

**PETROLEUM-HYDROCARBONS BIODEGRADATION BY *Pseudomonas* STRAINS
ISOLATED FROM HYDROCARBON-CONTAMINATED SOIL**

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ABSTRACT

Many indigenous microorganisms in water and soil are capable of degrading hydrocarbon contaminants. In this study, two bacterial strains were isolated from a contaminated soil of a refinery of Arzew (Oran). The isolated strains were identified as *Pseudomonas aeruginosa* (P3) and *Pseudomonas fluorescens* (P4). The capability of these isolates to degrade petroleum was performed by measuring the optical density, colony forming unit counts (CFU/ml) and concentration of total petroleum hydrocarbons (TPH). Degradation of Isomerate by these isolates was analyzed by gas chromatography with flame ionization detector (FID). Results indicated that the isolates can use petroleum as carbon source. Isolates P3 has the highest capability of hydrocarbons degradation (80.86% of biodegradation).

Keywords: Biodegradation, contaminated soil, Petroleum, Isomerate, *Pseudomonas* sp.

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1. INTRODUCTION

Pollution with petroleum hydrocarbons and their derivatives represent a serious problem for the environment and human health. Many petroleum hydrocarbons components are toxic, mutagenic and carcinogenic [1]. The refining, storage and distribution of crude oil and derivatives are important sources of soil and water pollution [2]. Petroleum hydrocarbons can be divided into four classes: The saturates, the aromatics, the asphaltenes, and the resins [3]. Soil is a key component of natural ecosystems because environmental sustainability depends largely on a sustainable soil ecosystem. When soil is polluted, the ecosystem is altered, and agricultural activities are affected. The soil remediation processes leading to the eventual removal of petroleum hydrocarbon and heavy metal, involve physical, chemical and biological techniques [4; 5]. However, physicochemical technologies are complex, expensive, environmentally unfriendly, and do not always result in complete neutralization of pollutants. On the other hand bioremediation technology has been recognized to be a valuable alternative for the detoxification and disposal of toxic substance. This is because of their simplicity, cost-effectiveness, environmental friendliness, and conservation of soil texture and characteristics [4]. The process of bioremediation is an evolving method for the removal and degradation of many environmental pollutants including petroleum hydrocarbons. Biodegradation by microorganisms represents one of the primary mechanisms by which petroleum hydrocarbons pollutants can be removed from the environment [1]. Many environmental factors affect the rate of biodegradation process, which are soil moisture and pH, availability of oxygen and nutrients, contaminant concentration [6]. The key role in bioremediation is played by microorganisms which are very diverse in nature and comprise bacteria, fungi, and yeast [5]. A number of bacterial species are known to degrade hydrocarbons including *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Mycobacterium* spp., *Haemophilus* spp., *Rhodococcus* spp., *Paenibacillus* spp. [9]. The biodegradation of petroleum hydrocarbons is carried under aerobic and anaerobic conditions. There are two main strategies of bioremediation: bioaugmentation and biostimulation. In the first, the addition of oil-degrading microorganisms increases the rates of biodegradation, while in the second, the growth of indigenous hydrocarbon-degraders is stimulated by the addition of nutrient salts, surfactant or organic matter [6]. However, only very few Algerian works have

been reported in the literature on the biodegradation of petroleum hydrocarbons with microorganisms isolated from contaminated soil [7; 8; 9].

The aim of the present work was to explore the hydrocarbon degradation capacity of microorganisms isolated from petroleum contaminated soil of a petroleum refinery. The identification of bacterial isolates was performed by biochemical analyses. In addition, the degradation capacity of petroleum by bacterial strains was performed by measuring the optical density, colony forming unit counts (CFU/ml) and concentration of total petroleum hydrocarbons (TPH). Degradation of Isomerate by isolates was characterized by GC/FID. In this study, we report bacterial isolates which are able to degrade efficiently a wide spectrum of hydrocarbons found in refined petroleum.

