available food source that bacteria can assimilate quickly [28, 34]. For these reasons, inclusion of bioemulsifiers in a bioremediation treatment of a hydrocarbon polluted environment could be really advantageous. In this sense, it has been reported [8, 19, 34] that biosurfactant and other natural emulsifying agents are important tools for biotreatment of hydrocarbon polluted environment.

In the present study, we describe the properties, characterization and efficiency of EPS emulsifier produced by *O. anthropi* as biostimulating agent to oil hydrocarbon biodegradation under experimental conditions such as soil microcosms and biopiles.

Materials and methods

Microorganism

Strain AD2 was isolated from an activated sludge plant (wastewater treatment plant of a Repsol YPF oil refinery located in Puertollano, Spain). Sludge samples (10 g) from the aerobic biological reactor were placed in 500-ml Erlenmeyer flask mixed with 100 ml of BH liquid medium and 1 ml of Kirkuk crude oil and incubated at 28 °C for a week. Samples for bacterial isolation (0.1 ml) were plated on BH agar medium [15] with the addition of 0.1% w/v naphthalene, phenanthrene or pyrene. For that purpose, the polycyclic aromatic hydrocarbon (PAH) was dissolved in ether and the solution was then spread onto the surface of the medium. Strain AD2 was selected for further studies due to its ability to grow and produce a clear halo on the surface of the BH medium supplemented with 0.1% of PAHs. The strain was long-term maintained in the laboratory by lyophilisation. For this study, stock cultures were grown on TSA (Difco) slants.

Culture media

BH medium used for isolation of hydrocarbon degrading microorganisms is a minimal medium with the following composition (g/l): $MgSO_4 \cdot 7H_2O$, 0.2; K_2HPO_4, 1; $CaCl_2 \cdot 2H_2O$, 0.02; $NH_4NO_3 \cdot 6H_2O$, 1; $FeCl_3$, 0.05 and agar, 20.

Growth studies of strain AD2 was determined in nutrient broth (NB medium) with the following composition (g/1): glucose, 10; yeast extract, 5; proteose-peptone, 5; and NaCl, 5. Bacteria were cultivated at 32 °C for 5 days under aerobic conditions in a rotatory shaker (150 rpm).

The effect of different hydrocarbons on growth and EPS emulsifier production by strain AD2 was evaluated by addition of octane, toluene, xylene, mineral light oil, mineral heavy oil or crude oil to glucose NB media at concentration of 1% v/v.

PAHs, octane, toluene xylene, mineral light oil, mineral heavy oil supplied by Sigma Co, were standard for HPLC and gas chromatography (≥99.7% of purity). Crude oil used was Kirkuk crude oil from the refinery of Repsol YPF (Puertollano, Spain).

Taxonomical characterization of strain AD2

Strain AD2 was identified by the analysis of the sequence of the gene encoding 16S RNA (16S rDNA). Primers fD1 and rD1 [43] were synthesized by Sigma-Genosis (Haverhill, UK) and used to amplify almost the full length of 16S rRNA gene. Freshly cultured colonies of strain AD2 were lysed by the addition of 20 µl of a mixture of NaOH (0.05 M)–SDS (0.25%, w/v) and then boiled for 15 min.

The lysates were adjusted to 200 µl with sterile bidistilled water and centrifuged at 2,500*g* for 5 min.

The cleared lysates (4-µl aliquots) were used as template for identification. PCR was performed adding to the lysates 1×PCR buffer (ABI; Perkin Elmer, Norwalk, CT), 1.5 mM $MgCl_2$ (ABI), 200 µM dNTPs (Roche Molecular Biochemicals, Mannheim, Germany), 20 pmol of each primer, and 1 U of Taq polymerase (ABI). The final volume in the reaction tubes was adjusted to 50 µl. Reactions were run in a Perkin Elmer GeneAmp PCR system 2400 (Perkin Elmer, Norwalk, CT). The temperature profile was the one previously described by Vinuesa et al. [41] except for the initial denaturation step, which was extended to 7 min. PCR products were run on 1% agarose gels and bands were purified using a Quiaex II kit (Quiagen, Hilden, Germany). The nucleotide sequence of the purified bands was determined by the deoxy chain terminator method, using the ABI-PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit, (Perkin Elmer) and automated sequencer Applied Biosystems ABI capillary system (Perkin Elmer). Sequence data were analyzed using gene Runner 3.05 software, and compared to sequences in the EMBL databank using the free he BLASTn [3] online software provide by the European Bioinformatics Institute (http://www.ebi.ac.uk.)

