Electronic Journal of Biotechnology 61 (2023) 24–32

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

journal homepage: www.elsevier.com/locate/ejbt

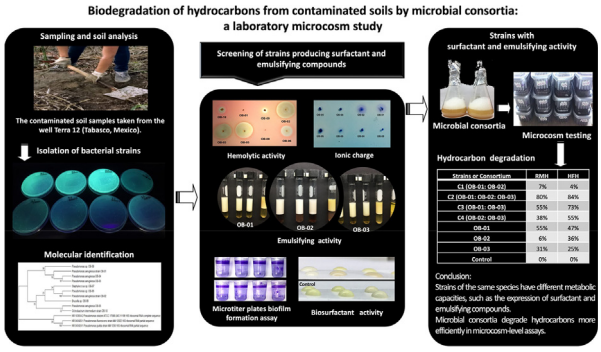
Research Article

Biodegradation of hydrocarbons from contaminated soils by microbial consortia: A laboratory microcosm study

Stephania Lázaro-Mass [a,b], Sergio Gómez-Cornelio [a], Mariana Castillo-Vidal [c], Carina Shianya Alvarez-Villagomez [c], Patricia Quintana [b], Susana De la Rosa-García [c,*]

[a] Universidad Politécnica del Centro, Carretera Villahermosa-Teapa Km 22.5, Tumbulushal, 86290 Villahermosa, Tabasco, Mexico
[b] Departamento de Física Aplicada, Centro de Investigación y de Estudios Avanzados, Unidad Mérida, Km 6 Antigua Carretera a Progreso. Apdo. Postal 73, Cordemex, 97310 Mérida, Yucatán, Mexico
[c] División Académica de Ciencias Biológicas, Universidad Juárez Autónoma de Tabasco, Carretera Villahermosa-Cárdenas Km. 0.5, Rancheria Emiliano Zapata, 86150 Villahermosa, Tabasco, Mexico

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 29 April 2022
Accepted 18 October 2022
Available online 25 October 2022

Keywords:
Biofilms
Bioremediation
Contaminated soils
Emulsifiers
Environmental impact
Hydrocarbons
Microbial consortium
Petrochemical industry
Pollution
Pseudomonas

ABSTRACT

Background: Compounds derived from hydrocarbons are essential for industry and our daily life; however, accidents such as spills or leaks occur regularly, causing severe environmental impact. Therefore, bacteria isolated from an oil well were characterized to assess their potential in the degradation of hydrocarbons under individual and consortium treatments. Soil samples, from a well contaminated with hydrocarbons in Tabasco, Mexico, were collected. The biosurfactant, emulsifying capacity, hemolytic activity, ionic charge, and biofilm formation were analyzed.
Results: Most of the isolated strains belong to the genus *Pseudomonas* according to 16S rDNA sequencing. In general, emulsification percentages greater than 60% in the different substrates evaluated were observed. In addition, these strains can form biofilms, and those selected to integrate the microbial consortia present anionic surfactants. Three strains were selected to evaluate the degradation potential of soil contaminated with hydrocarbons from the same site where the bacteria were isolated in a microcosm. The microbial consortia degraded the contaminated soil more efficiently than the strains were evaluated alone, mainly the three bacteria consortia, with percentages greater than 80%.
Conclusions: This study shows that, despite belonging to the same species, bacterial strains' metabolic capacity for the expression of surfactant compounds, emulsifiers, and the formation of biofilms are different. Furthermore, the more structured a community is, the greater the biodegradation process that

Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.
* Corresponding author.
E-mail address: susana.delarosa@ujat.mx (S. De la Rosa-García).

https://doi.org/10.1016/j.ejbt.2022.10.002

occurs when bacteria act individually on the substrate. Therefore, this study demonstrates that strains of the same species integrated microbial consortia improve the bioremediation processes of hydrocarbons in contaminated soils.

How to cite: Lázaro-Mass S, Gómez-Cornelio S, Castillo-Vidal M, et al. Biodegradation of hydrocarbons from contaminated soils by microbial consortia: A laboratory microcosm study. Electron J Biotechnol 2022;61. https://doi.org/10.1016/j.ejbt.2022.10.002.

1. Introduction

Hydrocarbon pollution represents a severe problem worldwide, the petrochemical industry being one of the primary sources of these persistent residues [1,2]. Polycyclic aromatic hydrocarbons (PAHs) are recalcitrant compounds generated from petroleum industry activities and incomplete combustion of diesel fuel, coal, and oil [3]; hence, their degradation is complicated. In addition to the environmental impact, they represent health risk due to their mutagenic and carcinogenic potential [1]. Conventional treatment methods are generally limited to spill containment with booms or synthetic surfactants that generate toxic byproducts [4]. Therefore, biotechnological methods through microorganisms represent a sustainable advantage by allowing the biodegradation of pollutants [5].

Microorganisms play an essential role in treating hydrocarbons through processes such as bioabsorption and bioaugmentation [5]. However, because of the variety and complexity of the molecules deposited in contaminated sites, a single strain will not be able to perform an efficient biodegradation process, achieving complete mineralization of the hydrocarbons to CO_2 and H_2O; hence, it is necessary to integrate a set of strains by a microbial consortium [6].

Microbial consortia have a higher degradative capacity than a single microbial species. Using a single microorganism would limit the effectiveness percentage since an organism's metabolism capacity is reduced to a certain amount of substrate [7]. These microbial clusters form a symbiosis that acts together to benefit and enable survival [8]. Synergistic work among consortium members improves their enzymatic skills [6], allowing them to carry out more complex tasks. Furthermore, the use of microbial consortia represents a potential increase in bioremediation efficiency and gives them greater resistance to abiotic stress [8].

Adverse effects have been recorded on the soil and water bodies from oil spills. These residues represent a potential danger to the environment and nearby human populations; therefore, we isolated microorganisms from areas contaminated by an oil well to evaluate the capacity of hydrocarbon degradation by microbial consortia and axenic cultures of bacteria, under the hypothesis that microbial consortia will have a higher percentage of biodegradation than a single bacterial strain.

2. Materials and methods

2.1. Sampling and soil analysis

The contaminated soil samples were taken from the well Terra 12, located in the Oxiacaque community in Nacajuca, Tabasco (18°15′11.314″N; 92°59′31.489″W), and the environmental parameters of relative humidity and temperature were measured *in situ*. In the laboratory, electrical conductivity (EC) and pH were measured by the paste pH technique [9], in which a mixture was prepared with a portion of distilled water (pH 6.5 and EC 6 μS/cm) and 2/3 parts of soil, and the calibrated EC and pH electrodes were introduced.

2.2. Isolation and identification molecular of bacteria

Serial dilutions from 10^{-1} to 10^{-6} (mg/mL) concentrations of the contaminated soil were inoculated by pouring (100 μL) in Petri dishes with soy trypticase agar at 10% and incubated at 30°C for 2–3 days. The microorganisms with different morphologies were purified. For the taxonomic determination, genomic DNA was extracted from each strains using the Wizard® Genomic DNA Purification kit (Promega). A PCR reaction was performed to amplify the 16S rDNA gene fragment using the universal nucleotides fD1 (AGAGTTTGATCCTGGCTCAG) and rD1 (AAGGAGGTGATCCAGCC). Amplification was performed in a gradient thermal cycler by an initial denaturation cycle at 95°C for 2 min, followed by 30 cycles (denaturation at 95°C for 20 s, annealing temperature of nucleotides at 53°C for 30 s, and extension at 72°C for 1 min), and one final cycle at 72°C for 10 min [10]. PCR products were purified from an electrophoresis gel using GeneJET Gel Extraction and DNA Cleanup kit (Thermo Scientific). The 1500 bp fragments were sequenced. The sequences obtained were compared in the GenBank database using the Blast software (http://www.ncbi.nlm.nih.gov/BLAST/). Using MEGA X software, a phylogenetic tree was generated using the neighbor-joining (NJ) method.

