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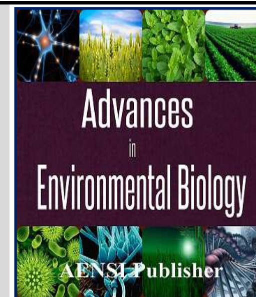

Biodegradation of diesel and isomerate by *pseudomonas aeruginosa* and *Brevibacillus laterosporus* isolated from hydrocarbons contaminated soil.

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ABSTRACT

Biodegradation of petroleum hydrocarbons is more effective, powerful and economical provident than physical and chemical techniques. The most important process uses newly isolated indigenous microorganisms from contaminated sites. Our study aims to isolate, characterize the diversity of microbial communities specifically involved in the biodegradation of diesel within polluted soil. Two bacterial strains were isolated from hydrocarbons-contaminated soils collected from the refinery of Arzew, Northern Algeria, using the enrichment technique. The morphological characteristics of the colonies, the cell structure, Gram staining and the observation of the bacterial spore were studied, the biochemical characterization were performed by the use of biochemical galleries. The bacterial strains were identified as *Pseudomonas aeruginosa* (P1) and *Brevibacillus laterosporus* (B2). The ability of *P. aeruginosa* (P1) and *B. laterosporus* (B2) to degrade diesel was performed by measuring the optical density, colony forming unit counts (CFU/ml), gravimetric method. The degradation of Isomerate by the two isolates was analyzed by gas chromatography with flame ionization detector (FID). Results indicated that the isolates can use diesel as sole source of carbon. Biodegradation of diesel by P1 and B2was indicated by an increase in the optical density of culture and colony forming unitcounts (CFU/ml)from $7\times10^4$and 2.3×10^3 CFU/ml to 5.9×10^9 CFU/ml and 3.4×10^8 CFU/ml on day 8 respectively. The biodegradation potential of diesel was 20% (for P1) and51.35% (for B2) during 12 days of incubation.Isolate P1have the highest capability of diesel degradation, thiswas consistent with the complete degradation in five days of four components of Isomerate(C3, I-C4, 2-3dimethylbutane and toluene) and more than 90% of 8 other components (I-C5, cyclopentane, 2methylpentane, 3methylpentane, hexane, benzene, Et. Benzene and O. Xylene). Isolate B2 completely degraded hexane and more than 80% of C3, I-C4, 2 methylpentane and toluene.

KEYWORDS: Biodegradation, diesel, Isomerate, *Pseudomonas aeruginosa, Brevibacillus laterosporus*.

INTRODUCTION

One of the major environmental problems today is hydrocarbon contamination resulting from the activities related to the petrochemical industry [31]. Hydrocarbon components are imposing serious threats to human health and are constantly affecting the surrounding environment due to their prolonged existence in the environment [17]. Hydrocarbons compounds belong to organic compounds with strong toxic, mutagenic, carcinogenic properties and can accumulate in the environment with persistence. Most PAHs (about 90 %) are accumulated in soil, which receives PAHs from combustion of wood and fossil fuels, emission of fumes from

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motor vehicles, or soil application of sewage sludge Despite such high accumulation of PAHs in soil, it is possible to remove these contaminants with increasingly advanced methods, including physicochemical, chemical, photocatalytic degradation , electrokinetic remediation , thermal , or biological pathway. Among the latter methods, next to phytoremediation, most attention is paid to bioremediation, as it is perceived to be a safe and economically viable method [26].