Production and characterization of EPS emulsifier

Production of EPS emulsifier was determined in NB medium. Erlenmeyer flasks (500 ml) containing 100 ml of NB medium were inoculated with 1 ml of an overnight culture, grown in the same medium. After incubation at 32 °C for 5 days under aerobic conditions in a rotatory shaker (150 rpm), the cultures were centrifuged at 36,000*g* in a Sorval RC-JB refrigerated centrifuge at 4 °C for 60 min. Supernatant obtained were precipitated with cold ethanol. The biopolymer precipitated from the supernatants was dissolved in distilled water, dialysed against distilled water during 24 h lyophilised, and weighed [10]. The EPS obtained were subjected to colorimetric analysis of proteins [9] and total carbohydrates [16].

Emulsification assays and surfactant activity test

Emulsifying activity of biopolymer synthesized by strain AD2 grown in glucose NB liquid media amended or unamended with hydrocarbon compounds was detected by a modified version of the method previously described by Cooper and Goldenberg [12]. Test tubes (105 × 15 mm) were amended with 3.0 ml of exopolymer diluted in distilled water (0.1%, w/v) and 3 ml of a hydrophobic substrate (*n*-octane, xylene, toluene, mineral light oil, mineral heavy oil or crude oil). Then the tubes were shaken vigorously to homogeneity using a vortex, and left to stand for 24 h. Emulsifying activity was expressed as the percentage of the total height occupied by the emulsion. Surfactant activity was determined by measurement of surface tension with a Krüs K11 digital tensiometer, using a plate method [7]. This property was searched in the bacterial culture and in bioemulsifier distilled water solution.

Microcosm assays

Soil microcosms were constructed with oil polluted soil samples (Table 1) incubated at room temperature in steady conditions for 21 days and weekly tested. The soil samples were obtained from a real contaminated soil; they were supplied by the Company AG Ambiental (Madrid, Spain) in order to perform assays of biotreatability. This soil was franc-clay soil (36% clay, 33% sand and 10% slime); it was polluted by a mixture of diesel and fuel oil at final total hydrocarbon concentration near 10,000 mg/Kg (See Table 1 for values of initial pollution).

Six microcosms (three replicate for each treatment) were constructed in order to evaluate the effect of the EPS emulsifier on hydrocarbon biodegradation. Each microcosm received a specific treatment: (A) NPK fertilizer (0.3 g/Kg of soil), (B) oleophilic compound S200 C (1 ml/Kg of soil), (C) EPS emulsifier (1.5 g/Kg of soil), (D) NPK fertilizer (0.3 g/Kg of soil) + EPS emulsifier (1.5 g/Kg of soil), (E) S200 C (1 ml/Kg of soil) + EPS emulsifier (1.5 g/Kg of soil) and (F) control microcosm without any addition.

The NPK fertilizer (Agroblem S.L) is a inorganic complex fertilizer composed of 18% total nitrogen (nitrate, 1.2%; ammonium, 3.1% and urea 13.7%), 8% of P_2O_5, 2% of MgO, 19% of K_2SO_3 and 0.5% of Fe. S200 C is an oleophilic fertilizer (IEP Europe) which stimulates the indigenous hydrocarbon microorganisms.

Biopile systems

The composting process was determined in biopile systems of 1 m height/1.25 m^3 total volumes. The biopiles were

Table 1 Concentration of hydrocarbons (mg of hydrocarbon/kg of soil) in oil polluted soil utilized in microcosms and biopiles assays

Hydrocarbon compound	Polluted soil of microcosms	Polluted soil of biopiles
TPH	8,530 ± 1438	11,000 ± 1458
Alkanes	5,726 ± 572	7,863 ± 171
Prystane	547 ± 81	219 ± 84
Phytane	468 ± 89	212 ± 56
Alkenes and alkynes	ND	997 ± 285
Naphthalenes	263 ± 45	1,663 ± 64
Pregnans	ND	45 ± 25

ND Not detected

constructed with 1,245 Kg of soil, contaminated with 13.05 Kg (14.5 l of Kirkuk crude oil) of hydrocarbons and 300 Kg of straw added as bulking agent. Homogenization of contaminant was carried out by turning over. Four different biopiles (in duplicate) were tested in order to understand the effects of activated sludge and EPS emulsifier on hydrocarbon biodegradation. The treatments assayed were as follows: (A) control biopile, (B) biopile amended with 325 Kg of activated sludge, (C) biopile amended with 325 Kg of activated sludge + 150 g of EPS emulsifier, (D) biopile amended with 150 g of EPS emulsifier. The total amount of EPS emulsifier was added in four doses: 60 g of EPS emulsifier at the beginning of experiment and 30 g after 15, 30 and 45 days of treatment. EPS emulsifier addition was done by irrigation with 1% EPS solution in distilled water. Table 1 shows values of initial pollution.

Enumeration of culturable bacteria in soil

Three replicate samples from each microcosm were withdrawn every week for enumeration of aerobic heterotrophic bacteria. 0.1 ml of serially diluted soil samples were plated in 1/10 diluted Trypticase Soy Agar (TSA, Difco) according to Avidano et al. [5]. Triplicate plates were incubated at 28 °C for 48 h before the colonies were counted.