2.3. Characterization of isolated strains

For subsequent assays, the bacterial isolated were cultured in trypticase soy broth (TSB) at 37°C overnight. The suspensions were adjusted to 1.5×10^8 CFU/mL in saline solution (0.85%) using 0.5 standard of the McFarland scale, and 10 mL of suspension bacterial was inoculated into 90 mL of TSB in Erlenmeyer flask and incubated in an orbital shaker (150 rpm) for 96 h at 37°C. The cultures from each flask were centrifuged at 6,000 rpm at 4°C for 20 min and then filtered through with a 0.45 μm membrane filter (Merck-Millipore, USA) to obtain a cell-free supernatant (CFS).

2.3.1. Biosurfactant activity

Biosurfactant activity was determined using the drop-collapse technique. This assay was carried out on the lid of a 96-well microplate according to Meliani and Bensoltane [11] by placing 2 μL of mineral oil on the surface of the lid, then 5 μL of CFS was deposited onto the mineral oil by octuplicate and 25 μL on a strip of parafilm-M by triplicate. Droplet collapse was observed 1 min later. Tween 1% and TSB (without inoculum) were used as positive and negative controls, respectively. Biosurfactant activity was considered positive, when a bacterial CFS collapsed the oil droplet [12].

2.3.2. Emulsifying activity (EI_{24})

The 24-h emulsification index (EI_{24}) was determined according to the method proposed by Cooper and Goldenberg [13] with modifications. Briefly, 2.5 mL of burned oil, hexadecane, diesel, xylene, and motor oil were mixed with 2.5 mL of CFS in test tubes with a vortex mixer. The emulsion was left to stand for 24 h in the dark to measure the stable area of emulsification and EI_{24} equation was applied [14] (**Equation 1**)

$$EI_{24} = \frac{emulsification\ \ height}{total\ \ height} \times 100 \quad \text{Equation 1}$$

The mixture of liquid hydrocarbons and water during bioremediation or fermentation processes generate emulsions; therefore, these compounds can be applied to stimulate bioremediation [15]. In the assays, the surfactants SDS and Tween 20 were used as positive controls. In addition, the stability of bioemulsifying in salinity conditions was evaluated by adding NaCl to the CFS to give concentration of 25 and 50 g/L of NaCl, and the emulsifying activity was measured as described above.

2.3.3. Hemolytic activity

The hemolytic activity test was performed to determine the presence or absence of biosurfactants through a qualitative test [12]. Detection is determined by the appearance of a staining area around the well; depending on the staining, three types of hemolysis could be recognized: Alpha hemolysis in a medium with dark greenish coloration, beta hemolysis in a medium with yellowish coloration or with evident hemolysis halo, and gamma hemolysis in an unaltered media [16]. For the hemolysis test, 5% blood agar plates were used. Wells of 9 mm diameter were drilled, and 2 µL of CFS from each strain were placed [17]. Triton X-100 at 1% was used as a positive control, and sterile TSB was used as a negative control. The plates were incubated for 24 h at room temperature.

2.3.4. Ionic charge

The ionic charge was assessed using a double diffusion technique [18]. Regularly spaced rows of wells were realized in 1% agar plates with a low degree of hardness. The wells were filled with the biosurfactant solutions from strains, using sodium dodecyl sulfate (SDS, 20 mM) as the anionic commercial surfactant and cetyltrimethylammonium bromide (CTAB, 20 mM) as the cationic surfactants. The plates were maintained at room temperature and monitored over a 48-h period; the appearance of precipitation lines between the wells determines the ionic nature of the biosurfactants.

2.3.5. Detection of glycolipid biosurfactant

The blue agar assay was used to detect the presence of extracellular glycolipids compounds by bacterial strains [19]. The blue agar plates were prepared by adding 0.2 g of CTAB and 0.005 g of methylene blue to 1 L of mineral salts medium. A solution of 2 µL of CFS was deposited in blue agar plates and incubated at 37°C for 24–48 h to observe the appearance of halos of intense blue or purple as indicators of anionic biosurfactants.

2.3.6. Microtiter plates biofilm formation assay

Each strain was grown in 10 mL of TSB (at 37°C) overnight. Biofilm production assays were performed under oligotrophic conditions in a minimum mineral medium with glucose 1% (MMG) and copiotrophic conditions (TSB) with minimal modification to Qi et al. [20]. Overnight cultures in TSB were adjusted at 1.5×10^8 UFC/mL and transferred (100 µL) to 10 mL of MMG or TSB. After vortexing, 125 µL were transferred to eight wells per strain in vinyl microtiter plates (Corning NY, USA), previously UV sterilized. Microplates were incubated at 37°C for 48 h. The cell turbidity was monitored using a Multiskan Go (Thermo scientific) at an optical density of 550 nm. After the incubation period, the medium was removed from the wells, and the microtiter plate wells were washed five times with phosphate buffer saline (PBS) to remove loosely associated bacteria. The microplates were air-dried for 30 min, and each well was stained with 125 µL of 1% crystal violet solution in water for 15 min. After staining, plates were washed with PBS for three times. The biofilms were visible as purple rings formed on the wall of each well.

2.4. Microbial consortia and microcosm testing

According to the results of the tests, three strains were selected to integrate four microbial consortia and determine the degradation of hydrocarbons in microcosm. The consortia and the three strains alone were inoculated by triplicate in plastic boxes with 500 g of contaminated soil with hydrocarbons from the same site where the bacteria were isolated, as control was used as an uninoculated triplicate. The treatments were manually homogenized and placed in the shade. Humidity, temperature, and gases (NO_X, CO_2, and CO) parameters were monitored with specialized sensors twice a week for 56 days.

The quantification of hydrocarbons was performed by extraction and gravimetry at the experiment's beginning and end. The extraction was realized by a Soxhlet with 4 cycles/hour for 4 h by using n-hexane as a solvent; the porous thimble was filled with a mixture of 10 g of contaminated soil and 10 g of sodium sulfate (Na_2SO_4). The flask with the extract was dried at 125°C; the extract was then weighed, and the removable material with hexane (RMH) was calculated using **Equation 2.**

$$RMH(mg\ \ kg^{-1}) = \frac{W_h * 1000 * 1000}{W_s} \quad \text{Equation 2}$$

W_s = Soil sample weight; $W_h = W_2 - W_1$ = Flask weight with dry extract – flask weight.

The RMH is the material extracted from a sample and determined by the fat and oil method. Subsequently, 85 mL of n-hexane were added to the flask with the extract to redissolve it, 3 g of silica gel were added for each 100 mg of RMH, and the solution was filtered in a previously tared distillation flask and distilled at 75°C. It was then dried at 125°C and weighed to obtain the heavy fraction of hydrocarbons. HFH is the RMH treated with silica gel; therefore, the nonpolar material that is not absorbed was calculated (**Equation 3**):

$$HFH(mg\ \ kg^{-1}) = \frac{W_2 - W_1(1000)(1000)}{W_s} \quad \text{Equation 3}$$

where W_s = Soil sample weight; W_1 = Flask weight with dry HFP extract; W_2 = Flask weight.

Finally, the data analysis was performed with one-way analysis of variance (ANOVA) considering $P < 0.05$ with Tukey's test, after checking for homoscedasticity and normality with Levene's and the Shapiro–Wilk tests.

