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Original Article

# Use of Biotechnology for Environmental Decontamination: Isolation of Bacteria Degrading Hydrocarbons From Soil Contaminated With Diesel Oil

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**Abstract**
To use biotechnology for environmental decontamination, the current study attempted to isolate bacterial strains capable of assimilating hydrocarbons. To this end, oil-contaminated soil samples were obtained from a gas station in Mascara (Alegria). Two bacterial strains were identified from the tainted soil. The results demonstrated the capacity of these strains to use hydrocarbon substrates as carbon sources, including diesel, benzene, naphthalene, and toluene. The strains' capacity to break down diesel oil at 1%, 2%, 3%, and 4% (v/v) concentrations was evaluated. According to the biochemical traits identified, the isolated strains S4 and S11 were associated with the gender of *Pseudomonas* and *Staphylococcus*, respectively. Based on these findings, both strains grew best when fed a 2% diesel oil substrate. Using oil diesel, benzene, naphthalene, and toluene as substrates, the isolates' growth measurement characteristics revealed that strain S4 degraded hydrocarbon substrates more effectively than strain S11. In summary, these bacterial strains can reduce petroleum pollution and aid in the bioremediation process.
**Keywords:** Decontamination, Biodegradation, Diesel oil, Soil pollution

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## 1. Introduction

The needs of daily life are currently met by petroleum hydrocarbons, as a substantial energy source. The extensive contamination of water and soil during the manufacture, processing, and distribution of petroleum products is a direct result of their use. However, monoaromatic and polyaromatic compounds have a high solubility in water, making it easier for them to migrate and quickly contaminate soil and subsurface water. Even at low concentrations, these compounds can have a major negative impact on the environment and human health (1). Considering that the constituents of petroleum hydrocarbons have the potential to be toxic and carcinogenic, pollution from these energy sources, such as diesel and aromatic hydrocarbons, has major consequences (2). About 1.7-8.8 million metric tons of diesel were discharged into land and marine environments (3). Hydrocarbon-polluted soils and marine environments are frequently remedied using technologies grounded in physical, chemical, and biological principles (4). One of the main methods for removing petroleum and other hydrocarbon pollutants from the environment is biodegradation by the natural populations of microorganisms (5). Furthermore, bacteria, yeasts, and fungi are known to be the primary microorganisms that consume petroleum hydrocarbons (6). Bacteria continue to be the most active species, both qualitatively and quantitatively, among those that can thrive on hydrocarbons (7, 8). *Pseudomonas, Acinetobacter, Alcaligenes, Vibrio, Flavobacterium, Achromobacter, Micrococcus, Nocardia,* and corynebacteria are the most common bacterial genders identified on this topic based on the frequency of isolation (9-11). Numerous significant parameters, including temperature, pollutant concentration, pH, microbe type, oxygen, nutrients, and pollutant bioavailability, have been found by researchers to influence the rates and efficiencies of the biological treatment of hydrocarbons in soils (12, 13). Thus, the current study seeks to separate and identify the possible bacteria that break down diesel from the soil contaminated with diesel and assess how well they could break down hydrocarbon pollutants such as diesel oil (alkanes and aromatic compounds), monoaromatic, and polyaromatic compounds.

## 2. Materials and Methods

### 2.1. Samples and Reagents

A gas service station in the Mascara region (35° north latitude and 18° south longitude) provided the soil polluted by the petroleum derivatives used in this investigation, which was utilized to isolate hydrocarbon-degrading bacteria. Rhbal et al reported the soil preparation and sampling process (14). The sampling was performed at a depth of 0–20 cm, where biological activity is the highest, and in three distinct places. It should be noted that these three locations were two meters apart, and the samples were collected under identical physical circumstances (temperature and humidity). Using a spatula, the samples were aseptically collected and placed in sterile bottles before being brought to the laboratory. Analytical-grade benzene, toluene, naphthalene, and other chemical reagents were employed, and diesel fuel was acquired from the gas station.

### 2.2. Isolation and Screening of Bacteria

Briefly, 5 g of the soil sample was transferred to flasks containing 100 mL of minimal salt medium (S.M.) as described by Bushnell and Haas (15). This medium contains $K_2HPO_4$ 1 g/L, $KH_2PO_4$ 1 g/L, $KNO_3$ 2 g/L, and $NH_4NO_3$ 1 g/L supplemented with 1% (v/v) of diesel oil. This process was followed to determine bacterial counts and isolate pure bacterial strains. For a week, the flasks were incubated at 30 °C with a pH rate of 6.5 while being shaken at 150 rpm. After finishing, the water-soil suspension was centrifuged for 15 minutes at 1500 rpm. Following the proper dilution, 0.1 of the supernatant was plated on the nutrient agar medium containing oil diesel as the only source of carbon and energy. The plate was then cultured for two weeks at 30°C to monitor the outcome. If cultures grew when diesel was present, it indicated that they had the ability to break down the diesel. Colonies were observed to track bacterial growth (16). The isolates that grew the fastest were chosen for additional examination.