TPH, *n*-alkanes and PAH determinations

Total petroleum hydrocarbons were extracted from the soil samples with a mixture of hexane: acetone 1:1 (v/v) and determined by gravimetric analysis according to.Aguilera-Vázquez et al. [1]. Analysis of *n*-alkanes and polycyclic aromatics hydrocarbons (PAHs) in the soil samples were determined from the extracted fractions above mentioned using a Hewlett–Packard 6890 GC system equipped with a HP-5-MS-capillary column (30 × 0.32 mm I.D.). Helium (1.6 ml/min) was utilized as carrier gas. The determinations were performed using the following temperature program: 40 °C held 1 min isothermal, heating rate 4 °C/min up to 310 °C, final temperature held for 1.5 min. Injector and detector temperatures were 250C and 300 °C, respectively. *n*-alkanes and PAH were detected using a mass detector 5872 (Hewlett–Packard) and the library utilized was Wiley 275.

Results

Taxonomical characterization of strain AD2

The initial characterization of strain AD2 revealed that they are Gram negative rods. Percentages similarity values obtained after pair-wise alignment of the sequence of 16S rDNA versus the EMBL data base demonstrated the affiliation of this strain to the species *Ochrobactrum anthropi* (over 100% ungapped sequence identity). Thus, our results showed that strain AD2 isolated from activated sludge of the wastewater treatment plant of the oil refinery of Repsol YPF should be classified as *O. anthropi*.

Production and characterization of bioemulsifier AD2

In the present study, assays were carried out to know the capacity of *O anthropi* strain AD2 to produce extracellular biopolymers with biosurfactant or bioemulsifiers activities. Our data showed that strain AD2 was able to produce exopolysaccharide growing on glucose NB liquid media and the capacity of this bacterium to grow and produce the EPS emulsifier in the presence of hydrocarbon compounds. Table 2, shows yield production and chemical composition, in terms of carbohydrates and proteins, of the EPS emulsifier synthesized by *O anthropi* strain AD2 in glucose NB medium and in glucose NB medium added with *n*-octane, xylene, toluene, mineral light and heavy oils and crude oil. Addition of *n*-octane, mineral light oil and mineral heavy oil enhanced the amount of EPS synthesized. In contrast, strain AD2 was unable to grow in presence of xylene and toluene, thus both hydrocarbons seem to be toxic for it. When a preliminary characterization of the exopolymers was performed, it was found that these water soluble substances contain high amounts of proteins and carbohydrates. However, the addition of hydrocarbons (*n*-octane, mineral light oil, mineral heavy oil or crude oil) affected the protein and carbohydrate composition. Thus, *O anthropi* strain AD2 produced in glucose-crude oil medium extracellular polymers with higher content of protein (32.57%) than polymers synthesized in glucose-mineral light oil (10.77%). In this context, our results show that *O anthropi* strain AD2 can produce significant amounts of extracellular polymers in the presence of different hydrocarbons although these compounds can affect the chemical composition of the extracellular polymers.

The extracellular biopolymers synthesized in glucose NB medium showed a wide emulsifying activity, crude oil being the substrate most effectively emulsified followed by mineral light oil (Table 3). Thus, we found that all extracellular biopolymers had a noticeable activity on crude oil; however, the specificity of biopolymers was modified by the conditions used for culture of the strain AD2. For example, extracellular biopolymers extracted from media with mineral light oil were most active on toluene. However, mineral light oil and mineral heavy oil were not emulsified by the polymers produced on crude oil. Finally, none of the isolated biopolymers produced by *O. anthropi* showed biosurfactant activity.

Table 2 Yield production (g/l of culture medium) and chemical composition (percentage of carbohydrates and proteins) of EPS synthesized by *Ochrobactrum anthropi* strain AD2 in glucose NB media amended with *n*-octane, xylene, toluene, mineral light oil, mineral heavy oil, and crude oil media

Carbon source of EPS culture media production	Yield production (g/l)	Chemical composition	
		Carbohydrates (%)	Proteins (%)
Glucose	0.5 ± 0.1	72.79 ± 3.91	18.3 ± 1.56
Glucose + *n*-octane	0.71 ± 0.1	60.82 ± 2.39	26.99 ± 1.72
Glucose + xylene	ND	ND	ND
Glucose + toluene	ND	ND	ND
Glucose + mineral light oil	0.65 ± 0.1	78.97 ± 4.38	10.77 ± 0.48
Glucose + mineral heavy oil	0.94 ± 0.1	66.14 ± 2.35	14.35 ± 1.91
Glucose + crude oil	0.47 ± 0.1	64.35 ± 1.26	32.57 ± 3.55

Values are mean ± standard error of three replicates

ND Growth and extracellular polymers production was not detected

Table 3 Emulsifying activity of EPS emulsifier produced by *O anthropi* strain AD2 in glucose media amended or un-amended (control) using n-octane, toluene, xylene, mineral light oil, mineral heavy oil and crude oil