3. Results

3.1. Sampling and soil analysis

In the sampling site, the temperature was 32°C and 74% of relative humidity. The pH and EC of the soil sample were 6.84 and 2026 µS/cm (2.026 mS/m), respectively, while in a control soil without contamination by hydrocarbons, a pH of 7.3 and EC of 875 µS/cm (0.875 mS/m) was recorded. EC indicates that the soil at the sampling site is moderately saline [21].

3.2. Identification and characterization of the isolated strains

Isolated strains with different morphologies were sequenced by 16S rDNA gene analysis; according to the phylogenetic tree with ten strains, seven shared significant (>92%) related to the genus *Pseudomonas* (Table 1 and Fig. 1). Even though most of the strains belong to the genus *Pseudomonas*, subsequent characterizations show different metabolic capacities. All isolated strains could grow in a medium supplemented with 5% of NaCl.

Table 1
Genetic identification of isolated bacterial strains.

Strain	Nearest phylogenetic neighbor	Identity %	GenBank accession
OB-01	*Pseudomonas aeruginosa*	99.04	MZ317466
OB-02	*Pseudomonas aeruginosa*	98.80	MZ317472
OB-03	*Pseudomonas aeruginosa*	99.00	MZ317475
OB-04	*Pseudomonas aeruginosa*	99.45	MZ317479
OB-05	*Pseudomonas aeruginosa*	99.45	MZ317480
OB-06	*Pseudomonas* sp.	95.73	MZ317483
OB-07	*Staphylococcus* sp.	88.00	MZ317486
OB-08	*Pseudomonas* sp.	92.70	MZ317489
OB-09	*Brucella* sp.	87.50	MZ359809
OB-10	*Ochrobactrum intermedium*	99.25	MZ317500

The emulsification assays were performed with CFS extracted at 24 h in dark conditions. The obtained results are shown in Table 2 and Fig. 2. OB-04 and OB-08 strains did not show emulsifying activity in evaluated substrates. On the other hand, the OB-02, OB-03, and OB-07 strains showed emulsification index between 60–66% in diesel and hexadecane, while for burned oil, OB-03 and OB-05 had an EI_{24} between 76–80%; however, although OB-05 had good emulsifying activity on other substrates, the EI_{24} in diesel was 3.21%. In motor oil, emulsification rates were 50–62%, and burned oil was up to 79%. It is essential to highlight that the emulsification activity produced by all of the isolates was followed in time for up to one year, and in all cases, the EI_{24} remained unchanged, which shows the remarkable stability of the produced emulsification for bacterial strains. Furthermore, the addition of NaCl from 25 at 50 g/L did not affect the emulsifying activity. The EI_{24} values were similar with NaCl than without NaCl (data not shown).

In the drop-collapse assay with parafilm-M and microplate lids, the OB-01, OB-02, and OB-03 strains showed consistent production of biosurfactant compounds on both surfaces. In parafilm-M, the drop-collapse was 7 mm, while in microplate lids, it was greater than 4 mm. The rest of the strains presented values lower than 3.5 in microplate-lids and of 5 in parafilm-M (Table 2). In the hemolytic activity, the OB-02, OB-03, and OB-05 strains had a halo of hemolysis greater than 28 mm, while for the rest of the strains, it was less than 18 mm, or in the case of OB-01, OB-04, and OB-08 no activity was detected (Table 2). Regarding the type of hemolytic activity, 40% of the strains showed alpha hemolysis, 20% showed beta hemolysis, and in the remaining strains, no hemolysis was detected. Alpha hemolysis was observed on strains OB-02, OB-03, OB-06, and OB-07, and beta hemolysis corresponds to strains OB-09 and OB-05.

The presence of anionic biosurfactants was detected for OB-01, OB-02, OB-03, and OB-06 strains being more evident for the strains *P. aeruginosa* OB-01 and 0B-02, with the most intense halos with diameters of 1.0 and 1.2 cm, respectively; while for OB-03 and OB-06 values of 0.6 and 0.4 were observed in the strains, respectively. Therefore, these data suggest that the surfactant activity of these strains is given by anionic glycolipid-type biosurfactants, which also correspond to high collapsed droplet values in microplates and parafilm.

The results show that the ten strains present the formation of biofilm in TSB and/or MMG (Fig. 3). In the TSB medium, the upper part was the most preferred for biofilm formation, except for OB-06, which showed biofilm formation throughout the well column. While in MMG, the strains with significant biofilm formation were OB-01, OB-03, OB-05, OB-06, and OB-08, mainly in the superficial part of the well.

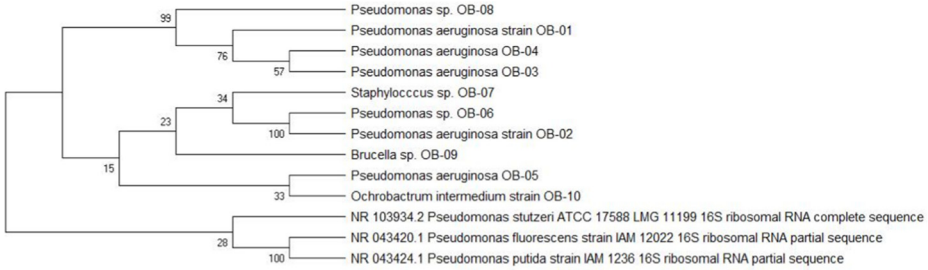


Fig. 1. Phylogenetic tree based on the nucleotide sequences of 16S rDNA gene fragment from ten strains isolated and other *Pseudomonas* species using the NJ method. Values at branch points represent percentage frequencies for tree topology after 1000 interactions.

Table 2
Results drop-collapse, hemolytic activity, and emulsifying activity of strains evaluated.

Strain	Drop-collapse (mm)		Hemolytic activity (mm)	Biosurfactant activity CTAB (mm)	Emulsifying activity (%)				
	Micro plate lids	Parafilm-M			Burned oil	Hexadecane	Diesel	Xylene	Motor oil
OB-01	4.5	7	(-)	1.0	62.20	53.86	61.52	61.53	61.54
OB-02	4.0	7	29	1.2	65.55	64.00	61.28	63.04	53.12
OB-03	5.0	7	29	0.6	76.92	61.28	65.55	66.30	60.24
OB-04	3.0	5	(-)	0	0	0	0	0	0
OB-05	3.0	5	28	0	79.54	54.94	3.21	39.75	51.61
OB-06	3.0	5	18	0.4	59.57	65.21	13.82	63.04	62.37
OB-07	3.5	5	18	0	65.55	63.33	63.33	55.91	55.69
OB-08	3.0	5	(-)	0	0	0	0	0	0
OB-09	3.0	5	8	0	61.81	61.81	59.34	63.33	52.75
OB-10	3.0	5	15	0	58.72	46.27	59.77	45.55	50.53

(-): hemolytic activity not observed.

