Isolation of Indigenous Bacteria from Oil-Contaminated Soil and Evaluation of Their Bioremediation Potential (Case Study: Kuwait Soil)

ABSTRACT

Soil contamination with petroleum hydrocarbons is a major environmental challenge in oil-producing regions, including the Persian Gulf. Conventional remediation methods are costly and may cause secondary pollution, highlighting the need for sustainable biological alternatives. This study aimed to isolate indigenous hydrocarbon-degrading bacteria from oil-contaminated soil of Kuwait (initial total petroleum hydrocarbons, TPH, 10%) and evaluate their bioremediation potential. Enrichment in minimal salt medium with crude oil (2% v/v) as the sole carbon source yielded 11 bacterial isolates (KK3, KK4, KK6, KK7, KK8, KK9, KK10, KK14, KK16, KK17, KK19). Their performance was compared with two reference strains (*Pseudomonas* sp. S21-1 and H712) in a 60-day microcosm experiment (completely randomized design, 14 treatments, triplicate) using naturally contaminated soil. TPH reduction was measured gravimetrically after Soxhlet extraction. Isolate KK17 achieved the highest degradation (38%), significantly outperforming reference strains (e.g., S21-1: 21.5%). However, KK17 grew poorly at 50°C, limiting its use in hot climates. In contrast, KK16 and KK14 showed lower degradation (29% and 28%, respectively) but grew well at 50°C, making them more suitable for field applications in the Persian Gulf region. Qualitative observations (soil color lightening, reduced odor) supported the quantitative findings. Although KK17 was the most effective degrader at moderate temperature, KK16 and KK14 are better candidates for field-scale bioremediation due to their thermotolerance. Future work should focus on molecular characterization, process optimization, and consortium development.

Keywords: Oil contamination, oil-degrading bacteria, bioremediation, biodegradation, petroleum hydrocarbons, Kuwait soil, thermotolerance

Introduction

Soil contamination with petroleum hydrocarbons is one of the most critical environmental challenges of the present era, posing serious threats to ecosystem health, food security, and human well-being (1, 2). This problem is particularly severe in oil-rich countries of the Persian Gulf region, including Iran, Iraq, Saudi Arabia, and Kuwait, due to intense extraction, refining, and transportation activities, as well as military conflicts such as the 1991 Gulf War. Large areas of soil in this region are contaminated with various petroleum pollutants, including light and heavy hydrocarbons and polycyclic aromatic hydrocarbons (PAHs), with long-term negative impacts on vegetation, groundwater, and biodiversity (3, 1).

The Persian Gulf region holds nearly half of the world's proven oil reserves and has been continuously exposed to oil-related pollution. Wars, especially the 1991 Gulf War, resulted in the release of millions of barrels of oil into the environment, causing widespread soil contamination (1). Furthermore, routine industrial activities such as extraction, refining, and transport, along with accidental leaks from pipelines and storage tanks, perpetuate and intensify this pollution. Kuwait, as one of the epicenters of this contamination, has vast areas of crude oil-polluted soil, some of which remain devoid of vegetation and active microbial life even decades later. These conditions strongly demand efficient remediation strategies adapted to the local environment.

Among various methods for cleaning oil-contaminated soils, bioremediation has gained attention as an environmentally friendly, cost-effective, and sustainable approach. This process

harnesses the metabolic capacity of microorganisms, especially bacteria that can use hydrocarbons as carbon and energy sources, to convert pollutants into less harmful or harmless compounds (2, 4, 5). Compared to expensive and often secondary-polluting physical and chemical methods (e.g., soil washing, incineration, or chemical oxidation), microbial bioremediation is more compatible with ecosystems and enables natural soil restoration (2, 6). Soil is a complex, dynamic ecosystem hosting rich and diverse microbial communities. Hydrocarbon contamination can significantly alter this community structure, reducing species diversity and abundance (7, 8). However, in response to such disturbance, specialized microbial populations adapted to harsh conditions can emerge. These microorganisms are capable of degrading and consuming hydrocarbons as carbon and energy sources (2, 9). Using naturally contaminated soil (rather than artificially spiked soil) has the advantage that the microbial community has already been subjected to natural selection and may include strains specifically adapted to complex, real-world crude oil (7). This increases the chance of finding efficient bacteria with high potential for field-scale bioremediation.