Growth medium for EPS emulsifier production	Emulsifying activity using following substrate (%)					
	Octane	Toluene	Xylene	Light oil[a]	Heavy oil[a]	Crude
NB[a]	37.5 ± 2.7	28.8 ± 1.3	22.5 ± 4.6	51.2 ± 2.4	28.8 ± 3	88.7 ± 4.2
NB-octane	38.1 ± 0.6	25 ± 1.3	23.2 ± 2	56.1 ± 1.2	31.1 ± 0.4	95.1 ± 3.3
NB- light oil[b]	8.16 ± 0.2	58.33 ± 3.9	10.42 ± 3.1	14.58 ± 0.7	12.5 ± 0.5	85.41 ± 5.1
NB- heavy oil[b]	0	53.19 ± 2.8	27.66 ± 0.6	32 ± 0.9	0	81.25 ± 7.2
NB- crude EPS	0	31.25 ± 0.3	14.58 ± 0.7	0	0	72.92 ± 1.4

[a] Nutrient broth

[b] Mineral oil

Microcosm assays

In this study, it has been evaluated the effectiveness of the biopolymer EPS emulsifier as biostimulating agent and its application in bioremediation processes using microcosm and biopile systems. In soil microcosms, EPS emulsifier was applied alone or amended with an inorganic NPK fertilizer or the S200 C biostimulating agent. Figure 1 shows the response of indigenous microbial population in treated and nontreated soil microcosms. In general, no differences were found among the assays tested, except for the microcosm treated with S200 C + EPS emulsifier. This treatment remarkably enhanced indigenous microbial population (Fig. 1) throughout the incubation period (21 days). On the other hand, GC/SM analyses showed that inoculation of EPS emulsifier enhanced hydrocarbon removal efficiency when compared to un-amended control soils (Fig. 2). Moreover, a significant increase in the elimination of TPH and *n*-alkanes was detected in the soil microcosm soil treated with NPK + EPS emulsifier compared to microcosms treated with inorganic NPK fertilizer alone. In the same way, addition of the EPS emulsifier bioemulsifier also stimulated the hydrocarbon biodegradation rate in microcosms containing S200 C. As it can be seen in Fig. 2, the percentage of hydrocarbon removed in microcosms treated with S200 C + EPS emulsifier was notably higher than in microcosms containing S200 C alone.

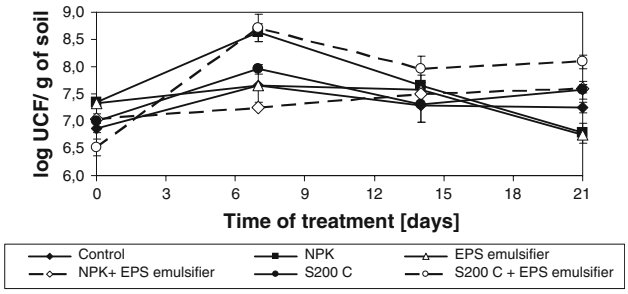

Fig. 1 Total heterotrophic bacteria in soil microcosms amended with NPK fertilizer, EPS emulsifier, S200 C product and un-amended microcosm (control)

Biopile systems

Composting processes have been usefully applied in bioremediation of soil polluted with hydrocarbon. In this context, the composting processes are able to increase the

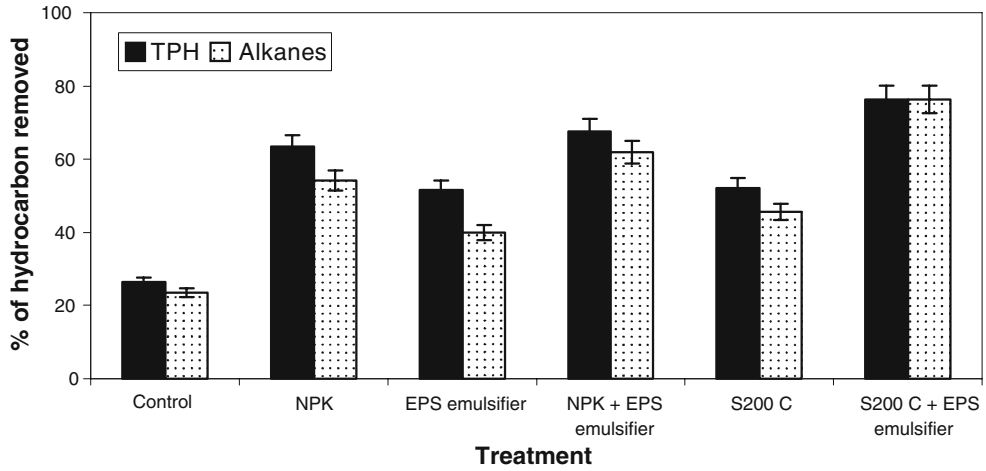

Fig. 2 Efficiency of TPH and *n*-alkanes removing in un-amended soil microcosms (control) or amended soil microcosms with NPK fertilizer, EPS emulsifier and S200 C, after 21 days of treatment

microbial biodegradation activities and consequently enhance the biotransformation of different nutrients such as hydrocarbons. Under this point of view we have studied the effect of the biopolymer EPS emulsifier in the biodegradation of hydrocarbon using composting technology (biopile systems).