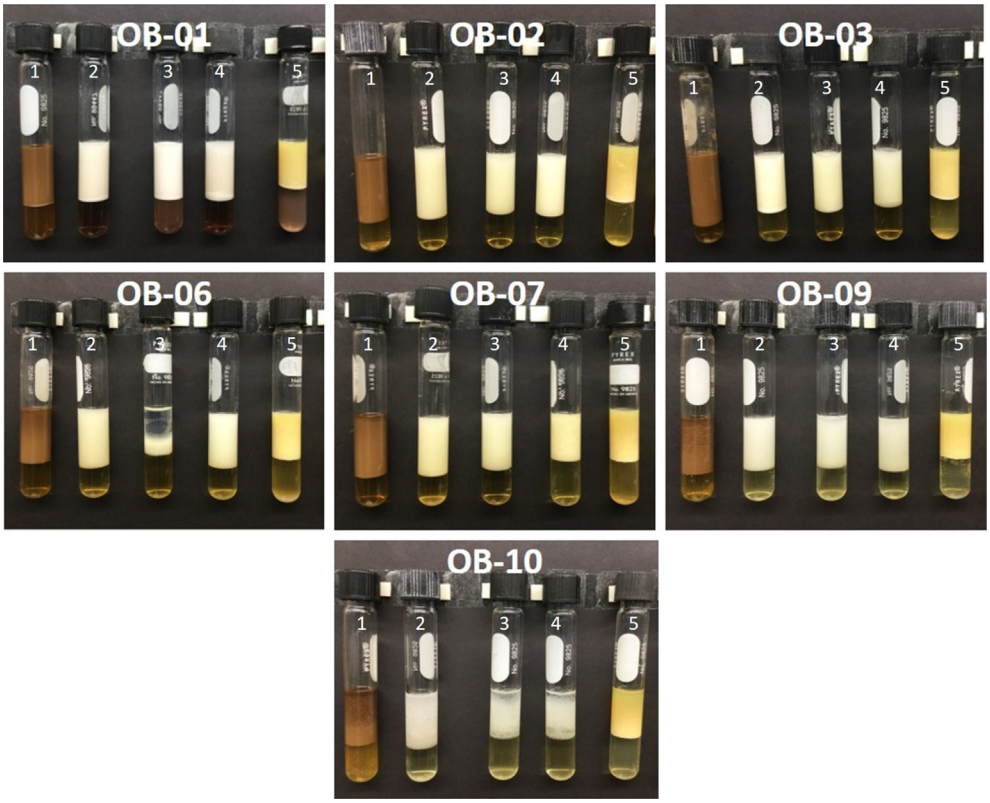


Fig. 2. Emulsifications test for each bacterial strain. 1) Burned oil, 2) Hexadecane, 3) Diesel, 4) Xylene, and 5) Motor oil.

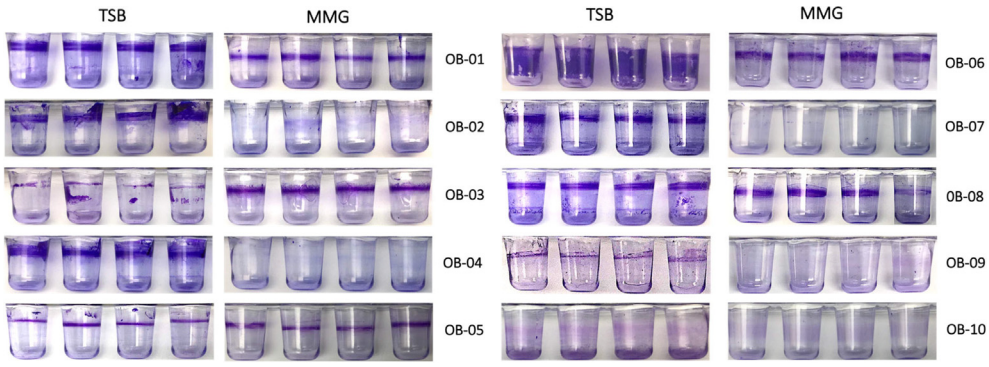


Fig. 3. Biofilm formation assay of strains from contaminated soil.

3.3. Microbial consortia and microcosm assays

Based on the results (Table 1, Fig. 2 and Fig. 3), the OB-01, OB-02, and OB-03 strains were selected for the formation of four consortia (C) and the evaluation of biodegradation process in microcosm: C1 (OB-01: OB-02), C2 (OB-01: OB-02: OB-03), C3 (OB-01: OB-03), and C4 (OB-02: OB-03). The humidity increased as a function of time during the first five days until it remained stable between 90 and 100%; the percentages were similar in all treatments, except on day 20, when the control registered a value lower than 75%. In temperature, a greater variation of the microenvironments was observed than in humidity; the consortiums and the individual treatments showed similar behavior, finding temperature values between 28 to 36°C.

The evaluated gases were carbon dioxide (CO_2), nitrogen oxides (NO_x), and carbon monoxide (CO). Fig. 4a shows the significant gas variation; the C2 consortium has a higher CO_2 release rate; therefore, this would be expected to be the most effective treatment in the degradation of the pollutant. Fig. 4b shows the NO_x values obtained as a function of time, with the C2 consortium corresponding to the highest values. The values corresponding to CO are shown in Fig. 4c finding that consortia 2 (OB-01: OB-02: OB-03) and 3 (OB-01 and OB-03) have a higher production of CO between days 30 and 40. For all three gases, the lowest values correspond to the triplicate tests without inoculating.

The removable material with hexane (RMH) and the heavy fraction hydrocarbons (HFH) were quantified at the beginning and end of the experiment to determine the hydrocarbon degradation

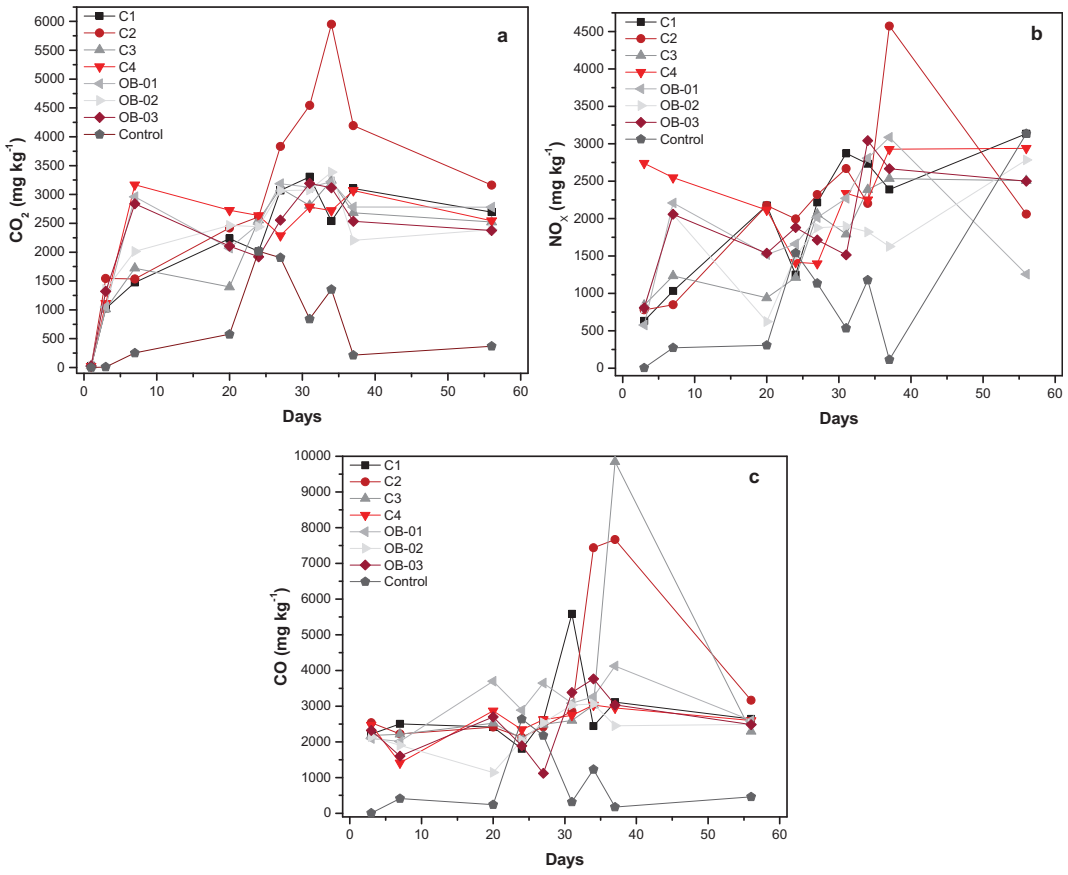


Fig. 4. (a) CO_2; (b) NO_X; and (c) CO concentration in mg kg^{-1} for 56 days.