Biodegradation using microorganisms is usually the preferred and major route of hydrocarbons removal from contaminated environments because of its cost effectiveness and complete clean-up [23]. Bacteria and fungi are primary mediators in hydrocarbon degradation; bacteria have been shown to be more versatile than fungi and therefore may play a greater role during biodegradation of hydrocarbons [8], 79 bacterial genera that can use hydrocarbons as carbon and energy sources, as well as 9 cyanobacterial genera, 103 fungal genera and 14 algalgenera that are able to degrade or transform hydrocarbons [19]. Bacteria and fungi are primary mediators in hydrocarbon degradation; bacteria have played a greater role during biodegradation of hydrocarbons. The most important hydrocarbon-degrading bacterial genera in soil environments include *Achromobacter*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Collimonas*, *Corynebacterium*, *Dietzia*, *Flavobacterium*, *Gordonia*, *Micrococcus*, *Mycobacterium*, *Nocardia*, *Nocardioides*, *Pseudomonas*, *Ralstonia*, *Rhodococcus*, *Sphingomonas*, *Variovorax*. Among the fungi, *Aspergillus*, *Candida*, *Cunninghamella*, *Fusarium*, *Mucor Penicillium*, *Phanerochaete Rhodotorula*, *Sporobolomyces* and *Trichoderma* are hydrocarbon degrading genera frequently isolated from soil [8].

Various studies haveisolated microorganisms able to degrade oil compounds from different locations in Algeria, including *Corynebacterium aquaticum* and *Pseudomonas aeruginosa* [2], *Natrialba* sp. [24], *Pseudomonas*, *Shewanella*, *Enterobacter* and *Serratia* [18], *Paenibacillus* sp [28] and *Pseudomonas citronellolis* KHA [36]. But there are not enough reports of biodegradation of Isomerate (Naphtha petroleum, Swigert et al. [39], which is a complex combination of hydrocarbons obtained by the fractional distillation of petroleum. This fraction boils in a range of approximately 20–135 °C [39]. The aim of the present work was to isolate the hydrocarbon degrading bacteria from oil-contaminated soil. In addition, the diesel degradation capacity of selected bacterial strains was assessed by measuring the optical density, colony forming unit counts (CFU/ml), gravimetric method .and the Isomerate biodegradation ability of the isolates was characterized by GC/FID.

MATERIAL AND METHODS

Soil samples:

Oil contaminated soil samples were collected from two sites in a refinery in Arzew, Northern Algeria (Sonatrach, Algerian Petroleum Company).

Isolation and characterization of microorganisms from contaminated soil:

Hydrocarbons-degrading bacteria were isolated on Bushnell-Haas medium (BH) [3], which contained per liter:1.0g KH_2PO_4,1.0gK_2HPO_4, 1.0g NH_4NO_3, 0.2g $MgSO_4 \cdot 7H_2O$, 0.05g $FeCl_3$ and 0.02g $CaCl_2 \cdot 2H_2O$ (pH 7). For enrichment, 10g contaminated soil sample was added to 100 ml of BH medium containing 2% (v/v) petroleum or diesel [15]. Cultures were incubated in the dark at 30°C on a rotary shaker 150 rpm. After one week, 2ml of enriched media was transferred into freshBH medium and incubated at the same conditions.After three consecutive transfers (each including a short incubation interval of 1 week), 100µl of culture were plated on BH agar, which were covered with 100 µl of petroleum or diesel and incubated at 30°C [11,37,18]. Colonies were isolated, purified and stored at 4° C on nutrient agar. For long duration, the isolates are stored in glycerol (30%) at -20° C.

Purecultures of bacterial isolates were identified on the basis oftheir colonial morphology, cellular morphology andbiochemical characteristicsby using API20 NE and API 20E (bioMérieux, Marcy-l'Etoile, France) according to the taxonomicscheme of Bergey's Manual of Determinative Bacteriology [14,6].

Diesel degradation assays:

Ability to degrade diesel was performed as described by [25] with modifications.Erlenmeyer Flasks of 250ml containing 100 ml of BH liquid medium with 2 ml of diesel were prepared.The isolates were incubated overnight in 5ml nutrient broth at 30°C. Then the cultures were centrifuged at 6000 rpm for 15min and cells collected. These were washed three times with BH liquid medium and re-suspended in a 5ml of the same medium [10], until OD_{600} was equivalent to 0.91 [29]. One ml of inoculum (0.91 OD_{600} equivalent) was transferred into the flasks and Non-inoculated flasks were prepared as controls [5]. The cultures were grown in the dark at 30°C for 12 days with constant shaking (160 rpm) [29].