The most important step for successful bioremediation is obtaining efficient microbial strains capable of degrading a wide range of hydrocarbons. Numerous studies have shown that indigenous bacteria isolated from contaminated soils, due to their adaptation to local climatic and environmental conditions, have higher potential for pollutant degradation. Well-known genera such as *Pseudomonas*, *Bacillus*, *Rhodococcus*, *Acinetobacter*, and *Stenotrophomonas* possess powerful enzyme systems (oxygenases and dehydrogenases) that enable them to degrade both aliphatic and aromatic compounds (4, 3, 5, 10, 11, 12).

Previous studies have extensively isolated oil-degrading bacteria from contaminated soils in various regions. Sarikhani et al. (13) evaluated nine bacterial isolates and identified *Pseudomonas* sp. S21-1 as the most effective, with 26% TPH degradation. Afsharnia et

al. (4) isolated 60 strains from contaminated soils around the Tabriz refinery and identified four promising genera: *Stenotrophomonas*, *Pseudochrobactrum*, *Arthrobacter*, and *Shewanella*. Ebrahimi et al. (5) isolated 19 bacterial strains belonging to 12 different genera from the Bushehr region (Iran) and confirmed their ability to degrade diesel oil.

Despite numerous studies on isolating oil-degrading bacteria from contaminated areas (14, 12), few have specifically focused on Kuwait's polluted soils while considering the region's unique climatic conditions (high temperature, salinity, and the heavy nature of local crude oil). Moreover, many previous studies have only isolated and identified bacteria without providing a comprehensive evaluation of their degradation efficiency under near-field conditions (i.e., using the original contaminated soil). Successful field-scale bioremediation requires selecting strains that not only have high degradation ability but also perform effectively in the complex soil matrix in the presence of other microorganisms.

To address the knowledge gap regarding locally adapted oil-degrading bacteria and to develop an effective cleanup strategy for the Persian Gulf region, this study aimed to isolate, purify, and identify indigenous hydrocarbon-degrading bacteria from Kuwait's contaminated soils, which have been naturally selected under extreme conditions of high temperature, salinity, and heavy crude oil. The research quantitatively and qualitatively evaluated the selected isolates' ability to remove petroleum hydrocarbons using original contaminated soil in laboratory microcosms, while also screening for thermotolerance at 50°C to ensure survival during harsh regional summers (45–50°C). A comparative analysis of degradation efficiency versus heat tolerance was conducted to identify optimal candidates that balance high breakdown capacity with environmental adaptability. The ultimate goal was to select and recommend the most robust, high-performing bacterial strains

for future field-scale bioremediation applications in Kuwait and other similarly hot, oil-polluted environments.

Materials and methods

Collection of oil-contaminated soil and measurement of physicochemical properties

The soil used in this study was naturally contaminated with petroleum hydrocarbons and originated from Kuwait. Before the experiment, soil samples were air-dried in the shade and passed through a 2-mm sieve. Physical and chemical properties were measured according to standard methods and are summarized in Table 1. Soil texture was determined by the hydrometer method (15); pH was measured in a 1:2 soil:water suspension using a pH meter (AZ 86502) (16); electrical conductivity (EC) was measured in a 1:2 suspension using an EC meter (AZ 8301) according to (17); calcium carbonate equivalent (CCE) was determined by titration with HCl (18); organic carbon was measured by the Walkley–Black oxidation method (19); available phosphorus was determined by the Olsen method (20); and available potassium was extracted with ammonium acetate (21). Soil properties are presented in Table 1.

Table 1. Selected physicochemical properties of the soil used in this study.

Soil texture (%)	Sand	Silt	Clay	Organic C (%)	CCE (%)	Available P (mg kg^{-1})	Available K (mg kg^{-1})	pH	EC (dS m^{-1})
Sandy loam	95.0	1.6	3.4	8.75	1.0	2.0	12.0	7.72	3.85

Initial TPH concentration, measured by Soxhlet extraction (22), was approximately 10% (w/w). After determining the initial contamination level, 20-g aliquots of soil were placed into sterile glass Petri dishes without any further pretreatment. All treatments were performed in three independent replicates.