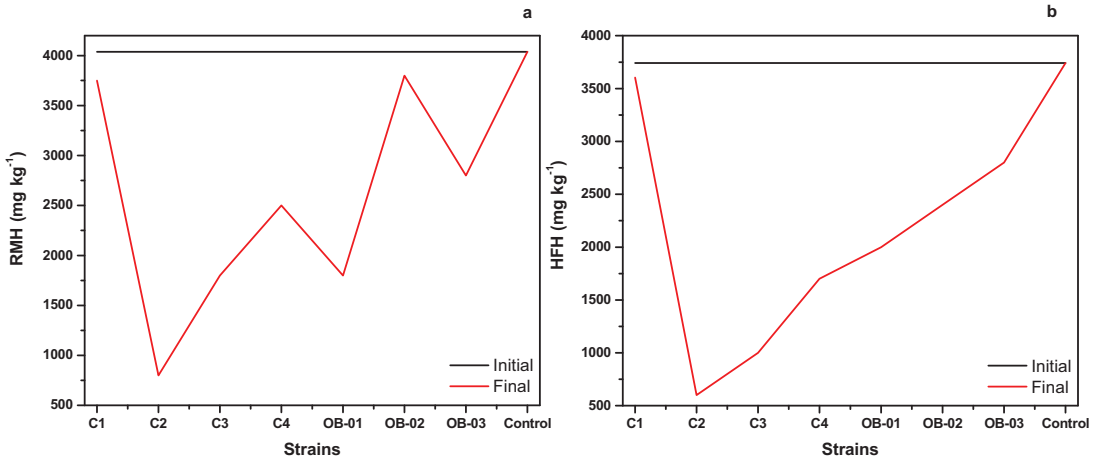


Fig. 5. (a) RMH and (b) HFH at the beginning (day 1) and the end (day 56) by microbial consortia and individual treatments.

Table 3
RMH and HFH degradation efficiencies per treatment.

Strains or Consortium	RMH	HFH
C1 (OB-01: OB-02)	7%	4%
C2 (OB-01: OB-02: OB-03)	80%	84%
C3 (OB-01: OB-03)	55%	73%
C4 (OB-02: OB-03)	38%	55%
OB-01	55%	47%
OB-02	6%	36%
OB-03	31%	25%
Control	0%	0%

process by the different treatments. The results are shown in Fig. 5. Although in the CTAB and drop-collapse tests, good results of biosurfactant activity were observed by the OB-01 and OB-02 strains, when they integrate the consortium C1, they do not present an efficient degradation of RMH and HFH, finding the results lower among all consortiums and even in individual treatments. Significant differences between the HFH dry weight at the experiment's beginning and end were found. Table 3 shows the degradation efficiencies of each treatment. Consistent with what was observed in gas monitoring, the C2 consortium showed the best results in both MEH and HFH decreases.

4. Discussion

Polycyclic aromatic hydrocarbons (PAHs) represent a unique class of petroleum hydrocarbons due to their pyrogenic nature and the complexity of their chemical structure. There are several reports of bioremediation of high-molecular-weight polyaromatic hydrocarbons (PAHs); such studies can help adopt efficient and predictable bioremediation strategies [11,22,23]. The most reported strains with biosurfactant activity in the literature are *Pseudomonas* spp., *Ochrobactrum* spp., and *Bacillus* spp. [24,25,26]. Hydrocarbon contamination has been shown to increase the EC of soil due to the presence of inorganic salts and microbial activity [27]. Therefore, soils contaminated with hydrocarbons are usually saline soils where *Pseudomonas* spp. have shown a high capacity for tolerance to salt [28], and in this study, a concentration of NaCl 5% was very well tolerated. In addition to that, *Pseudomonas* species can generally degrade hydrocarbons at different pH values [29].

Axenic cultures were characterized by various methods to determine their potential bioremediation of oil-contaminated soils. The drop-collapse method is a sensitive and easy assay to evaluate biosurfactant production [30,31]. Therefore, drops of CFS on a solid microplate surface would result in either stable or spreading, or even collapsing droplets depending on the presence of biosurfactant. The droṕs stability depends on the biosurfactant concentration and correlates with surface and interfacial tension [32]. Therefore, the high values of biosurfactants activity found in methods of microplate and parafilm collapsed droplet tests correspond to the OB-01, OB-02, OB-03, and OB-06 strains, which are phylogenetically related to the genus *Pseudomonas*. Similar activity has been reported for the PP3 and PP4 strains of *P. aeruginosa* by the drop-collapse method, with emulsifying activity values (E24) of 42% and 57%, respectively, using crude oil as substrate [33].

The emulsification activity or index (EI_{24}) was used to characterize emulsifying properties of biosurfactants; it is often used as an indirect method to screen biosurfactant production [34]. In general, OB-01, OB-02, and OB-03 show the best emulsifying results 53–77% in different hydrocarbon substrate. Comparable results with species of *Pseudomonas* have been previously reported [6], showing high emulsifying activity with petroleum (70%) and diesel (80%); Aparna et al. [35] reported an EI_{24} of 69% for hexadecane. Also, Morales-Guzmán et al. [17] report an EI_{24} of 74.2% in diesel from bacteria isolated from contaminated sites in Tabasco. Emulsification indices and stability vary according to the substrate used. Monteiro et al. [36] purified the DAUPE 614 surfactant produced by *P. aeruginosa*, which showed an EI_{24} of 70% in kerosene and 86.4% in toluene and behaved differently in each compound; in kerosene, it maintained stability for 30 days. However, in toluene, the 30-day emulsification decreased by at least 20% when compared with EI_{24}, which is considered a stable compound with potential applications in bioremediation [37].

In the hemolytic and biosurfactant activity tests, the OB-08 and OB-04 strains did not show activity, while OB-05, OB-07, OB-08, OB-09, and OB-10 strains showed hemolytic activity but no biosurfactant, and the OB-01 strain showed biosurfactant activity but no hemolytic activity. Chandankere et al. [38] recommend using hemolytic activity only as a primary method for detecting biosurfactant-producing strains because the hemolysis phenomenon can be more complex and be associated with the production of other metabolites or enzymes possibly related to the antimicrobial activity; therefore, our results indicate that 60% of the strains produce biosurfactants.

The blue agar technique is widely used to detect extracellular rhamnolipids and other anionic glycolipids [39]. This technique is based on the property of anionic surfactants in aqueous solutions to form pairs of insoluble ions when interacting with cationic substances. As a result, precipitates of pairs of insoluble ions with cetyltrimethylammonium bromide and methylene blue are formed on the agar plate, which gives a dark blue color against the light blue background. In addition, various authors have observed that there is a relationship between the diameter of the dark blue region and the concentration of the rhamnolipid biosurfactants produced; hence, it is a semiquantitative test [39], which allows selecting those strains that produce anionic biosurfactants of those that do not produce them [40,41]. Biosurfactants can produce up to four distinct types of rhamnolipids [42]. Rhamnolipid-containing glycolipid is a crystalline acid composed of β-hydroxy fatty acids and connected to a carboxyl terminus of a rhamnose sugar. It is produced mainly by *Pseudomonas* spp. Some bacteria can produce only mono-rhamnolipids and less frequently produce only di-rhamnolipids, while very few bacteria produce both mono- and di-rhamnolipids [43]. In that sense, the anionic surfactants have the greatest foaming, emulsifying, wetting properties, excellent emulsification properties, efficiently remove crude oil from contaminated soil, and facilitate oil spills [44,45], if compared with the other types of biosurfactants.