2. EXPERIMENTAL METHOD

2.1. Soil samples

Hydrocarbon contaminated soil samples were collected from the Arzew refinery in northern Algeria (Sonatrach, Algeria Petroleum Corporation). The refinery receives crude oil from the production site of Hassi Messaoud (located at 900 km in the south of Algiers). The refinery represents one of the largest oil industrial zones of Africa [10]. The samples were taken (in October 2014) using a clean spatula at a depth of 20 cm. The soil is removed in sterile glass vials and was stored in the refrigerator at 4°C. Petroleum was filtered with Filter unit 0.22 µm.

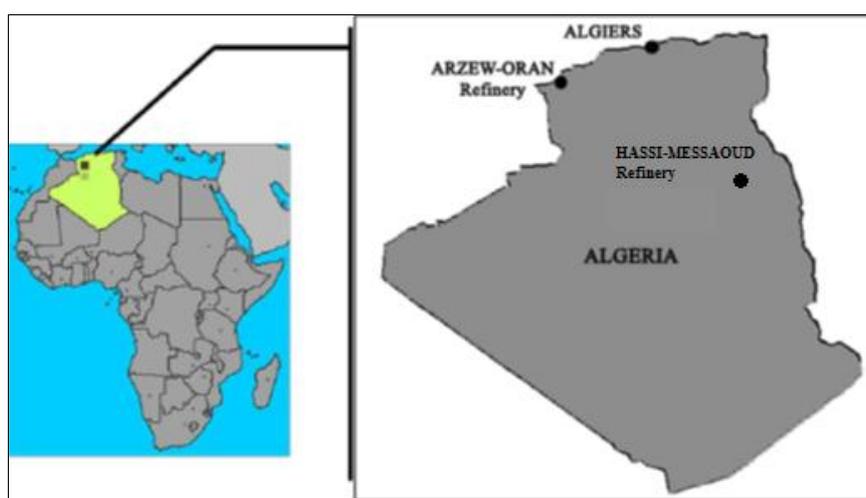


Fig.1. Map of Algeria, showing the localization of the petroleum refinery of ARZEW where the hydrocarbon contaminated soil samples was obtained [10].

2.2. Enrichment and Isolation of aerobic hydrocarbons degrading bacteria

Hydrocarbons-degrading bacteria were isolated on Bushnell-Haas medium (BH) [11], which contained per liter: 1.0g of KH_2PO_4 , 1.0g of K_2HPO_4 , 1.0g of NH_4NO_3 , 0.2g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05g of FeCl_3 and 0.02g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (pH 7). For enrichment, 10g contaminated soil was added to 100 ml of BH medium containing 2% (v/v) Petroleum [12]. Cultures were incubated in the dark at 30°C and 150 rpm. After one week, 2ml of enriched media was transferred into fresh BH medium and incubated at the same conditions as described above. After three consecutive transfers (interval of 1 week), 100µl of culture were plated on BH agar, which were covered with 100 µl of Petroleum and incubated at 30°C [13; 14]. Colonies were isolated, purified and stored at 4° C on nutrient agar. For long duration, the isolates are stored in glycerol (30%) at -50° C.

Pure cultures of bacterial isolates were identified on the basis of their colonial morphology, cellular morphology and biochemical characteristics using API20 NE (bio Mérieux, Marcy-l'Etoile, France) according to the taxonomic scheme of Bergey's Manual of Determinative Bacteriology [15;16].

2.3. Kinetic of HydrocarbonsDegrading Isolates

The ability to degrade petroleum was performed as described by Koolivand et al., (2013) [17] with modifications. Erlenmeyer Flasks of 250ml containing 100 ml of BH Liquid medium with 2ml (2%) of petroleum were prepared. The isolates were incubated overnight in 5ml Nutrient Broth at 30°C. After incubation, 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 small volume (5ml) of the same medium [18], until OD600 was equivalent to 0.91[19].

One ml of inoculum (0.91 OD600 equivalent) was transferred into the flasks and Non-inoculated flasks were prepared as controls [20].

The cultures were grown in the dark at 30°C for 12 days with constant shaking (160 rpm) [19]. The growth patterns were measured using the optical density at 600 nm [21] and the total viable counts (CFU/ml) [22] of the isolates at intervals of 2 days.