### 2.3. Morphological and Biochemical Characteristics

The chosen bacterial isolates were described based on their biochemical and physical traits. Physiological test kits were used to test and identify bacteria in accordance with the API 20E Analytical Profile Index Micromethods (17).

### 2.4. Utilization of Different Hydrocarbons by the Isolate

The chosen strains' capacity to absorb hydrocarbons as developing bacterial strains was evaluated using various hydrocarbon pollutants in different flasks, including diesel oil (alkanes and aromatic compounds), as well as monoaromatic and polyaromatic compounds such as benzene, toluene, and naphthalene. Seven 250 mL Erlenmeyer flasks (corresponding to 0 days, 3 days, 6 days, 9 days, 12 days, and 15 days of the experiment) were utilized for the biodegradation assay, which was conducted over two weeks. Each flask included 50 mL of the mineral medium and 10% of acclimated inoculums under aseptic conditions. Strains S4 and S11 were cultivated in Erlenmeyer flasks with 50 mL of S.M. with 1% hydrocarbon compounds as the only carbon and energy source for the induction and hydrocarbon degradation investigations by employing nonproliferating cells. The flasks were then incubated in a rotary shaker at 150 rpm at 30°C. A sterile pH 7 buffer was used twice to wash the cells. Growth on the individual substrate was periodically removed to compare the concentration of the following changes in optical density at 600 nm of washed cells against biotic (without substrate) and sterile (without bacteria) controls and test their bioremediation capacity (18).

### 2.5. Kinetic Analysis of Stains Growth on Diesel

In flasks holding 90 mL of the S.M. broth medium supplemented with diesel concentrations of 1%, 2%, 3%, and 4% (v/v), tests were conducted in triplicate under ideal growth circumstances. Following a 10-mL inoculation with one of the acclimated strains, the flasks were cultured for seven days at 30°C. The optical density at 600 nm was employed to track bacterial growth over a 24-hour incubation period. Similar preparations were made for controls devoid of diesel oil (18).

### 2.6. Statistical Analysis

Three replicates were used throughout the experiments, and the mean values with standard deviations were calculated using Microsoft Excel.

## 3. Results and Discussion

Overall, 14 strains of bacteria that break down diesel were identified from enrichment cultures. The energy sources were obtained, and diesel could be utilized as carbon by all isolates. Two isolated strains, S4 and S11, were chosen for additional research out of the isolates because they had a greater development rate. The isolated hydrocarbon-degrading strains S4 and S11 were related to the gender of *Pseudomonas* and *Staphylococcus*, respectively, according to the morphological (Fig. 1), biochemical, and physiological features (Table 1).

Some researchers (19, 20) claimed that bacterial strains from these two genera might break down the components of hydrocarbons. The ability of the strains isolated on diesel agar plates to use various hydrocarbon compounds, including diesel (alkanes and aromatic compounds), as well as monoaromatic and polyaromatic compounds, including toluene, benzene, and naphthalene, underwent investigation. Figs. 2 and 3 show the biomass growth curves for diesel, benzene, toluene, and naphthalene for both strains.

In line with the results of a previous study (21), our findings revealed that both strain isolates were capable of breaking down hydrocarbon pollutants during the 15 days of incubation and using them as the only source

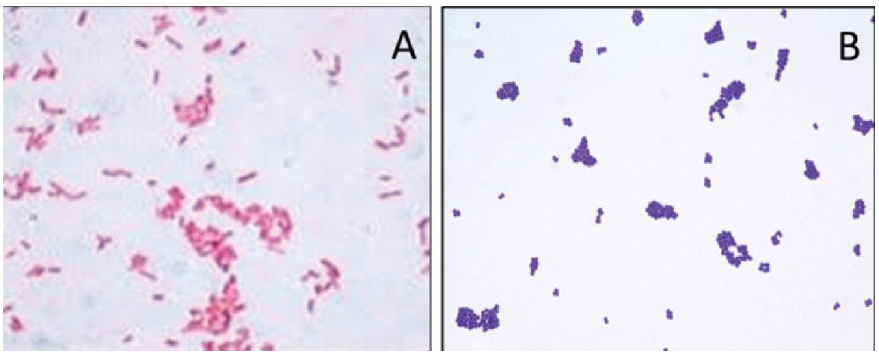


**Fig. 1.** Cells Photomicrographs After Gram-Staining of Hydrocarbon-Degrading Strains S4 (A) and S11 (B) Isolated From Soil in Mascara, Algeria

