ARTICLE / INVESTIGACIÓN

# Isolation and characterization of fungi and bacteria able to grow on media containing gasoline and diesel fuel

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**Abstract:** Petroleum products are significant environmental pollutants. This study aimed to isolate microorganisms able to grow on media containing gasoline and diesel fuel. Microorganisms were isolated from soils sampled near gasoline and diesel pumps. Bacterial isolates were characterized and tested on media containing concentrations ranging from 10 to 100 % gasoline and diesel fuel and combinations of both 50/50 % and 25/25/50 % (gasoline/ diesel/ Mueller Hinton broth). Results showed that microbial isolates belong to the genera *Pseudomonas, Bacillus, Staphylococcus, Micrococcus, Flavobacterium, Actinobacteria, Penicillium, Hansfordia* and *Alternaria*. *Pseudomonas spp*. and *Bacillus spp*. showed the ability to grow on both products up to the concentration of 80 %. However, no growth was noticed above that concentration and on both mixtures. Throughout this study, it has been shown that using a selective screening method for microorganisms able to grow on pollutants can present a significant advantage for bioremediation.

**Key words:** Pollution; gasoline; diesel; microorganisms.



## Introduction

Petroleum-based products are the primary energy source for industry and daily life; however, they are also considered major environmental toxic pollutants. Leakages and accidental spills occur regularly during large-scale exploration, production, refining, transport, and storage of petroleum and derived products[1]. Pollution due to petroleum hydrocarbons and their derivatives, including diesel fuel, gasoline, heavy oil, motor oil, fuel residues and mineral oil, has an increasing impact on the environment leading to air, soil and groundwater pollution as to the contamination of the food chain[2,3].

Diesel oil spills represent one of the leading environmental pollution problems due to their extensive production and use. Diesel oil, a product of petroleum distillation, is formed of up to 4,000 hydrocarbons. It is a mixture of regular, branched and cyclic alkanes and aromatic compounds (e.g., polycyclic aromatic hydrocarbons PAHs), which are persistent pollutants with high mutagenic, carcinogenic and reprotoxic potential[4].

Petroleum hydrocarbon spills, including diesel oil and gasoline, have been shown to hurt soil's biochemical and physicochemical characteristics and be toxic to plants[5]. They reduce soil fertility and nitrogen fixation and cause erodibility leading to a more significant loss of soil and nutrients and a unbalance in soil fauna and flora[6].

Several physicochemical methods have been developed to treat hydrocarbon-contaminated soil. However, these limitations include expensive costs, incomplete removal of pollutants, and toxic environmental impact[7,8]. Microorganisms can metabolize hydrocarbons, such as diesel oil, as the sole carbon and energy source, into nontoxic, biodegradable products. These microorganisms have catabolic genes that synthesize metabolizing enzymes involved in hydrocarbon degradation[1]. The initial intracellular reaction is an oxidative process in which the activation and incorporation of oxygen is the key enzymatic reaction catalyzed by oxygenases and peroxidases[9]. Microorganisms have the potential to detoxify hazardous organic compounds using polymerization, mineralization, or transformation[3].

Hydrocarbon degradation by microbial communities depends on the composition of the community and its adaptive response to the presence of petroleum hydrocarbon. The susceptibility of hydrocarbons to microbial degradation can be generally ranked as follows: n-alkanes > branched alkanes > low molecular weight aromatics > cycloalkanes > polyaromatic hydrocarbons (PAHs)[10,11].

Microbial degradation, known as bioremediation, is one of the essential methods of decontamination of pollutants in both terrestrial and aquatic environments[4]. Bioremediation is an efficient, cost-effective, and environmentally friendly approach for decontaminating polluted soils[12].

Microorganisms are critical bioremediation agents and can effectively degrade a wide range of contaminants[13]. Besides, bacteria are the most active agents in petroleum degradation and work as primary degraders of spilled oil in the environment[10].