We have investigated the effects of the addition of biosurfactant EPS emulsifier and activated sludge on biopile systems for composting of soil contaminated with petroleum hydrocarbons. Both microbial populations and total hydrocarbon biodegradation (Figs. 3, 4) were enhanced in biopile amended with activated sludge plus biosurfactant compared to biopiles amended with activated sludge, AD2 or un-amended control. Thus, our data showed that the addition of both activated sludge and EPS emulsifier to biopile systems clearly increases the petroleum hydrocarbon biodegradation.

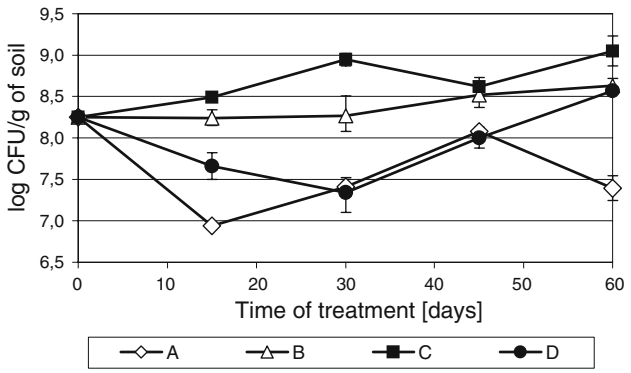

Fig. 3 Evolution of total heterotrophic bacteria in control (un-amended) composting biopiles and composting biopiles amended with activated sludge and EPS emulsifier bioemulsifier, after 60 days of treatment *A* control, *B* sludge, *C* sludge + EPS emulsifier, *D* EPS emulsifier

Discussion

O. anthropi is a Gram negative bacterium belonging to α Proteobacteria with capacity for bioremediation due to its capability of degrading a high number of pollutants such as organophosphorus pesticides, phenol compounds, and petroleum wastes [31]. Its ability of removing heavy metals by producing high amount of exopolysaccharide has also been described [31]. Studies of the microbial biodiversity during long-term storages of crude oil at different layers of the tank storage, have demonstrated that *Ochrobactrum* spp. were isolated from all the samples collected at each sampling time, suggesting the presence of these microorganisms in a viable state [44]. Similarly, Katsivela et al. [24] have reported that in a 14 months land farming treatment, although microbial diversity was decreased with increased degradation of petroleum hydrocarbons, the genera *Enterobacter* and *Ochrobactrum* were always detected in the land farming treated soil [24].

O. anthropi strain AD2 was isolated in our laboratory from activated sludge of the wastewater treatment plant of an oil refinery and it could be considered as an indigenous crude oil microorganism grown in the refinery oil compounds. This strain was selected among other isolates because it was able to grow in the hydrocarbon culture media and due to its property of producing high amount of exopolymers with emulsifying activity against different hydrocarbon compounds. Thus, *O. anthropi* strain AD2 produces polymers under different culture conditions and in the presence of hydrophobic substances. However, culture medium modifies chemical composition and emulsifying activity of strain AD2 extracellular biopolymers (EPS emulsifier). Most polymers studied here have shown high emulsifying activities. This suggests that *O. anthropi* could produce the emulsifier polymer after being inoculated into

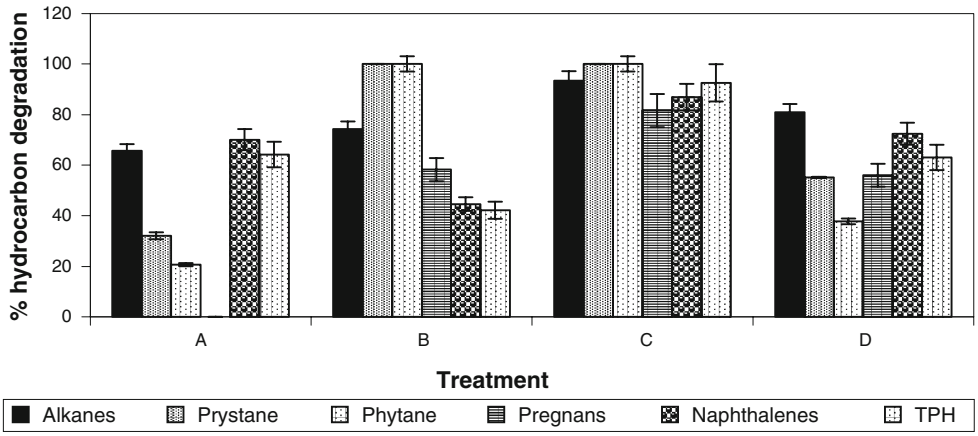

Fig. 4 Efficiency of hydrocarbons removing in control (un-amended) biopile systems, and biopiles systems amended with activated sludge and EPS emulsifier, after 60 days of treatment. *A* control, *B* sludge, *C* sludge + EPS emulsifier, *D* EPS emulsifier, Naphthalene