The formation of biofilms and cell aggregates is a style of adherence and survival for many bacteria; the further growth of the biofilm depends on the rate of colonization of microorganisms, in addition to being essential for the biodegradation of heavy hydrocarbon compounds, due to the association between degradation and the production of extracellular polymeric substances (EPS) [46,47]. The EPS promotes the establishment process on the surface and has various compositions that include exopolysaccharides, nucleic acids, proteins, glycoproteins, and phospholipids [47]. All strains isolated from oil-contaminated soil show a good capacity for biofilm formation, which places them at an advantage during the degradation process.

Due to the increase in the concentration of hydrocarbons by oil spills, environmental stressors increase at the site; therefore, the biofilm formation by the endemic microorganisms of the site is crucial to protect the cells from the stressful environment and to be able to use its metabolic machinery efficiently for the degradation of compounds. In the process of biofilm formation, the presence of rhamnolipids is essential for the initial colonization, formation of channels in the biofilm, and the dispersion of microbial cells; they are also excellent biosurfactants that reduce the surface tension of hydrocarbons, even a higher percentage of remediation than commercial surfactants [48].

In microbial consortia of *Pseudomonas stutzeri* and *Acinetobacter baumannii*, a percentage of PAHs degradation of 52.6% was reached; however, by adding biosurfactants produced by *Bacillus subtilis* and iron nanoparticles, the percentage of degradation was greater than 85% [49]. The consortia (except C1) have HFH degradation efficiencies of 55–84%, while in individual cultures was of 25–47%. Ghazali et al. [50] obtained comparable results *in vitro* oil degradation test for 60 d; the consortium with the highest number of strains had a 43% degradation for aliphatic compounds compared to the consortium with fewer strains. Varjani et al. [1] reported reductions of 92.97% in 56 days of pollutants using a consortium with *P. aeruginosa*, and the hydrocarbons were quantifiers by the gravimetric method. In other studies, Elumalai et al. [51] reported high percentages of enzymatic biodegradation of long-chain hydrocarbons using thermophilic bacteria (*Geobacillus thermoparaffinivorans* IR2, *Geobacillus stearothermophillus* IR4, and *Bacillus licheniformis* MN6) in axenic cultures and mixed consortia, finding better results (90%) for the mixed consortia compared to the axenic cultures in C32 alkanes; while for C40 alkanes, a greater degradation was obtained with pure strains.

The OB-02 strain results indicate that, even though some microorganisms in the consortium do not have excellent degrada-

tive efficiency, they fulfill their function within the microbial consortium, facilitating the degradation. In this process, gaseous byproducts of microbial metabolism, such as H_2S, SO_2, and CO_2 are often generated [52]; therefore, the gas concentration in the treatment is proportional to the degradative capacity of each consortium; the treatments without inoculation had the lowest CO_2 concentrations, and the C2 consortium, made up of the three strains, had the highest. The CO_2 detachment is closely related to the degradation process and can be considered a parameter sensitive to the changes that occur in the transformation process, mainly during mineralization [53]. NO_X emission is common in soils due to the processes of mineralization of organic matter [54]; the highest values of CO_2 and NO_X were those of the C2 consortium, followed by the C3 on day 34. On the other hand, the microbial activity can be considered proportional to the CO detachment; hence, the greater the amount of compound released, the greater the microbial activity occurs [55], the highest release of CO was recorded on day 37.

The obtained results by the OB-01 and OB-02 strains in the characterization tests indicate a good biosurfactant and emulsifying activity. Although both belong to the same species, in the C1 consortium, the degradation process was inefficient, which can occur in an antagonistic process between the strains due to the metabolic compounds they produce, as reflected in the lack of degradative capacity; however, in other consortia, it was observed that they produce substances that help in the degradation process, as reported by Röling and Van Bodegom [56]. Except for this consortium, the remaining three had a more significant decrease in HFH than axenic cultures; in microbial consortia, the degradation processes have greater efficiency than axenic cultures since an individual population demands more resources and energy than a mixed population that ideally distributes the work. These results show that microbial consortia in the degradation processes had higher efficiency than axenic cultures because an individual strain demands more resources and energy than a mixed population that ideally distributes the functional and metabolic work.

5. Conclusions

In this study, bacteria with potential degradation isolated from soil contaminated with hydrocarbons were characterized, which according to the 16S rDNA gene analysis, mostly *Pseudomonas* species. The ability of isolated bacteria to express biosurfactant and bioemulsifying properties was demonstrated. Emulsifications for burned and motor oil were greater than 50% in the strains positive. Anionic biosurfactants were detected for the three strains that made up the consortium and the formation of biofilms in vitro. Even though five strains showed high similarity (<98%) with *P. aeruginosa*, the results show high metabolic variability between the strains with different hemolytic, biosurfactant, bioemulsifying activity, anionic surfactant, and biofilms formation. In microcosm assays, the consortia presented high values in the degradation efficiency of RMH and HFH compared to the individual treatments and the C1 consortium. In addition, a correlation was observed between the hydrocarbon degradation process and microbial respiration in the consortia. Therefore, our results suggest that although the strains belong to the same species, the formation of microbial consortia can contribute to the remediation process of soil contaminated by hydrocarbons.

Author contributions

- Study conception and design: S Lázaro-Mass; S Gómez-Cornelio; S De la Rosa-García

- Data collection: S Lázaro-Mass; M Castillo-Vidal; CS Alvarez-Villagomez

- Analysis and interpretation of results: S Lazaro-Mass; S Gómez-Cornelio; P Quintana; S De la Rosa-García

- Draft manuscript preparation: S Lázaro-Mass; S Gómez-Cornelio; S De la Rosa-García

- Revision of the results and approved the final version of the manuscript: S Lázaro-Mass; S Gómez-Cornelio; M Castillo-Vidal; CS Alvarez-Villagomez; P Quintana; S Gómez-Cornelio

Financial support

This research work was financially supported by Consejo Nacional de Ciencia y Tecnología (CONACYT, MX), Grant No 283643.

Conflicts of interest

The authors declare no conflict of interest.

References

[1] Varjani SJ, Gnansounou E, Pandey A. Comprehensive review on toxicity of persistent organic pollutants from petroleum refinery waste and their degradation by microorganisms. Chemosphere 2017;188:280–91. https://doi.org/10.1016/j.chemosphere.2017.09.005. PMid: 28888116.

[2] Mnif I, Sahnoun R, Ellouz-Chaabouni S, et al. Application of bacterial biosurfactants for enhanced removal and biodegradation of diesel oil in soil using a newly isolated consortium. Process Saf Environ Prot 2017;109:72–81. https://doi.org/10.1016/j.psep.2017.02.002.

[3] Patel AB, Shaikh S, Jain KR, et al. Polycyclic aromatic hydrocarbons: Sources, toxicity, and remediation approaches. Front Microbiol 2020;11:562813. https://doi.org/10.3389/fmicb.2020.562813. PMid: 33224110.

[4] Pacwa-Płociniczak M, Płaza GA, Piotrowska-Seget Z, et al. Environmental applications of biosurfactants: recent advances. Int J Mol Sci 2011;12 (1):633–54. https://doi.org/10.3390/ijms12010633. PMid: 21340005.