Moreover, the rate of microbial degradation of hydrocarbons in soils is affected by several physicochemical factors such as soil's particles size, nutrients, oxygen, pH, quality and quantity of the contaminants and temperature, which plays a significant role in controlling the bioavailability of low-solubility hydrocarbons and hence the nature and the extent of microbial metabolism[14].

This study aimed to isolate and identify microorganisms (bacteria and fungus) from polluted soils near gasoline and diesel fuel pumps. Afterward, the isolates were tested for

**Citation:** Meknassi, K.; Aït Abderrahim, L.; Taïbi, K.; Sassi, M.; Boussaid, M. Isolation and characterization of fungi and bacteria able to grow on media containing gasoline and diesel fuel. Revis Bionatura 2023;8 (1)26. http://dx.doi.org/10.21931/RB/2023.08.01.26
**Received:** 23 October 2022 / **Accepted:** 15 January 2023 / **Published:** 15 March 2023

their ability to grow on media containing pure gasoline and diesel fuel in a perspective of bioremediation approach.

## Materials and methods

### Soil and petroleum hydrocarbons samples

Several soil samples were aseptically collected at different depths (5 to 20 cm) from various sites near gasoline and diesel pumps of gas stations in Tiaret, Algeria. The collected samples were kept in sterile bottles and stored in the refrigerator at 4 °C before the manipulation. Samples of gasoline and diesel fuel were obtained directly from a gas station.



### Isolation and purification of microorganisms

Suspensions from each soil sample were prepared by mixing 1 g of soil in 10 ml of sterile distilled water. After mixing, the suspensions were left to settle then volumes ranging between 0.1 and 0.5 ml were taken from the supernatant and inoculated into Petri dishes containing culture media i.e., nutrient agar, Sabouraud agar, King A and King B agar[15]. After inoculation, Petri dishes were incubated at 30°C and observed after 24 h, 48 h, until 7 days. Colonies that formed on the culture media were subsequently transferred to new media, and successive subcultures were carried out until pure cultures were obtained.

### Identification of isolates

The identification of isolates was based on their morphological and biochemical characteristics.

### Morphological characterization

After purification, the microbial isolates are observed macro- and microscopically to check their purity and to visualize colonies and cell structures and disposition as a first step to their identification. The colonies' color, shape, aspect and transparency were examined and recorded as colony morphological characteristics. Whereas the cell's shape, size, arrangement and type of cell wall were examined under the microscope using simple staining and Gram stain for bacteria.

### Biochemical characteristics

Several biochemical tests were performed on the microbial isolates to help identify these: citrate utilization, triple sugar iron agar, catalase, oxidase, O-nitrophenyl-β-D-galactopyranoside (ONPG), mannitol mobility tests and respiratory type.

### Test of viability of the microbial isolates on diesel and gasoline

After isolation and purification of the microbial strains, each strain is inoculated separately in glass Petri dishes containing different concentrations of gasoline and diesel fuel ranging from 10 % to 100 % mixed with Mueller Hinton agar. Each strain is also inoculated in a mixture of the two hydrocarbons at concentrations of 50/50 % (gasoline/diesel) and 25/25/50 % (gasoline/ diesel/ Mueller Hinton broth) in test tubes. Incubation is done at 37 ºC from 24 h to 72 h after microbial growth is recorded.

## Results

### Microorganisms isolated from polluted soil samples

After the purification and identification of microbial isolates from soil samples (Table 1), we noted the presence of several bacteria belonging mainly to the genera: *Pseudomonas, Bacillus, Staphylococcus, Micrococcus, Flavobacterium* and *Actinobacteria* (Figure 1) whereas the fungal isolates belong to the genera *Penicillium sp.*, *Hansfordia sp.* and *Alternaria sp.* (Figure 2).