polluted environmental sites, thus, be useful for in situ bioremediation treatment. The ability of all the polymers studied to emulsify crude oil is also noteworthy, as they are its emulsifying activity comparing to chemical surfactants. Thus, the emulsifying capacity of EPS emulsifier was always higher than that compared to Tween 20, Tween 80 and Triton X100 chemical surfactants [27].

The persistence of hydrocarbons within the ecosystem is due to the low aqueous solubility that limits their availability to hydrocarbon degrading microorganisms; bioemulsifiers can emulsify hydrophobic compounds, form stable emulsion, increase hydrocarbon solubility and consequently enhance the bioavailability in the environment [34]. Thus, they can stimulate the growth of hydrocarbon degrading bacteria, improving their capacity to utilize these compounds [39].

There is a useful diversity of biosurfactants and bioemulsifiers due to the wide variety of producer microorganisms [6]. The EPS emulsifier is an exopolysaccharide which is produced during the stationary phase of growth (after 7 days of incubation). This biopolymer can be included in the high molecular weight exopolymer group [35] and it is able to efficiently emulsify petroleum hydrocarbons, but unable to reduce surface tension.

It has been described that chemical composition and functional properties of exopolymers could be influenced by the nutritional composition of culture media where the bacteria are growing [37]. Thus, yield production, chemical composition and emulsifying activity of the exopolymers depend not only on the producer strain but also on the culture conditions [14, 29]. These chemical modifications could be responsible of the different emulsifying activity detected in the EPS emulsifier according to the growth media where the strain AD2 was cultured.

The effectiveness of EPS emulsifier as a biostimulating agent in oil bioremediation processes was evaluated in the present study using oil contaminated soil microcosms. The EPS emulsifier was applied alone or amended with other biostimulating products such as an inorganic NPK fertilizer and the S200 C oleophilic commercial product. The primary limiting factors in the microbial degradation of petroleum are nitrogen and phosphorous concentrations. These nutrients are important to cellular production and their supplementation increases the hydrocarbon degradation effectiveness [38]. The results obtained have shown that the application of NPK fertilizer stimulated microbial population at the beginning of the soil treatment (until 7 days of incubation). However, at the end of the soil treatment (21 days) microbial count decreased below un-amended controls. In contrast, combined treatment of NPK and EPS emulsifier enhanced steadily bacterial growth but it was able to maintain higher number of microorganisms at the end of experiments (21 days). This result suggests that EPS emulsifier could be considered as a useful emulsifier that increases the bioavailability of hydrocarbons to bacteria, favoring the use of hydrocarbon as carbon and energy source. This agrees with the highest percentage of hydrocarbons eliminated in microcosms added with inorganic fertilizer and EPS emulsifier.

According to commercial indications, S200 C product selectively stimulates hydrocarbon degrader microorganisms and not other type of bacteria, consequently the specific natural selection of indigenous hydrocarbon degraders promotes a quicker degradation of contaminants. Our experiments have revealed that the addition of S200 C to soil microcosm systems enhanced the percentage of TPH removed compared to un-amended control 52.2% (4,452 ppm eliminated) and 26.3% (2,243 ppm), respectively. However, significant differences in the number of total heterotrophic bacteria were not observed. Finally, when the bioemulsifier EPS emulsifier was added in combination with S200 C, stimulation in microbial growth and oil hydrocarbon biodegradation was detected. Thus, under these experimental conditions, the amount of TPH removed at the end of the treatment (21 days) was near 6,500 ppm (76.3%).

Bioremediation is not a new strategy for oil hydrocarbon removal but in many cases in situ remediation shows a

small rate of degradation. This could be associated with the microorganism's availability to degrade, to low solubility of the contaminant and to specific nutrient limitation. The results obtained suggest that EPS emulsifier usefully enhances the solubility and consequently the bioavailability of hydrocarbon compounds to specific oil degrader microorganisms previously stimulating by the S200 C product. Thus, the use of this combined treatment may be useful for the study of oil hydrocarbon degradation and for bioremediation purposes.

Biopiles are used to reduce the concentration of petroleum constituents in excavated soils through the use of biodegradation. This technology can be performed onsite and involves keeping contaminated soils into piles and stimulating aerobic microbial activity within the soils through the addition of oxygen, minerals, nutrients, and moisture. The enhanced microbial activity results in the breakdown of the petroleum constituents in the soil.