[5] Samsu ZA, Jeffry FN, Azizan WN. Isolation and screening of potential biosurfactant-producing bacteria from used engine oil-contaminated soil. Mater Today: Proc 2020;31(1):A67–71. https://doi.org/10.1016/j.matpr.2020.12.438.

[6] Varjani SJ. Microbial degradation of petroleum hydrocarbons. Bioresour Technol 2017;223:277–86. https://doi.org/10.1016/j.biortech.2016.10.037. PMid: 27789112.

[7] Patowary K, Patowary R, Kalita M, et al. Development of an efficient bacterial consortium for the potential remediation of hydrocarbons from contaminated sites. Front Microbiol 2016;7:1092. https://doi.org/10.3389/fmicb.2016.01092. PMid: 27471499.

[8] Großkopf T, Soyer OS. Synthetic microbial communities. Curr Opin Microbiol 2014;18:72–7. https://doi.org/10.1016/j.mib.2014.02.002. PMid: 24632350.

[9] Daniels WL, Zipper CE, Orndorff ZW, et al. Predicting total dissolved solids release from central Appalachian coal mine spoils. Environ Pollut 2016;216:371–9. https://doi.org/10.1016/j.envpol.2016.05.044. PMid: 27323343.

[10] Weisburg WG, Barns SM, Pelletier DA, et al. 16s ribosomal DNA amplification for phylogenetic study. J Bacteriol 1991;173:697–703. https://doi.org/10.1128/jb.173.2.697-703.1991. PMid: 1987160.

[11] Meliani A, Bensoltane A. Enhancement of hydrocarbons degradation by use of Pseudomonas biosurfactants and biofilms. J Pet Environ Biotechnol 2014;5 (1):168. https://doi.org/10.4172/2157-7463.1000168.

[12] Ferreira INS, Rodríguez DM, Campos-Takaki GM, et al. Biosurfactant and bioemulsifier as promising molecules produced by *Mucor hiemalis* isolated from Caatinga soil. Electron J Biotechnol 2020;47:51–8. https://doi.org/10.1016/j.ejbt.2020.06.006.

[13] Cooper DG, Goldenberg BG. Surface-active agents from two *Bacillus* species. Appl Environ Microbiol 1987;53:224–9. https://doi.org/10.1128/aem.53.2.224-229.1987. PMid: 16347271.

[14] Boraha D, Yadavb RNS. Bioremediation of petroleum-based contaminants with biosurfactant produced by a newly isolated petroleum oil degrading bacterial strain. Egypt J Pet 2017;26(1):181–8. https://doi.org/10.1016/j.ejpe.2016.02.005.

[15] Ron EZ, Rosenberg E. Role of Biosurfactants. In: Steffan R, editor. Consequences of microbial interactions with hydrocarbons, oils, and lipids: Biodegradation and Bioremediation. Handbook of Hydrocarbon and Lipid Microbiology. Cham: Springer; 2019. https://doi.org/10.1007/978-3-319-50433-9_6.

[16] Shah N, Nikam R, Gaikwad S, et al. Biosurfactant: Types, detection methods, importance, and applications. Indian J Microbiol 2016;3(1):5–10. https://doi.org/10.5958/2394-5478.2016.00002.9.