2.4. Determination of used hydrocarbons degradation by concentration of total petroleum hydrocarbons

The direct measurement of the dissipation of hydrocarbons in the culture medium is a clear means to evaluate the biodegradation of hydrocarbons [2]. Every 4 days, samples were taken from cultures Flasks for residual of TPH values (Total Petroleum Hydrocarbons) [22; 23] the TPH was measured with Oil Content Analyzer (Infrared spectroscopy, HORIBA, OCMA-310) (Method of NF M07-203). The extraction of the hydrocarbons can be done by polychlorotrifluoroethylene (HORIBA S-316 Solvent).

2.5. Isomerate degradation assay by GC/FID

The ability of isolates to degrade Isomerate (Naphta Petroleum, Refinery of Arzew, SONATRACH) in BH at 30°C with agitation at 160 rpm was examined. Erlenmeyer flasks containing 100ml of BH, supplemented with 2% of Isomerate, were inoculated with strains to a final OD600 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 15 days of incubation, inoculated flasks and un-inoculated controls were extracted [24; 25]. The extracts were analyzed by Gas Chromatography (DANI Master GC Fast Gas Chromatograph System, DANI Instruments Sp. A., 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 (split/splitless mode) temperature 250°C; FID temperature 300°C. Carrier gas constant flow rate (helium) 40ml/min.

3. RESULTS

3.1. Isolation and identification of isolates

Two strains (P3 and P4) were isolated and characterized to have petroleum degrading capabilities. The isolates were respectively identified as *Pseudomonas* sp. (P3 and P4).

3.2. Determination of hydrocarbons degradation by turbidometry and bacterial counts:

Growth rate of the two isolates in BH broth medium supplemented with 2% petroleum was evident from the significant increase in cell density at 600 nm and alternate colony counting after 12 days of incubation (Fig 2). The increase in optical density indicates the ability of cultures strains to utilize and degrade petroleum as source of energy. More specifically, this was observed between day 4 and 8 after which the growth became limited. The cell-concentration increased from 1.3×10^5 and 1.32×10^5 CFU/ml in BH with Petroleum to 2×10^9 CFU/ml on day 4 and 1.5×10^9 CFU/ml on day 6, for the isolates P3 and P4 respectively (Fig 2).

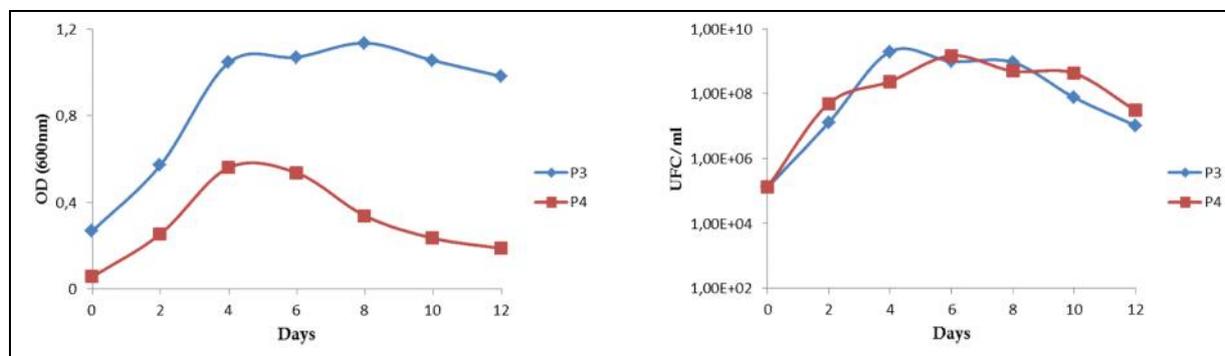


Fig.2. Growth rate of the isolates in BH broth medium supplemented with Petroleum (2%) while 12 days of incubation at 30°C.