The growth patterns were obtained by measuring the optical density at 600 nm [20] and total viable counts (CFU/ml)of the isolatesat 2 day intervals [4].

Gravimetric method:

Residual oil was extracted by liquid-liquid extraction as described by [33]. 4 ml of hexane and 2 ml of acetone were added to broth culture in flask and shaken thoroughly [11]. After removing the aqueous phase with separating funnel, hexane and acetone was evaporated using rotary evaporator at 50°C [13,27]. Control flasks were also extracted similarly. After the complete evaporation, the hydrocarbon residue obtained was weighed and taken as the gravimetric value for further calculation. The percentage of diesel oil degraded was determined from the following formula [41,33,27]:

Percentage of degraded diesel = $(W_c - W_r)/ W_c \times 100\%$
W_r: weight of residual diesel in cultures
W_c: weight of residual diesel in controls

Analysis of Isomerate biodegradation by gas chromatography:

The ability of isolates to degrade Isomerate (Naphtal Petroleum, Refinery of Arzew, SONATRACH) in BH at 30°C with agitation at 160rpm was examined. Erlenmeyer flasks containing 100ml of BH, supplemented with 2% of Isomerate, were inoculated with strains to a final OD_{600} of 0.91, parallel Erlenmeyer flasks, which were not inoculated with strains, were set up as controls to monitor loss of the hydrocarbons due to volatilization. After 5 days of incubation, inoculated flasks and un-inoculated controls were extracted [41, 32].

The extracts were analyzed by Gas Chromatography (DANI Master GC Fast Gas Chromatograph System, DANI Instruments Spa., Milan), with CP-Sil PONA CB Column (50mm, 0.21mm, 0.5µm) and Flame Ionization Detector (FID). All runs were carried out under the following conditions: initial temperature 40°C for 1min; temperature rate 10°C/min and final temperature 150°C for 1min; injector (spilt/splitless mode) temperature 250°C; FID temperature 300°C. Carrier gas constant flow rate (helium) 40ml/min.

Results:

Isolation and characterization of isolates:

Two strains were isolated from the contaminated soil (P1 and B2) with diesel degrading capabilities. The isolates were respectively identified as *Pseudomonas aeruginosa* and *Brevibacillus laterosporus*. Tables 1 and 2 show the characterization of isolates and the closest strains with similarity percentage of identity.

Table 1: characteristics of the P1 isolate from contaminated soil

	GRAM	oxidase	Catalase	Respiration	Motility	KingA	kingB	Mcc	NO3	TRP	GLU (fermentation)	ADH	URE	ESC	GEL	PNPG	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC	Tentative identity	% Similarity (%)
P1	-	+	+	Obligate aerobe	+	+	+	-	+	-	-	+	+	-	+	-	+	-	-	+	+	-	+	+	+	+	+	-	*Pseudomonas aeruginosa*	99.9

Table 2: characteristics of B2 isolate from contaminated soil

	GRAM	oxidase	Catalase	Respiration	Motility	NO3	GLU (fermentation)	ADH	URE	GEL	ONPG	ARA	MAN	LDC	ODC	TDA	IND	VP	INO	CIT	SOR	RHA	SAC	MEL	AMY	H2S	hemolysis on blood	Amylase	Lecithinase	Caseine	Spore forming	Tentative identity	% Similarity (%)
B2	+	-	+	Facultative anaerobe	+	-	+	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	Gamma	-	-	+	+	*Brevibacillus laterosporus*	94.1

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Determination of hydrocarbons degradation by turbidometry and bacterial counts:

Microorganism's growth in BH broth medium supplemented with 2% dieselwas evident from the significant increase in cell density at 600 nm and alternate colony counting after 12 days of incubation (figure1 and 2). The increase in optical density indicates the ability of cultures strains to utilize and degradediesel as source of carbon and energy(figure 1).More specifically, this was observed between day 4 and 8 after which growth became limited.