Activated sludges can be considered as an useful source of microorganisms with a wide range of metabolic activities. Our results demostrated that the addition to biopiles of activated sludge enhanced the bioremediation process; however, this positive effect can be increased by the addition of activated sludge plus biosurfactant EPS emulsifier, suggesting that the application of the composting technology for the bioremediation of oil contaminated soil could be improved with the addition of both products. In conclusion, our results suggest that a combined treatment including bioemulsifiers such as EPS emulsifier could be an efficient tool to improve the yield of hydrocarbons degradation in bioremediation processes.

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References

1. Aguilera-Vázquez L, Soto-Cruz NO, Saucedo-Castañeda G, Gutiérrez-Rojas MA (2001) Model system for composting hydrocarbon contaminated soil by using water activity and porosity as response variables. Chem Eng J 81:197–202
2. Alburquerque CDC, Filetti AMF, Campos-Takaki GM (2006) Optimizing the medium components in bioemulsifiers production by *Candida lypolitica* with response surface method. Can J Microbiol 52:575–583
3. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402
4. Ashtaputre AA, Shah AK (1995) Emulsifying property of a viscous exopolysaccharide from *Sphingmonas paucimibilis*. World J Microbiol Biotechnol 11:219–222
5. Avidano L, Gamalero E, Cossa GP, Carraro E (2005) Characterization of soil health in an Italian polluted site by using microorganisms as bioindicators. Appl Soil Ecol 30:21–33
6. Banat RS, MakkarRS, Cameotra SS (2000) Potential commercial applications of microbial; surfactants. Appl Microbiol Biotechnol 53:495–508
7. Barathi S, Vasudevan N (2001) Utilization of petroleum hydrocarbons by *Pseudomonas fluorescens* isolated from a petroleum-contaminated soil. Environ Int 26:413–416
8. Barkay T, Nayon-Venezia S, Ron EZ, Rosenberg E (1999) Enhancement of solubilization and biodegradation of polyaromatic hydrocarbons by the bioemulsifier alasan. Appl Environ Microbiol 65:2697–2702
9. Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. Anal Biochem 72:248–254
10. Calvo C, Martínez-Checa F, Toledo FL, Porcel J, Quesada E (2002) Characteristics of bioemulsifiers synthesized in crude oil media by *Halomonas eurihalina* and their effectiveness in the isolation of bacteria able to grow in the presence of hydrocarbons. Appl Microbiol Biotechnol 60:347–351
11. Calvo C, Toledo FL, Pozo C, Martínez-Toledo MV, González-López J (2004) Biotechnology of bioemulsifiers produced by microorganisms. J Food Agric Environ 2:238–243
12. Cooper DJ, Goldenberg BG (1987) Surface active agents from two *Bacillus* species. Appl Environ Microbiol 54:224–229
13. Chen SY, Lu WB, Wei YH, Chen WM, Chang JS (2007) Improved production of biosurfactant with newly isolated *Pseudomonas aeruginosa* S2. Biotech Progr 23:661–666
14. Desai JD, Banat M (1997) Microbial production of surfactant and their commercial potential. Microbiol Mol Biol Rev 61:47–61
15. Déziel E, Paquette G, Villemur R, Lépine F, Bisaillon JG (1996) Biosurfactant production by a soil *Pseudomonas* strain growing on polycylic aromatic hydrocarbons. Appl Environ Microbiol 62:1908–1912
16. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356
17. Fietcher A (1992) Biosurfactants: moving towards industrial application. Trends Biotechnol 10:208–217
18. Guerra-Santos LH, Kappeli O, Flechter A (1986) Dependence of *Pseudomonas aeruginosa* continuous culture biosurfactant production on nutritional and environmental factors. Appl Microbiol Biotechnol 24:443–448
19. Herman DC, Zhang Y, Miller R (1997) Rhamnolipid (biosurfactant) effects on cell aggregation and biodegradation of residual hexadecane under saturated flow conditions. Appl Environ Microbiol 63:3622–3627
20. Iyer A, Mody K, Jha B (2006) Emulsifying properties of a marine bacterial exopolysaccharide. Enzyme Microbiol Technol 38:220–222
21. Jacques RJS, Santos EC, Bento FM, Peralba MCR, Pedro A, Selbach EL, Camargo FAO (2005) Anthracene biodegradation by *Pseudomonas* sp. isolated from a petrochemical sludge landfarming site. Int Biodeterior Biodegradation 56:143–150
22. Javaheri MJ, Jenneman GE, McInnerney MJ, Knapp RM (1985) Anaerobic production of a biosurfactant by *Bacillus liqueniformis* JF-2. Appl Environ Microbiol 50:698–700
23. Kanaly R, Harayama S (2000) Biodegradation of high-molecular weight polycyclic aromatic hydrocarbons by bacteria. J Bacteriol 182:2059–2067
24. Katsivela E, Moore ERB, Maroukli D, Strömpl C, Pieper D, Kalogerakis N (2005) Bacterial community dynamics during in-situ bioremediation of petroleum waste sludge in land farming sites. Biodegradation 16:169–180