**Table 1.** Biochemical and Growth Characteristics of Isolated Bacterial Cultures

| Characteristics | S4 | S11 |
|---|---|---|
| Gram coloration | - | + |
| Respiratory metabolism | Strictly aerobic | Aerobic |
| Catalase | + | + |
| Oxidase | + | - |
| Nitrate reductase | + | - |
| TSI | - | + |
| MR | + | - |
| V.P. | - | + |
| ONPG | + | - |
| Mannitol | - | + |
| Mobility | + | + |
| Citrate de Simons | + | - |
| Urea indole | - | - |
| ADH | + | - |

*Note*. ADH, Antidiuretic hormone; TSI, Triple Sugar Iron; MR, Methyl Red; VP, Voges-Pproskauer Reaction; ONPG, Ortho-Nitrophenyl-B-Galactoside
+: Gram positive
-: Gram negative

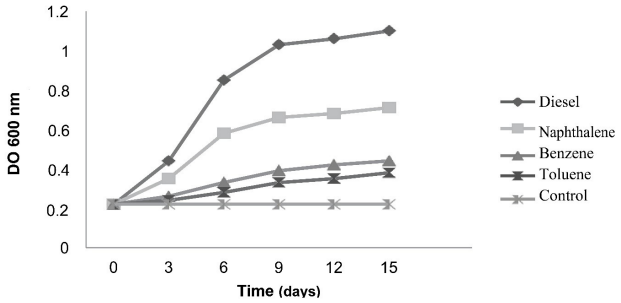


**Fig. 2.** Growth of Selected Isolates on a Mineral Medium Concerning Uninoculated Controls Over 15 Days of Degradation of *Pseudomonas* sp.

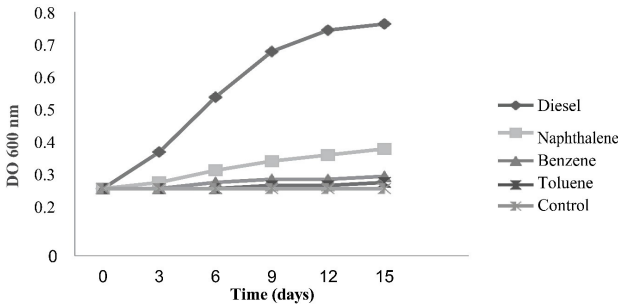


**Fig. 3.** Growth of Selected Isolates on Mineral Medium With Respect to Uninoculated Controls Over 15 Days of Degradation of *Staphylococcus* sp

of carbon. With optical density values of 1.18 ± 0.12 and 0.79 ± 0.06 (Figs. 1 and 2, respectively), two isolates, *Pseudomonas* sp. and *Staphylococcus* sp., demonstrated greater activity on the degradation of diesel than aromatic compounds. This could be because the isolates have enzyme systems that can break down and use diesel as a substrate (22). Furthermore, in contrast to isolate *Staphylococcus* sp., isolate *Pseudomonas* sp. represented strong diesel degraders. According to numerous investigations, *Pseudomonas* sp. exhibits strong diesel degradation (23-25). Based on the findings of this study, the *Staphylococcus* strain was not as effective at degrading polyaromatics as the *Pseudomonas* strain. Numerous studies have noted that *Pseudomonas* sp. has the capacity to break down a wide variety of polyaromatic chemicals (26, 27). Additionally, these strains grew after seven days when naphthalene was employed as a hydrocarbon source, and after fifteen days when benzene and toluene were utilized, the strains grew weakly. None of them displayed the lag phase of development, indicating that neither strain required a period of substrate adaptation for diesel, while *Pseudomonas* sp. did not require a period of adaptation for naphthalene.

Plotting the curve of A against time, which showed the start of the exponential development phase during the initial moments of cultivation, can demonstrate this issue. Plotting the curve of lnX against time, which, according to earlier research, marked the start of the exponential growth phase during the initial moments of cultivation, can be used to confirm this issue (28). Stepwise elimination was observed in the instance of a mixture of monoaromatic chemicals and naphthalene, with naphthalene being metabolized first, followed by toluene and benzene. Figs. 4 and 5 display the speeds at which both stains degrade in diesel oil at different diesel oil concentrations.