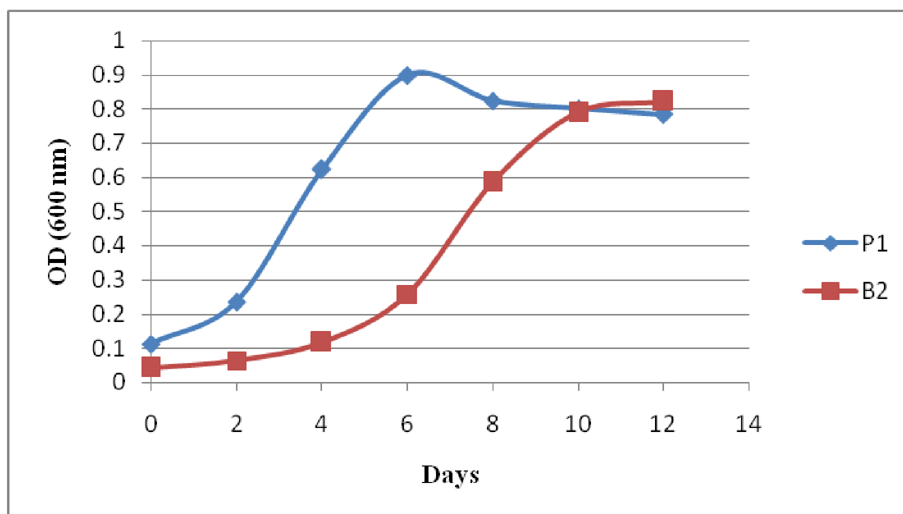


Fig. 1: Growth curve of the isolates P1 and B2 in BH broth medium supplemented with 2% diesel.

The cell count of P1 and B2 isolates on BH broth supplemented with 2% diesel increased from 7×10^4 and 2.3×10^3 CFU/ml in day 1 to 5.9×10^9 CFU/ml and 3.4×10^8 CFU/ml respectively after 8 days of incubation (figure 2).

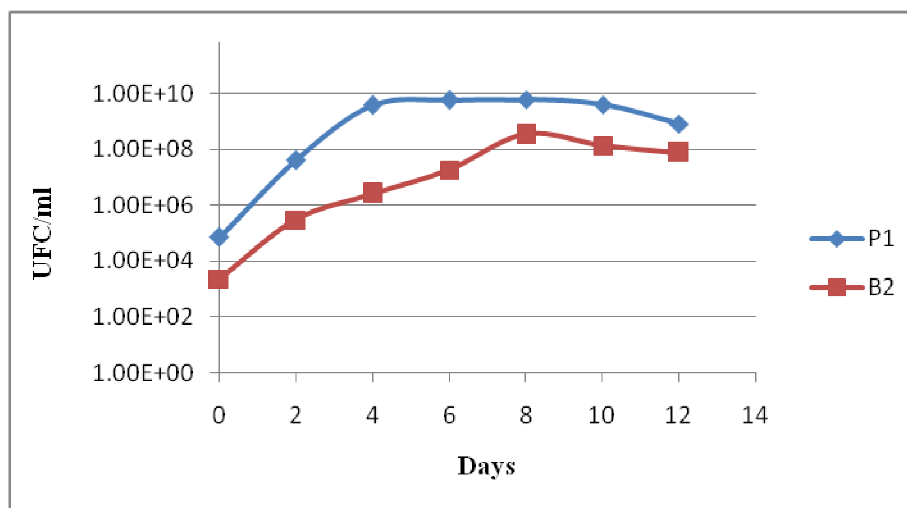


Fig. 2: Growth rate of the isolates P1 and B2 in BH broth medium supplemented with 2% diesel