### Growth of the bacterial isolates on diesel and gasoline

Among the isolated microbial strains, two bacterial strains belonging to the genus Bacillus and two others belonging to the genus Pseudomonas were tested for their ability to grow on gasoline and/or diesel fuel containing media. Results revealed that all the tested bacterial strains are able to grow on concentrations of diesel fuel and gasoline up to 80 % (Figure 3). However, beyond this concentration no bacterial growth was recorded. In addition, the bacterial isolates were not able to grow on both tested concentrations of the mixture gasoline-diesel.

| Bacteria/ test | *Bacillus* sp. | *Bacillus* sp. | *Pseudomonas* sp. | *Flavobacterium* sp. | *Staphylococcus* sp. | *Micrococcus* sp. |
|---|---|---|---|---|---|---|
| Catalase | + | + | + | + | + | + |
| Citrate | + | / | / | +[1] | + | - |
| Mannitol | + | + | + | + | + | + |
| ONPG | - | - | - | - | - | - |
| Oxidase | + | + | + | + | - | - |
| TSI | / | Glu+, Sac/Lac+ | / | Sac and/or Lac+ | Glu+, Sac/Lac+ | Glu+, Sac/Lac+ |
| Mobility | + | - | + | - | - | - |
| Gram stain | + | + | - | - | + | + |
| Respirtory type | Aeroan-aerobe | Aeroan-aerobe | Aerobe | Obligate aerobe | Aeroanaerobe | Aerobe |

+ positive result / - negative result

*Glu: glucose, Sac: Saccharose, Lac: Lactose.