[17] Morales-Guzmán D, Martínez-Morales F, Bertrand B, et al. Microbial prospection of communities that produce biosurfactants from the water column and sediments of the Gulf of Mexico. Biotechnol Appl Biochem 2020;68(6):1202–15. https://doi.org/10.1002/bab.2042. PMid: 32969539.
[18] Meylheuc T, van Oss CJ, Bellon-Fontaine MN. Adsorption of biosurfactant on solid surfaces and consequences regarding the bioadhesion of *Listeria monocytogenes* LO28. J Appl Microbiol 2001;91(5):822–32. https://doi.org/10.1046/j.1365-2672.2001.01455.x. PMid: 11722659.
[19] Singh P, Tiwary BN. Isolation, and characterization of glycolipid biosurfactant produced by a *Pseudomonas otitidis* strain isolated from Chirimiri coal mines, India. Bioresour Bioprocess 2016;3:42. https://doi.org/10.1186/s40643-016-0119-3.
[20] Qi L, Li H, Zhang C, et al. Relationship between antibiotic resistance, biofilm formation, and biofilm-specific resistance in *Acinetobacter baumannii*. Front Microbiol 2016;7:483. https://doi.org/10.3389/fmicb.2016.00483.
[21] Boulding JR. Description and sampling of contaminated soils: A field manual. 2nd ed. CRC Press, Routledge; 2017. p. 238. ISBN 0367449536.
[22] Chandra S, Sharma R, Singh K, et al. Application of bioremediation technology in the environment contaminated with petroleum hydrocarbon. Ann Microbiol 2013;63:417–31. https://doi.org/10.1007/s13213-012-0543-3.
[23] Rajasekar A. Biodegradation of petroleum hydrocarbon and its influence on corrosion with special reference to petroleum industry. In: Heimann K, Karthikeyan O, Muthu S, editors. Biodegradation and bioconversion of hydrocarbons. Environmental footprints and eco-design of products and processes. Singapore: Springer; 2017. p. 307–36. https://doi.org/10.1007/978-981-10-0201-4_9.
[24] Kumar CG, Sujitha P, Mamidyala SK, et al. Ochrosin, a new biosurfactant produced by halophilic *Ochrobactrum* sp. strain BS-206 (MTCC 5720): purification, characterization, and its biological evaluation. Process Biochem 2014;49(10):1708–17. https://doi.org/10.1016/j.procbio.2014.07.004.
[25] Barros FFC, Simiqueli APR, de Andrade CJ, et al. Production of enzymes from agroindustrial wastes by biosurfactant-producing strains of *Bacillus subtilis*. Biotechnol Res Int 2013;2013:103960. https://doi.org/10.1155/2013/103960. PMid: 23533780.
[26] Patowary K, Patowary R, Kalita MC, et al. Characterization of biosurfactant produced during degradation of hydrocarbons using crude oil as sole source of carbon. Front Microbiol 2017;8:279. https://doi.org/10.3389/fmicb.2017.00279. PMid: 28275373.
[27] Liu L, Li W, Song W, et al. Remediation techniques for heavy metal-contaminated soils: Principles and applicability. Sci Total Environ 2018;633:206–19. https://doi.org/10.1016/j.scitotenv.2018.03.161. PMid: 29573687.
[28] Zhang X, Bao D, Li M, et al. Bioremediation of petroleum hydrocarbons by alkali–salt-tolerant microbial consortia and their community profiles. J Chem Techon Biotechnol 2021;96(3):809–17. https://doi.org/10.1002/jctb.6594.
[29] Rajasekar A, Maruthamuthu S, Ting YP. Bacterial degradation of petroleum hydrocarbons. In: Singh S, editor. Microbial degradation of xenobiotics. Environ Sci Eng. Berlin, Heidelberg: Springer; 2012. p. 307–36. https://doi.org/10.1007/978-3-642-23789-8_13.
[30] Bodour AA, Drees KP, Maier RM. Distribution of biosurfactant producing bacteria in undisturbed and contaminated arid southwestern soils. Appl Environ Microbiol 2003;69(6):3280–7. https://doi.org/10.1128/AEM.69.6.3280-3287.2003. PMid: 12788727.
[31] Płaza G, Zjawiony I, Banat IM. Use of different methods for detection of thermophilic biosurfactant-producing bacteria from hydrocarbon-contaminated and bioremediated soil. J Petrol Sci Eng 2006;50(1):71–7. https://doi.org/10.1016/j.petrol.2005.10.005.
[32] Morikawa M, Hirata Y, Imanaka T. A study on the structure–function relationship of lipopeptide biosurfactants. Biochim Biophys Acta 2000;1488 (3):211–8. https://doi.org/10.1016/S1388-1981(00)00124-4.
[33] Muthukumar M, Parthipan P, AlSalhi MS, et al. Characterization of bacterial community in oil-contaminated soil and its biodegradation efficiency of high molecular weight (>C40) hydrocarbon. Chemosphere 2022;289:133168. https://doi.org/10.1016/j.chemosphere.2021.133168. PMid: 34890617.
[34] Pele MA, Ribeaux DR, Vieira ER, et al. Conversion of renewable substrates for biosurfactant production by *Rhizopus arrhizus* UCP 1607 and enhancing the removal of diesel oil from marine soil. Electron J Biotechnol 2019;38:40–8. https://doi.org/10.1016/j.ejbt.2018.12.003.
[35] Aparna A, Srinikethan G, Smitha H. Production, and characterization of biosurfactant produced by a novel *Pseudomonas* sp. 2B. Colloids Surf B Biointerfaces 2012;95:23–9. https://doi.org/10.1016/j.colsurfb.2012.01.043. PMid: 22445235.
[36] Monteiro SA, Sassaki GL, de Souza LM, et al. Molecular and structural characterization of the biosurfactant produced by *Pseudomonas aeruginosa* DAUPE 614. Chem Phys Lipids 2007;147(1):1–13. https://doi.org/10.1016/j.chemphyslip.2007.02.001. PMid: 17382918.
[37] Varjani SJ, Upasani VN. Biodegradation of petroleum hydrocarbons by oleophilic strain of *Pseudomonas aeruginosa* NCIM 5514. Bioresour Technol 2016;222:195–201. https://doi.org/10.1016/j.biortech.2016.10.006. PMid: 27718402.
[38] Chandankere R, Yao J, Choi M, et al. An efficient biosurfactant-producing and crude-oil emulsifying bacterium *Bacillus methylotrophicus* USTBa isolated from petroleum reservoir. Biochem Eng J 2013;74:46–53. https://doi.org/10.1016/j.bej.2013.02.018.
[39] Rekadwad B, Maske V, Khobragade CN, et al. Production and evaluation of mono- and di-rhamnolipids produced by *Pseudomonas aeruginosa* VM011. Data Br 2019;24:103890. https://doi.org/10.1016/j.dib.2019.103890. PMid: 31011602.
[40] Chong H, Li Q. Microbial production of rhamnolipids: opportunities, challenges, and strategies. Microb Cell Factories 2017;16:137. https://doi.org/10.1186/s12934-017-0753-2. PMid: 28779757.
[41] Wild M, Caro AD, Hernandez AL, et al. Selection and partial characterization of a *Pseudomonas aeruginosa* mono-rhamnolipid deficient mutant. FEMS Microbiol Lett 1997;153(2):279–85. https://doi.org/10.1111/j.1574-6968.1997.tb12586.x. PMid: 9271853.
[42] Thakur P, Saini NK, Thakur VK, et al. Rhamnolipid the glycolipid biosurfactant: emerging trends and promising strategies in the field of biotechnology and biomedicine. Microb Cell Factories 2021;20:1. https://doi.org/10.1186/s12934-020-01497-9. PMid: 33397389.
[43] Vaishnavi J, Parthipan P, Prakash AA, et al. Biosurfactant-assisted bioremediation of crude oil/petroleum hydrocarbon contaminated soil. In: Prasad MNV, editor. Handbook of Assisted and amendment: enhanced sustainable remediation technology, vol. 1. John Wiley & Sons; 2021. p. 313–29. https://doi.org/10.1002/9781119670391.ch16.
[44] Patowary K, Saikia RR, Kalita MC, et al. Degradation of polyaromatic hydrocarbons employing biosurfactant-producing *Bacillus pumilus* KS2. Ann Microbiol 2015;65:225–34. https://doi.org/10.1007/s13213-014-0854-7.
[45] Karlapudi AP, Venkateswarulu TC, Tammineedi J, et al. Role of biosurfactants in bioremediation of oil pollution-a review. Petroleum 2018;4(3):241–9. https://doi.org/10.1016/j.petlm.2018.03.007.
[46] Li W, Yu HQ. Insight into the roles of microbial extracellular polymer substances in metal biosorption. Bioresour Technol 2014;160:15–23. https://doi.org/10.1016/j.biortech.2013.11.074. PMid: 24345430.
[47] Parthipan P, Elumalai P, Karthikeyan OP, et al. A review on biodegradation of hydrocarbon and their influence on corrosion of carbon steel with special reference to petroleum industry. J Environ Biotechnol Res 2017;6(1):12–33.
[48] Sharma D, Ansari MJ, Al-Ghamdi A, et al. Biosurfactant production by *Pseudomonas aeruginosa* DSVP20 isolated from petroleum hydrocarbon-contaminated soil and its physicochemical characterization. Environ Sci Pollut Res 2015;22:17636–43. https://doi.org/10.1007/s11356-015-4937-1. PMid: 26146372.
[49] Parthipan P, Cheng L, Dhandapani P, et al. Impact of biosurfactant and iron nanoparticles on biodegradation of polyaromatic hydrocarbons (PAHs). Environ Pollut 2022;306:119384. https://doi.org/10.1016/j.envpol.2022.119384. PMid: 35504349.
[50] Ghazali FM, Rahman RNZA, Salleh AB, et al. Biodegradation of hydrocarbons in soil by microbial consortium. Int Biodeterior Biodegr 2004;54(1):61–7. https://doi.org/10.1016/j.ibiod.2004.02.002.
[51] Elumalai P, Parthipan P, Karthikeyan OP, et al. Enzyme-mediated biodegradation of long-chain *n*-alkanes (C_{32} and C_{40}) by thermophilic bacteria. 3. Biotech 2017;7:116. https://doi.org/10.1007/s13205-017-0773-y. PMid: 28567628.
[52] Rajasekar A, Balasubramanian R, Kuma JVM. Role of hydrocarbon degrading bacteria *Serratia marcescens* ACE2 and *Bacillus cereus* ACE4 on corrosion of carbon steel API 5LX. Ind Eng Chem Res 2011;50(17):10041–6. https://doi.org/10.1021/ie200709q.
[53] Mortazavi B, Horel A, Beazley MJ, et al. Intrinsic rates of petroleum hydrocarbon biodegradation in Gulf of Mexico intertidal sandy sediments and its enhancement by organic substrates. J Hazard Mater 2013;244–245:537–44. https://doi.org/10.1016/j.jhazmat.2012.10.038. PMid: 23228451.
[54] Sánchez-García M, Alburquerque JA, Sánchez-Monedero MA, et al. Biochar accelerates organic matter degradation and enhances N mineralization during composting of poultry manure without a relevant impact on gas emissions. Biotechnol Technol 2015;192:272–9. https://doi.org/10.1016/j.biortech.2015.05.003. PMid: 26038333.
[55] Guerrero-Ortiz P, Quintero-Lizaola R, Espinoza-Hernández V, et al. Respiración de CO2 como indicador de la actividad microbiana en abonos orgánicos de lupinus. Terra Latinoam 2012;30:355–62.
[56] Röling WFM, Van Bodegom PM. Toward quantitative understanding on microbial community structure and functioning: A modeling-centered approach using degradation of marine oil spills as example. Front Microbiol 2014;5:125. https://doi.org/10.3389/fmicb.2014.00125. PMid: 24723922.