3.3. Determination of total petroleum hydrocarbons (TPH)

The growth of bacteria in BH medium supplemented with 2% petroleum increased progressively with decrease in TPH throughout the monitored period (12 days). The total amounts of TPH in the first day of incubation were 260 and 262 mg/L medium with petroleum for strains P3 and P4 respectively. The initial amount of TPH of the control was 282 mg/L (Fig 3). These curves indicate the degradation of petroleum by bacterial isolates

because the amount of TPH decreased after 12 days of incubation to 49mg/L, and 169mg/L in BH with petroleum for P3 and P4, respectively. At the beginning, the degradation rate was high but decreased with time due to the substrate exhaustion resulting from the use of the substrate by the isolates, as already hypothesized by Ichor et al., (2014) [26].

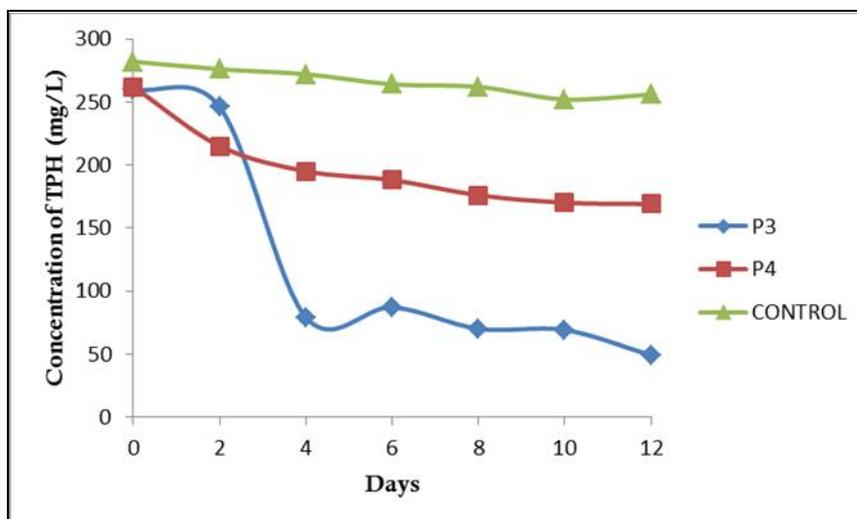


Fig.3. TPH degradation by isolated strains in BH broth medium supplemented with 2% of Petroleum while 12 days of incubation at 30°C.

3.4. Gas chromatographic analysis of Isomerate

GC analysis of Isomerate inoculated with isolate P3 and P4 after 15 days of incubation (Fig 4). The results showed that the level of hydrocarbon has been significantly reduced with the appearance of degradation products (C1, C2, N-C4, N-C5 and 2-2 dimethyl-butane) comparing with the control (Fig 4A). Fig 4B shows the disappearance of cyclopentane, Et-benzene and O-xylene were not visible after treatment with isolate P3. On the other hand, the treatment with isolate P4 (Fig 4C) favored the disappearance of I-C4 and hexane. According to results from GC-FID analysis, the percentage of Isomerate compounds's degraded was determined (Fig 5).