Gravimetric method:

The biodegradation assay was carried out to determine the diesel degradation capabilities of the indigenous microbial cultures that were isolated from contaminated soil. The isolates were separately inoculated in BH broth containing 2% diesel as sole of carbon source to estimate the potential of biodegradation (figure 3). According to our results B2 had a high degradation rate of51.35%, compared to P1 having a degradation rate of 20%. According to Oudot et al. [33] a microorganism is considered as petroleum and diesel degrading if the rate of its degradation is superior or equal to 5%.

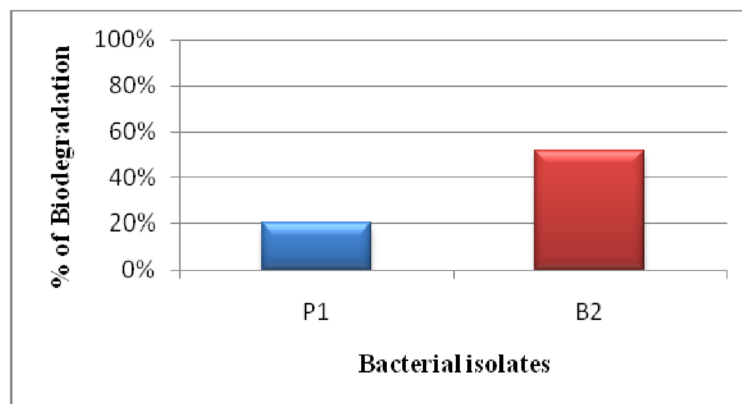
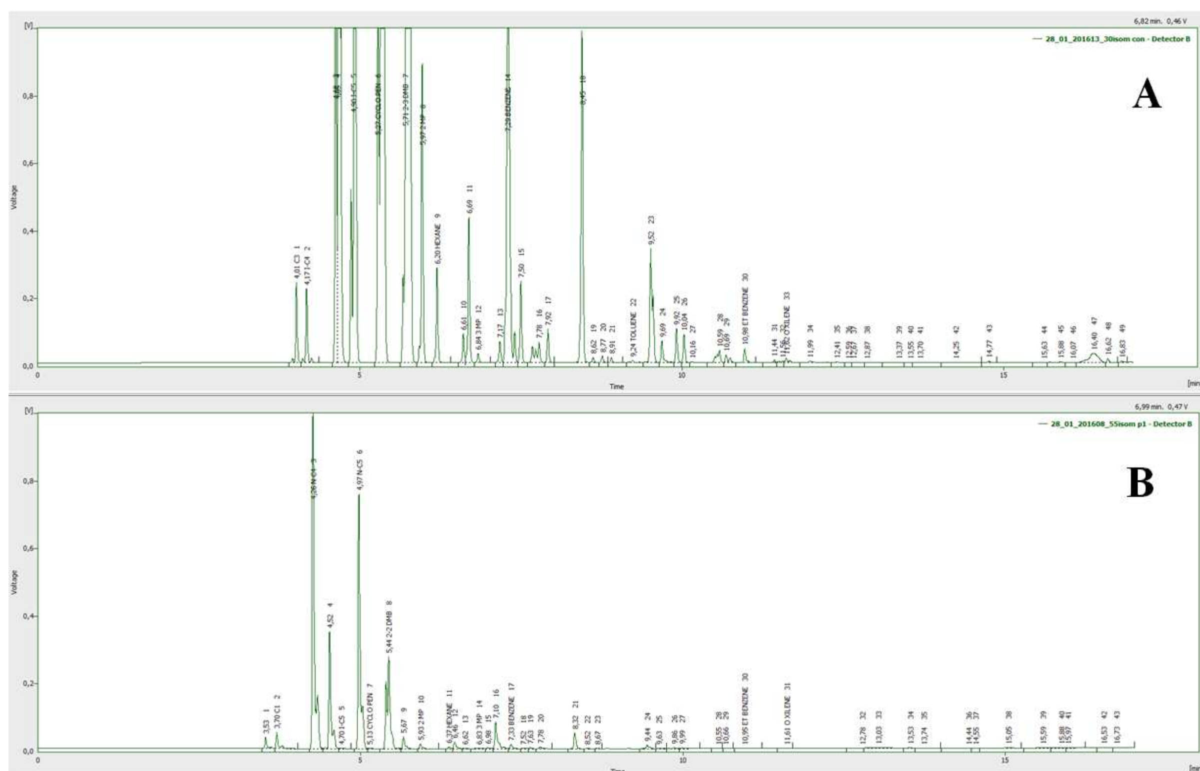