**Table 1.** Results of the biochemical tests on the bacterial isolates.

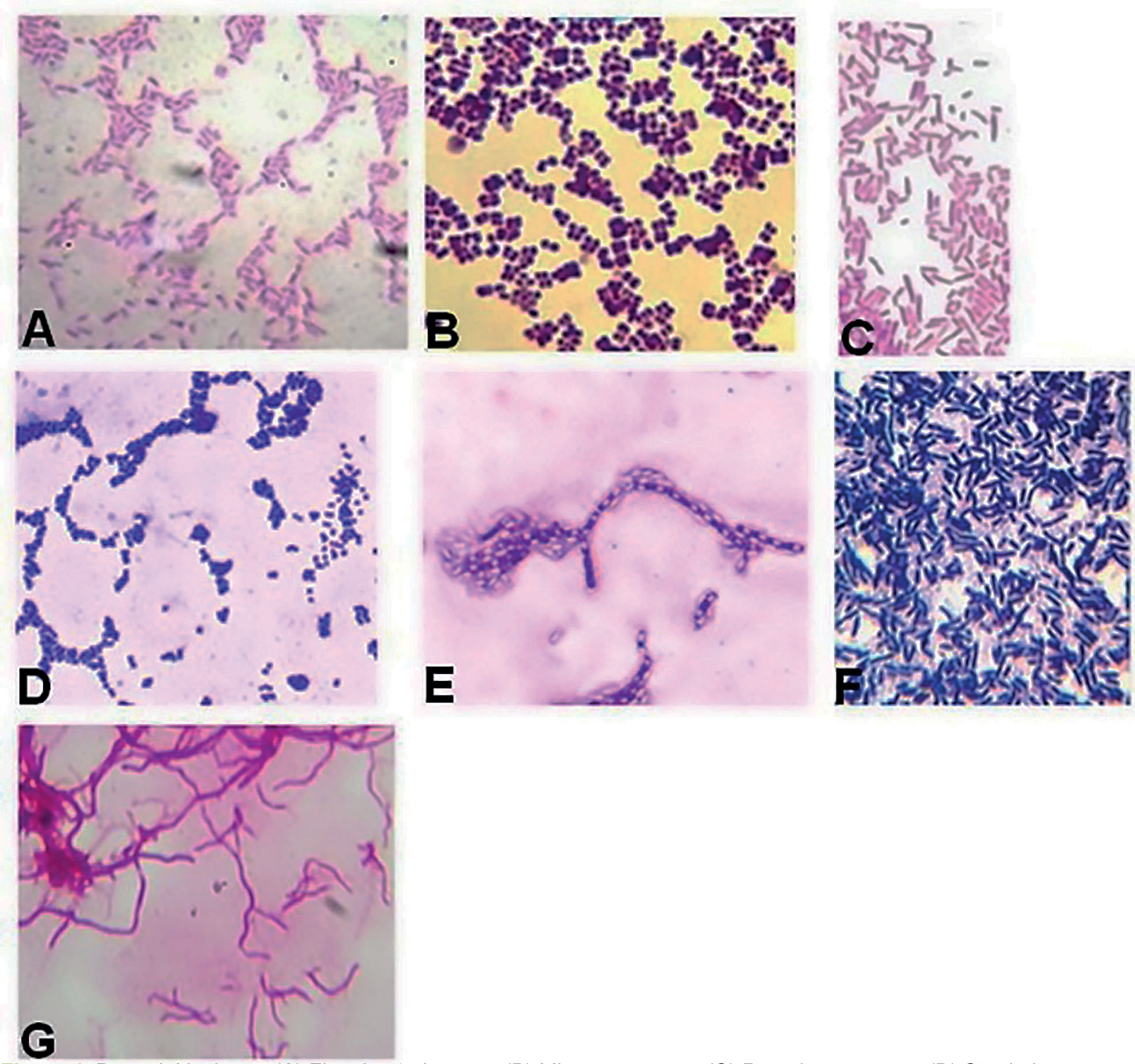


**Figure 1.** Bacterial isolates; *(A) Flavobacterium sp., (B) Micrococcus sp., (C) Pseudomonas sp., (D) Staphylococcus sp., (E) Bacillus sp., (F) Bacillus sp., (G) Actinobacteria.*

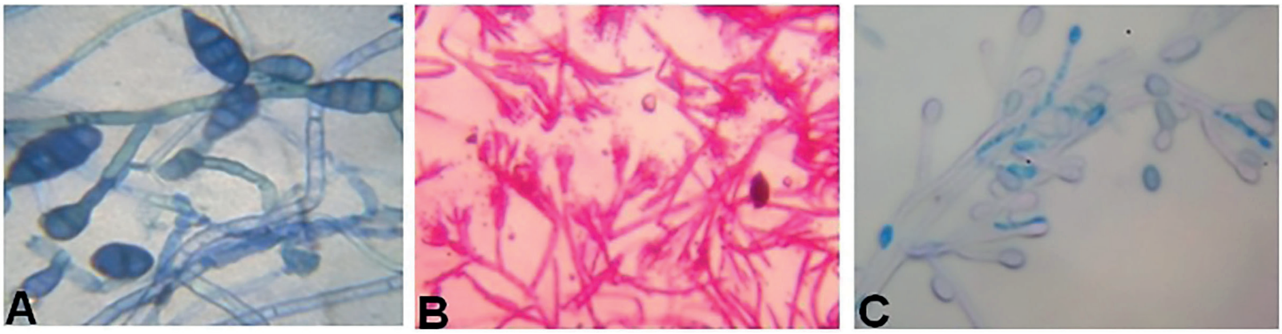


**Figure 2.** Fungal isolates; (a) *Alternaria sp.*, (b) *Penicillium sp.*, (c) *Hansfordia sp.*



## Discussion

Besides the fact that petroleum-derived products are used as the principal source of energy nowadays, they act as a globally environmental toxic pollutant[16]. This study aimed to isolate and characterize microbes capable of using petroleum hydrocarbons for their growth from the perspective of their use in the bioremediation process.

Throughout the present study, we could isolate several bacterial and fungal strains belonging mainly to the genera *Pseudomonas, Bacillus*, and *Staphylococcus* from gasoline and diesel fuel-polluted soils, *Micrococcus, Flavobacterium, Actinobacteria, Penicillium, Hansfordia* and *Alternaria*.