25. Lang S, Wullbrandt D (1999) Rhamnose lipids-biosynthesis, microbial production and application potential. Appl Microbiol Biotechnol 51:22–32
26. Leahy JG, Colwell R (1990) Microbial degradation of hydrocarbons in the environment. Microbiol Rev 54:305–315
27. Martínez-Checa F, Toledo FL, Vilchez R, Quesada E, Calvo C (2002) Yield production, chemical composition and functional properties of emulsifier H28 synthesized by *Halomonas eurihalina* strain H28 in media added with several hydrocarbons. Appl Microbiol Biotechnol 58:358–363
28. Menezes Bento F, Oliveira Camargo A, Okeke BC, Jr Frankenberger (2005) Diversity of biosurfactant producing microorganisms isolated from soils contaminated with diesel oil. Microbiol Res 160:249–255
29. Mulligan CN, Gibbs BF (1989) Correlation of nitrogen metabolism with biosurfactant production by *Pseudomonas aeruginosa*. Appl Environ Microbiol 53:3016–3019
30. Navon-Venezia S, Zosim Z, Gottlieb A, Legmann R, Carmeli S, Ron EZ, Rosenberg E (1995) Alasan a new bioemulsifier from *Acinetobacter radioresistens*. Appl Environ Microbiol 61:3240–3244
31. Ozdemir G, Ozturk T, Ceyhan N, Isler R, Cosar T (2003) Heavy metal biosorption by biomass of *Ochrobactrum anthropi* producing exopolysaccharide in activated sludge. Bioresour Technol 90:71–74
32. Peypoux F, Bonmatin JM, Wallach J (1999) Recent trends in the biochemistry of surfactin. Appl Microbiol Biotechnol 51:553–563
33. Plaza GA, Ulfig K, Brigmon RL (2005) Surface active properties of bacterial strains isolated from petroleum hydrocarbon-biorremediated soil. Pol J Microbiol 54:161–162
34. Ron EZ, Rosenberg E (2002) Biosurfactants and oil bioremediation. Curr Opin Biotechnol 13:249–252
35. Rosenberg E, Ron EZ (1999) High- and low-molecular-mass microbial surfactants. Appl Microbiol Biot 52:154–162
36. Rosenberg E, Rubinovitz C, Gottlieb A, Rosenhak S, Ron EZ (1988) Production of biodispersan by *Acinetobacter calcoaceticus* A2. Appl Environ Microbiol 54:317–322
37. Sutherland IW (2001) Microbial polysaccharides from Gram-negative bacteria. Int Dairy J 11:663–674
38. Ting YP, Hu HL, Tan HM (1999) Bioremediation of petroleum hydrocarbons in soil microcosms. Resour Environ Biotechnol 2:197–218
39. Toledo FL, Calvo C, Rodelas B, González-López J (2006) Selection and identification of bacteria isolated from waste crude oil with polycyclic aromatic hydrocarbons removal capacities. Syst Appl Microbiol 29:244–252
40. Toledo FL, González Lopez J, Calvo C (2007) Production of bioemulsifier by *Bacillus subtilis*, *Alcaligenes faecalis* and *Enterobacter* species in liquid culture. Bioemulsifier Bioresour Technol 99(17):8470–8475. doi:10.1016-j.biortech.2007.08.055
41. Vinuesa P, Rademaker JLW, De Bruijin FJ, Werner D (1998) Genotypic characterization of *Bradyrhizobium* strains nodulating endemic woody legumes of the Canary Islands by PCR-restriction fragment length polymorphism analysis of genes encoding 16S rRNA (16S rDNA) and 16S–23S rDNA intergenic spacers, repetitive extragenic palindromic PCR genomic fingerprinting, and partial 16S rDNA sequencing. Appl Environ Microbiol 64:2096–2104
42. Wang L, Tang Y, Wang S, Liu RL, Liu MZ, Zhang Y, Liang FL, Feng L (2006) Isolation and characterization of a novel thermophilic *Bacillus* strain degrading long-chain *n*-alkanes. Extremophiles 10:347–356
43. Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697–703
44. Yoshid N, Takahashi N, Hiraishi A (2005) Phylogenetic characterization of a polychlorinated dioxin-dechlorinating microbial community by use of microcosm studies. Appl Environ Microbiol 71:4325–4334
45. Zuckerberg A, Diver A, Peeri Z, Gutnick DL, Rosenberg E (1979) Emulsifier of *Arthrobacter* RAG-1: chemical and physical properties. Appl Environ Microbiol 37:414–420