Fig. 3: The rate of diesel degradation by the bacterial isolates after 12 days of incubation

Gas chromatography analysis of Isomerate:

The two isolates were incubated in BH medium supplemented with 2% Isomerate for 5 days. Gas chromatography analysis results of P1 isolate showed that the level of Isomerate has been significantly reduced accompanied with the appearance of degradation products (C1, N-C4, N-C5 and 2-2 dimethyl-butane)(Figure 4B);these compounds could possibly be the results of the enzymatic degradation induced by the microbial strains. Figure 4Bshows also the disappearance of C3, I-C4, 2-3dimethylbutane and toluene. On the other hand, B2 isolate caused the total disappearance of hexane and a decrease in the other Isomerate components (Figure 4C).



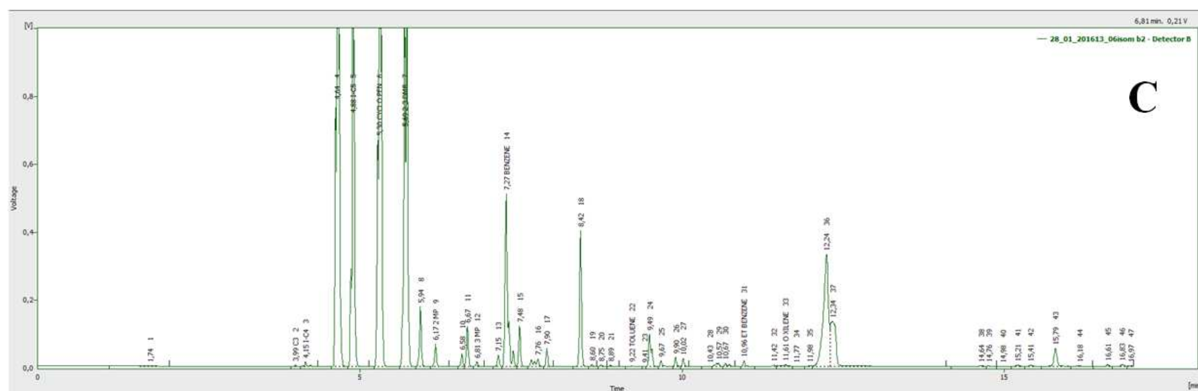


Fig. 4: the GC-FID analysis of Isomerate obtained before (**A**) and after treatment with microbial isolates for 5 days of incubation: P1 (**B**) and B2 (**C**).

Discussion:

Biodegradation of petroleum hydrocarbons is a complex process that depends on the nature and on the amountof the hydrocarbons present. Microbial degradation is the major and ultimate naturalmechanism by which one can clean-up the petroleum hydrocarbon pollutants from the environment [31].