These microorganisms are supposed to be adapted to

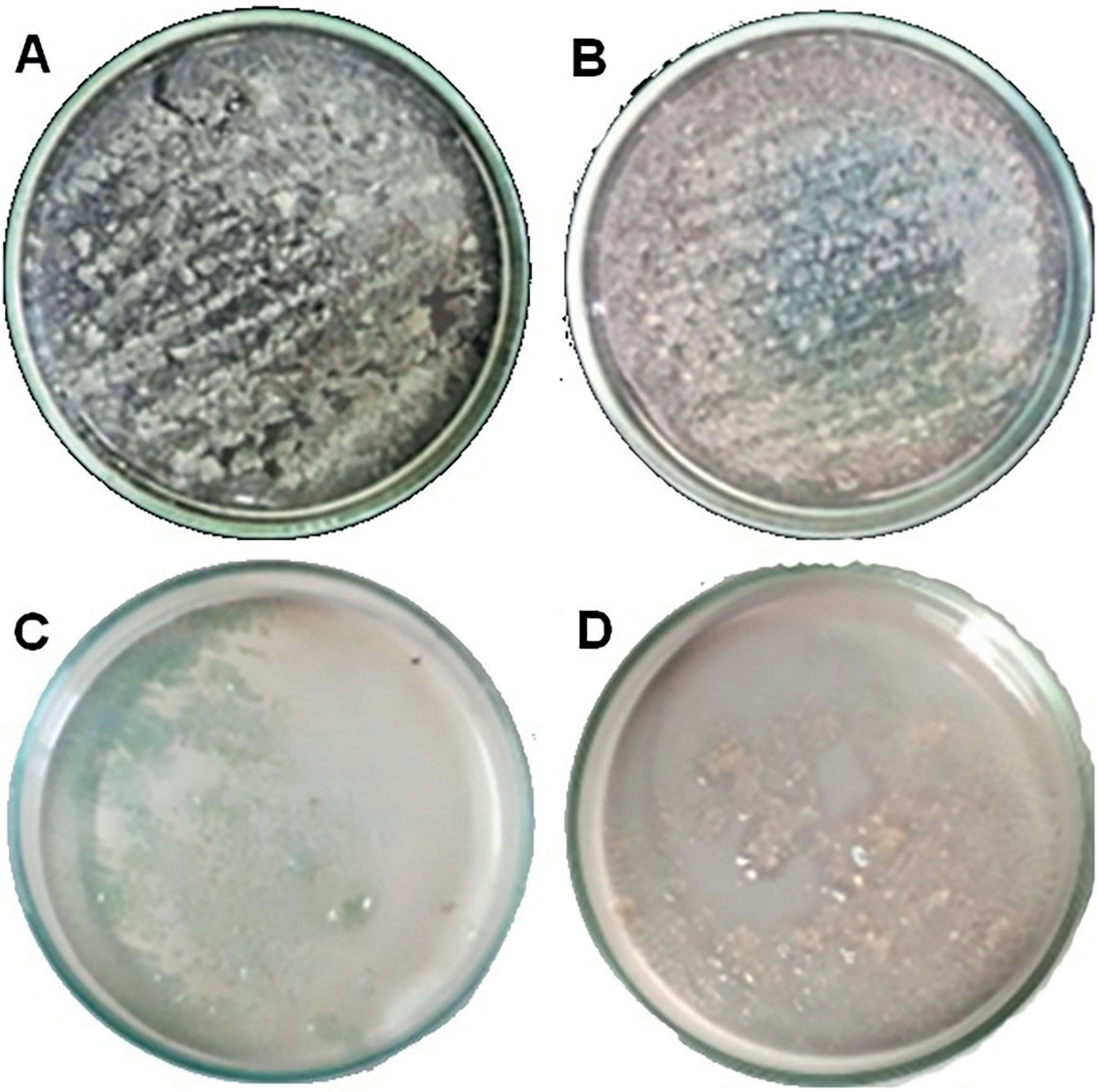


**Figure 3.** Growth of the bacterial strains on media containing (1) diesel fuel and (2) gasoline. (a) *Bacillus sp.*, (b) *Pseudomonas sp*.