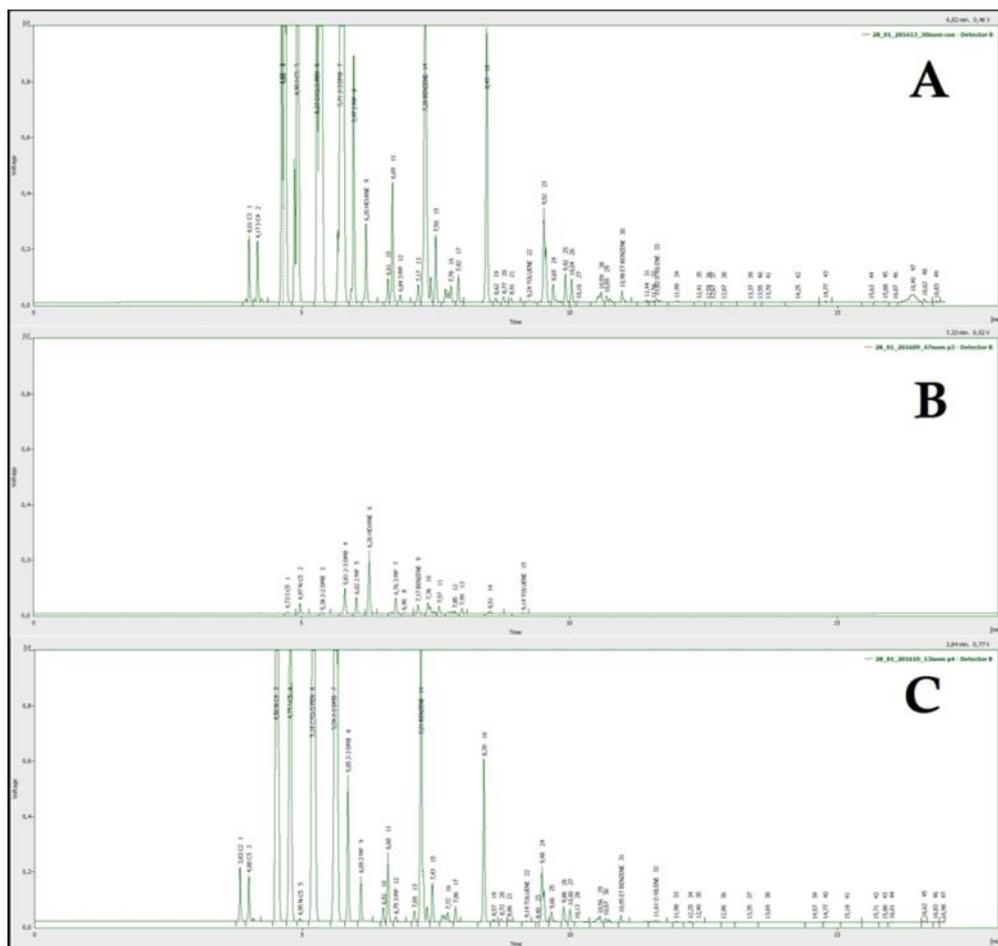


Fig.4. GC-FID analysis of Isomerate obtained without (A) and with treatment with microbial isolates: P3 (B) and P4 (C) for 15 days of incubation

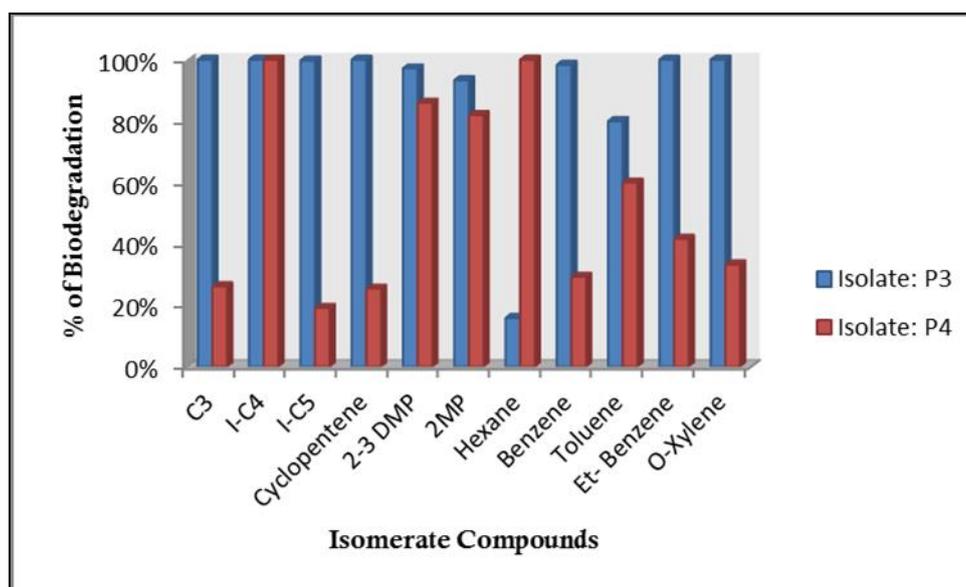


Fig.5. Percentage of Isomerate compounds’s biodegradation by bacterial isolates (P3 and P4) after 15 days of incubation.

4. DISCUSSION

Two bacterial strains used for this study were isolated by application of an enrichment technique from the contaminated soil of the refinery of Arzew, Northern Algeria. In the view of investigating their ability to retain their biodegradation potential of petroleum and Isomerase and employed those in bioremediation strategies of hydrocarbons contaminated sites. After morphological identification and number of biochemical tests, these isolates were included *Pseudomonas* sp. (P3 and P4).