Many microbial isolates from hydrocarbons contaminated soil have been found to degrade petroleum and diesel. They include *Bacillus cereus* [40], *Acinetobacter, Bacillus, Pseudomonas,Bosea, Azospirillum, Nitratireductar* [1], *Candida tropicalis* [16], *Fusarium oxysporum*, *Aspergillus niger* and *Aspergillus flavus* [33]. In our study, two bacterial strains were isolated from oil-polluted soil from the refinery of Arzew, Northern Algeria, by the application of an enrichment technique. After morphological identification and number of biochemical tests, these isolates were included; *Pseudomonas aeruginosa* (P1) and *Brevibacillus* sp(B2).

In this study, the degradation of diesel was observed by the significant increase in the population of the strains in BH medium supplemented with 2% of diesel. The growth rate observed in P1 (5.9×10^9 UFC/ml) agree with the observation of John et al. [22] that *Pseudomonas aeruginosa* exhibited greater ability to degrade hydrocarbons.

The results indicate that the isolates have the capability to utilize diesel (2%) as carbon source. B2 (51.35%) and P1 (20%) have the best levels of diesel biodegradation after 12 days of incubation. Petroleum and diesel degradation has been analyzed for several fungal and bacterial species, Panda et al. [34] have observed that percentage of diesel degradation of *Pseudomonas aeruginosa* isolated from the contaminated soils, collected from Oil Jetty, Paradip Port (Orissa), was49.93% after 20 days incubation period. Other work (Chithra and Hema, 2014) using *Pseudomonas* sp. found that its degradation rate of oil (92.3%) was better than other isolated species including *Bacillus* (83.7%) and *Micrococcus* (35.5%) after 25 days of incubation period.

In the present study, isolate P1proved to be better hydrocarbon degraders than the isolate B2. In the presence of P1 four Isomerate components (C3, I-C4, 2-3dimethylbutane and toluene) were completely degraded in five days of incubation. Moreover, isolate P1has degraded more than 90% of the following components; I-C5, cyclopentane, 2methylpentane, 3methylpentane, hexane, benzene, Et. Benzene and O. Xylene. Benzene can be used as sole carbon and energy source for aerobic growth by many bacteria especially *Pseudomonas* species [42]. *Pseudomonas* species was successfully used to degrade various components of BTEX, such as benzene, toluene, ethylbenzene, and mixture of xylenes [7]. The results obtained in the study of Sutton et al. [38] indicated that the capability of *Pseudomonas* sp. to use hydrocarbons as the only sources of energy and organic carbon was explained by the production of lipidic biosurfactant that increase the ability of biodegradation. Biosurfactants are a structurally diverse group of surface-active substances produced by microorganisms that exhibit the ability to reduce surface and interfacial tension. They may enhance hydrocarbon bioremediation by two mechanisms. The first includes the increase the substrate availability for microorganisms, while the second involves interaction with the cell surface, which increases the hydrophobicity of the surface, allowing hydrophobic substrates to associate more easily with bacterial cells [35].

Isolate B2 has completely degraded hexane and more than 80% of C3, I-C4, 2 methylpentane and toluene. Gram-positive isolates belonged to the genera *Brevibacillus* were found to be able to utilize diesel and a wide range of hydrocarbons [30].

Conclusion:

In conclusion, in this study we have isolated and identified two hydrocarbon-degrading bacteria from oil-contaminated soil. The degradation capacity of isolated bacterial strains was proven by the growth on diesel-containing media as sole carbon source and by their capacities to deplete or reduce the Isomerate components. The results of this study showed that isolate P1 (*Pseudomonas aeruginosa*) have the highest capability of diesel

and isomerate degradation compared to isolate B2 (*Brevibacillus laterosporus*). However, further studies are still required to confirm the environmental significance of *Pseudomonas* sp. and its bioremediation potential. Overall, this study provides that these isolates could be useful in hydrocarbon degradation, bioremediation strategies of contaminated soil, because of their wide range of oil derivatives degradation. The knowledge of the efficiency and the activities of bacteria in oil-polluted sites may be helpful for the bioremediation of oil spills and can be planned in order to clean up oil pollution in soil and water.

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