the hydrocarbons present in their living environment. Hydrocarbon degradation by microbial communities depends on the composition of the specific microbial population current and its adaptive response to the presence of petroleum hydrocarbon[10]. Thanks to their enzymatic activity, many microorganisms, such as bacteria, fungi, and yeast, can use hydrocarbons as a sole carbon source. The structural similarities between xenobiotics and complex molecules in living organisms 16 can explain the presence and abundance of microorganisms in polluted environments.

Several studies on the subject demonstrated the presence of many Gram-negative and Gram-positive bacteria in soils polluted with hydrocarbons, such as *Acinetobacter, Pseudomonas, Enterobacter, Corynebacterium, Arhrobacter, Micrococcus, Staphylococcus, Rhodococcus, Bacillus* and *Sphingomonas*[17]. Likewise, several fungal genera and species have been characterized by their ability to propagate in soils and to produce extracellular enzymes allowing the use of hydrocarbons such as *Aspergillus niger*, *Aspergillus terreus*, *Rhizopus sp.*, *Alternaria* and *Penicillium sp.*[18].

Microorganisms play a crucial role in eliminating petroleum hydrocarbons and another organic pollutants from the environment. These pollutants are used as carbon sources to provide energy for microbial growth and are transformed into non-polluting substances or fully mineralized into carbon dioxide and water by microorganisms[19].

The predominance of bacteria over fungi can be explained by the fact that bacteria are much more versatile organisms and have a wider field of action and thus remain qualitatively and quantitatively predominant for metabolizing various substrates even if the fungi, thanks to their hyphae and enzymatic paraphernalia, manage to fix efficiently complex and large amounts of pollutants[20].

Moreover, with their multiple metabolic pathways (both aerobic and anaerobic), bacteria are the most active agents in diesel degradation and act as the main degraders of petroleum hydrocarbons[1,19,21]. Bacteria belonging to the genera *Pseudomonas, Bacillus, Staphylococcus* and *Streptococcus* have been shown to be able to utilize and/or degrade hydrocarbons[22]. Bhuvaneswar *et al.*[23] reported that synergistic mixed culture of Pseudomonas and Staphylococcus could degrade the diesel oil.

Furthermore, Titah *et al.*[24], isolated thirteen bacterial strains from diesel-contaminated areas, of which only *Mi-*

*crococcus* and *Staphylococcus* displayed the best resistance and highest growth in the diesel-polluted medium at different concentrations.

Al-Dhabaan[15] described hydrocarbon-degrading strains of Bacteria from contaminated sites in Khurais oil field (Dhahran, Saudi Arabia); these are *Bacillus subtilis, Pseudomonas aeruginosa* and *Bacillus cereus*. The ability to form spores when nutrients are limiting makes species of *Bacillus* self-sustainable bioremediation tools[7]. In another study, many species belonging to the genus *Bacillus; B. coagulans, B. subtilis, B. megaterium* and *B. cereus* have been isolated from petroleum–contaminated soils[2]5.

In this study, fungal strains belonging to the genera *Penicillium, Alternaria* and *Hansfordia* were isolated from diesel fuel and gasoline-contaminated soils. Indeed, several fungal species can use petroleum hydrocarbons as carbon and energy sources and assimilate into fungal biomass[26]. Fungi have several advantages in biodegradation compared to the other microorganisms because of their filamentous form and their ability to cultivate on a large group of substrates by secreting extracellular hydrolytic enzymes, which can penetrate contaminated soil and remove pollutants in a process such as co-metabolism[27]. In addition, fungal cell membranes are permeable to many organic pollutants, and these can be degraded by intracellular enzymes, ex., cytochrome P450 which is considered as an efficient candidate for the potential degradation of polyaromatic hydrocarbons[28].