Due to the presence of a complex enzymatic system the genus *Pseudomonas* exhibits a wide variety of metabolic and physiological properties [28]. They are also the most predominant group of microorganisms that degrade xenobiotic compounds [28]. In this study, the degradation of petroleum is observed by the significant increase in the population of isolates P3 and P4 in BH medium supplemented with 2% of petroleum as compared to the initial population. However, the higher growth rate was observed in P3 (2×10^9 UFC/ml with petroleum) compared to P4 (1.5×10^9 UFC/ml with petroleum). These results agree with the observation of John et al., 2011 [29] that *Pseudomonas aeruginosa* exhibited greater ability to degrade hydrocarbons.

Biodegradation results demonstrated rapid utilization of the petroleum substance by the bacterial inoculums (Fig 3). From this result, it was clear that the P3 and P4 were able to degrade petroleum as sole source of carbon and energy since there was also loss in concentrations of total petroleum hydrocarbons (TPH). The best reduction in TPH (petroleum) was observed for isolates P3 as compared to control implying that the volatile components of the petroleum had evaporated. TPH loss was significant with time for isolates P4. Indeed, several *Pseudomonas* strains isolated from contaminated soil have been reported to grow on and/or degrade hydrocarbons or to remediate petroleum contaminated soils [27].

The growth of the isolates in BH supplemented with petroleum increased progressively with decrease in TPH (80.86% for P3 and 33.98% for P4) throughout the 12 days period monitored. Ying et al., (2011) [30] and Jinlan, (2012) [31] reported a correlation between the significant decreases of TPH concentration to an increase in biomass bacterial indicating that the microorganisms were responsible for the degradation which corroborates our results.

In the present study, isolate P3 proved to be better hydrocarbon degraders than isolate P4. P3 caused a complete degradation in fifteen days of incubation of five hydrocarbons were C3, I-C4, Cyclopentene, Et.benzene and O.xylene (Fig 4). Isolate P3 degraded more than 80% of the following hydrocarbons; I-C5, 2-3dimethylbutane, 2methylbutane, benzene and toluene (Fig 5). Benzene can be used as sole carbon and energy source for aerobic growth by many bacteria especially *Pseudomonas* species [32]. Whyte et al., (1997) [24] isolated three hydrocarbon-degrading bacteria from petroleum-contaminated Arctic soils and characterized two of the strains as *Pseudomonas* spp. The results obtained by Sutton et al., (2011) [33] indicate that the capability of *Pseudomonas* spp. to use hydrocarbons as the only sources of energy was explained by the production of lipidic biosurfactant that increase the ability of biodegradation. Isolate P4 has completely degraded I-C4 and hexane and more than 60% of 2-3 dimethylbutane, 2 methylbutane and toluene (Fig 5). Isolate P4 was identified as *Pseudomonas fluorescens*. This is a well-known degrader of benzene and toluene [34; 35]. The relative differences in patterns of biodegradation of these substances can be explained by their molecular weights and chemical characteristics. Low molecular weight compound can be degraded relatively easily, while compounds of higher molecular weight require more time to be mineralized. Molecular structure also affects microbial degradation [36]. Highly branched alkanes and multi-ring cycloalkanes are slower to degrade than their straight-chain analogues [36].

5. CONCLUSION

In conclusion, in this study we have isolated and identified from contaminated soil two bacterial strains identified as *Pseudomonas* sp. with the ability to degrade hydrocarbons. The microbial degradative capabilities of hydrocarbons were monitored by different microbial methods including enumeration of hydrocarbon degraders, determination of total petroleum hydrocarbons (TPH) and gas chromatographic analysis of Isomerate. Petroleum and Isomerate were efficiently degraded by isolates P3 and P4. The results of this study showed that isolates P3 have the highest capability of hydrocarbons degradation compared to isolates P4. These isolates could be useful in hydrocarbon degradation and bioremediation of contaminated soil and have desirable oil bioremediation characteristics.

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