According to the results, the content of diesel oil affects the rates of biodegradation. The ideal diesel content was 2% (v/v) under test circumstances since the hydrocarbon-degrading strains tended to use diesel as

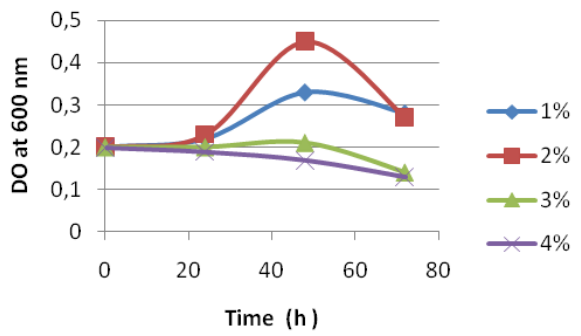


**Fig. 4.** Growth of *Pseudomonas* sp. Using Diesel Oil as the Sole Carbon and Energy Source at Different Concentrations (1%, 2%, 3%, and 4%)

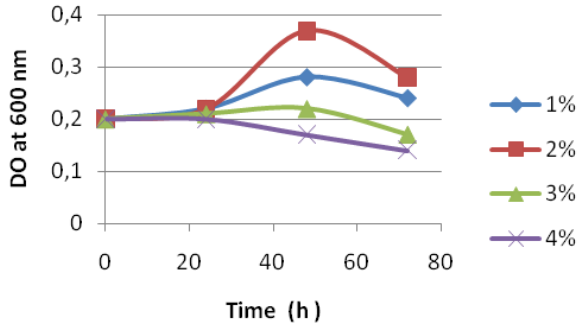


**Fig. 5.** Growth of *Staphylococcus* sp. Using Diesel Oil as the Sole Carbon and Energy Source at Different Concentrations (1%, 2%, 3%, and 4%)

a nutrient (Figs. 4 and 5). Palanisamy et al (18) found that *Acinetobacter baumannii* grew to its maximum at 4% of diesel, which contradicts our results. The rates of deterioration rose with the concentration of diesel, peaked at 2%, and then declined with an increase in the concentration. The limited growth shown at greater diesel oil concentrations was explained by Hawrot-Paw et al (29), who found that the lower concentration of diesel oil was less hazardous in terms of biomass output.

Although relatively low, 2% (v/v) of the tested diesel oil seemed to be the ideal substrate concentration, providing both strains S4 and S11 the best specific growth and biological activity against diesel oil (μ max = 0.009 h- and μ max = 0.006 h-, respectively). Earlier research on the bioremediation of petroleum-contaminated soil revealed similar findings (30, 31).

It should be mentioned that both strains reached their active growth phase at 2% (v/v) of diesel oil following a brief growth lag phase that indicates a period of substrate adaption, implying that the cells have produced all the enzymes they require for growth. Conversely, the isolates needed a significantly longer lag phase at diesel oil concentrations of 1% (v/v). After 20 hours of consumption, the microorganisms' maximum amount of substrate was used during the exponential development phase. The growth rate and biomass production decreased when the initial diesel concentration rose because it took more energy to keep the cultures alive. Nevertheless, there was no discernible variation in each strain's development across the examined diesel oils.

According to Figs. 4 and 5, bacterial growth decreases with increasing diesel concentration in the medium. This is brought on by the toxicity of elevated diesel concentrations, which inhibit bacterial development by causing stress and shock (32). Diesel can serve as a carbon and energy source for microorganisms, thus each one has a different tolerance level. Diesel oil contains a variety of aromatic compounds in addition to linear and branched alkanes with varying chain lengths. Many of these substances are known to be easily biodegradable since they are extremely linear alkanes. However, because these chemicals are poorly soluble in water, their slow rate of dissolution, desorption, or transport frequently limits their biodegradation. Generally speaking, the transport mechanism to microbial cells, the sorption properties, and the rates of dissolution or partitioning affect the bioavailability of hydrophobic substances (33). Future research is required to elucidate parameters influencing the ability and efficiency of diesel oil degradation in order to boost the viability of bacterial isolates as possible strains with biodegradation activity. However, the findings of this study demonstrated that *Pseudomonas* sp. strains were suitable for the bioremediation of oil contamination close to petrol stations since they could thrive and tolerate those substrates.

## 4. Conclusion

The findings revealed that the isolated diesel-degrading strains S4 and S11 were associated with the genders of *Pseudomonas* and *Staphylococcus*, respectively, according to the identified biochemical and physiological traits. These findings demonstrated the viability of using contaminated petroleum oil degrader sites to break down diesel and aromatic hydrocarbons. Future research must investigate factors influencing the capacity and effectiveness of petroleum oil degradation in order to increase the viability of bacterial isolates as possible strains with biodegradation activity.

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### Authors' Contribution

**Conceptualization:** Zouaoui Benattouche, Hamza Belkhodja.
**Data curation:** Hamza Belkhodja, Ahmed Hariri.
**Formal analysis:** Ahmed Hariri.
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**Methodology:** Ahmed Hariri.
**Project administration:** Djilali Bouhadi.
**Resources:** Zouaoui Benattouche.
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**Supervision:** Zouaoui Benattouche.
**Validation:** Ahmrd Hariri.
**Visualization:** Djilali Bouhadi.
**Writing-original draft:** Ahmed Hariri.
**Writing-review & editing:** Djilali Bouhadi.

**Competing Interests**
The authors of the study declare that there is no conflict of interests.

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