Numerous studies have shown that fungi, including Penicillium spp., Absidia spinosa and Cladosporium spp. can degrade various hazardous contaminants[29]. Al-Hawash *et al.*[30] reported that *Penicillium* sp. RMA1 and RMA2 isolated from the Rumaila oil field performed effective crude oil-degrading activity and emulsification. Some fungal strains, namely *Alternaria sp., Acromonium sp., Aspergillus terreus* and *Penicillium sp.* were isolated from petroleum-polluted areas of Arak refinery (Iran) where Alternaria sp. showed the highest growth ability in the petroleum-containing media[31]. Mohammadian *et al.*[32] have reported that *Alternaria obovoidea* and *Emericellopsis pallida* were isolated from petroleum contaminated soils in Khuzestan (Iran).

Besides, in this study, we were able to demonstrate that four bacterial strains belonging to the genera *Pseudomonas* and *Bacillus* were able to grow on media containing concentrations up to 80 %; above that concentration, no growth was noticed. This indicates either the ability of bacterial isolates to utilise diesel and gasoline for their growth or at least the tolerance threshold of these bacteria to the toxicity of the pollutant. Raju *et al.*[33] suggested that microbial degradation of diesel is greatly affected by its concentration. They also indicated that two selected strains *B. thuringiensis* B3 and *B. cereus* B6, have great potential in degrading polycyclic aromatic hydrocarbons in diesel. Microorganisms prefer to grow when the concentration of hydrocarbons is low, but every organism has its tolerance level; in addition, bacterial degradation is possible when the concentration of the contaminant is below the threshold of toxicity[34]. Oyewole et al. 21 observed that the highest degradation occurred at a diesel concentration of 1 % and 5 % for both isolates *Bacillus subtilis* and *Bacillus cereus*. The maximum bacterial growth was found in 20 % (v/v) of diesel. The bacterial growth increased with increasing diesel concentration but decreased at 25 % of diesel[34]. *B. cereus* utilizes the hydrocarbons as a sole carbon source for their growth by degrading the hydrocarbon due to the production of biosurfactant[35]. *B. pumilus* produces biosurfactants and has shown very high degradation potential for diesel oil and waste engine oil hydrocarbons[36]. Lipopeptide biosurfactants are commonly produced by bacteria belonging to the genus *Bacillus*[37].

Besides, *Pseudomonas* is among the most typical bacterial genera known for its capacity to degrade hydrocarbons and produce biosurfactants that can increase the solubilization and degradation of hydrophobic compounds[38]. The ability of *Pseudomonas* sp. to degrade petrol, diesel and engine oil was observed by Veerapagu *et al.*[39].

## Conclusions

Throughout the present study, several microbial strains were isolated from diesel fuel and gasoline-contaminated soils; these were seven bacterial strains belonging to the genera *Pseudomonas, Flavobacterium, Bacillus, Staphylococcus* and *Actinobacteria*, in addition to three fungal strains belonging to the genera *Penicillium, Alternaria* and *Hansfordia*.

Four bacterial strains belonging to the genera *Pseudomonas* and *Bacillus* have demonstrated their ability to grow on media containing gasoline and diesel fuel up to the concentration of 80 %, showing either their aptitude to use these pollutants for their growth or simply their tolerance threshold to the toxicity of the contaminant.

Further studies should be performed to identify and characterize the degradation potential of the isolates and the tools they use for petroleum hydrocarbon degradation for their future use in the bioremediation process.



### Author Contributions

All authors have read and agreed to the published version of the manuscript. All authors have contributed substantially to work reported.

### Conflicts of Interest

The authors declare no conflict of interest.

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