Isolation and purification of bacteria from contaminated soil

For isolation and enrichment of indigenous oil-degrading bacteria, CFMM mineral medium was used (5). The CFMM medium contained (per liter): KH_2PO_4 0.8 g, Na_2HPO_4 2.2 g, NH_4NO_3 3.0 g, $CaCl_2 \cdot 7H_2O$ 0.005 g, $MgSO_4 \cdot 7H_2O$ 0.01 g. The pH was adjusted to 7.0–7.5. First, 1 g of contaminated soil was aseptically added to a 250-mL Erlenmeyer flask containing 50 mL of CFMM medium and incubated for 24 h at 28°C on a rotary shaker at 150 rpm.

Then, 5 mL of this primary microbial suspension was transferred to 50 mL of fresh CFMM medium containing 2% (v/v) crude oil as the carbon source. Enrichment continued for one week under the same conditions (28°C, 150 rpm) (Figure 1). After visible turbidity (microbial growth), serial dilutions of the enriched suspension were prepared. From 10^{-4} to 10^{-7} dilutions, 100 µL aliquots were spread onto Petri dishes containing solid CFMM medium (amended with crude oil) or nutrient agar (NA). Plates were incubated at 28°C for 24–48 h (Figure 2). Selected colonies were re-streaked on NA and then on CFMM medium; isolates that grew well on CFMM were chosen for purification and further tests.

Colonies were examined for morphological characteristics (shape, margin, texture, color, size). Bacteria were purified by successive streaking on NA (Figure 3). For preliminary identification, standard biochemical tests including Gram staining, endospore staining, catalase test, and oxidase test were performed on each isolate (23) (Table 2).

Figure 1. Initial enrichment culture for isolating bacteria from oil-contaminated soil.

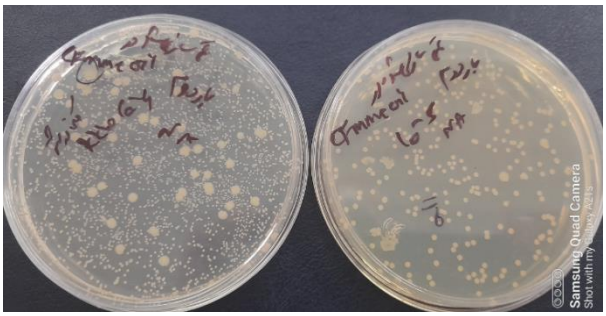

Figure 2. Examples of bacteria obtained after the initial enrichment phase on NA medium.

Figure 3. Purification of bacteria obtained after the initial enrichment phase on NA medium.

Bacterial cultivation and preparation of inoculum

Eleven bacterial isolates obtained in this study (KK3, KK4, KK6, KK7, KK8, KK9, KK10, KK14, KK16, KK17, KK19), along with two reference strains (H712 and *Pseudomonas* sp. S21-1) from the microbial bank (13), were used for the bioremediation experiment. Overnight cultures were prepared in NB medium. After reaching the desired population (OD = 1, equivalent to approximately 1×10^9 CFU/mL), the cultures were stored at 4°C. Prior to inoculation, they were diluted 10-fold and added to the soil.

Comparison of bacterial growth at 28°C and 50°C

Given the high ambient temperatures in the Persian Gulf region, the ability of isolates to grow at elevated temperature was assessed. Bacterial growth on NA solid medium was evaluated at 50°C (24). Streak cultures were prepared on plates and incubated at both 28°C and 50°C (Figure 4, Table 2). For quantitative assessment, spot cultures were used, and colony diameter was measured at regular intervals (Figure 5).

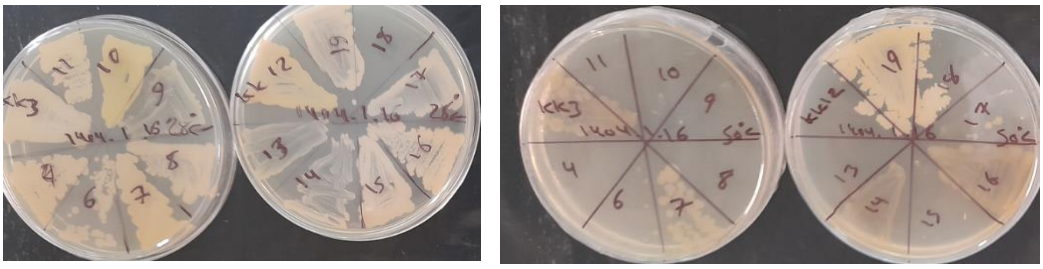

Figure 4. Evaluation of bacterial growth ability at 50°C. Streak culture of the studied bacteria at two different temperatures: 50°C (right) and 28°C (left).

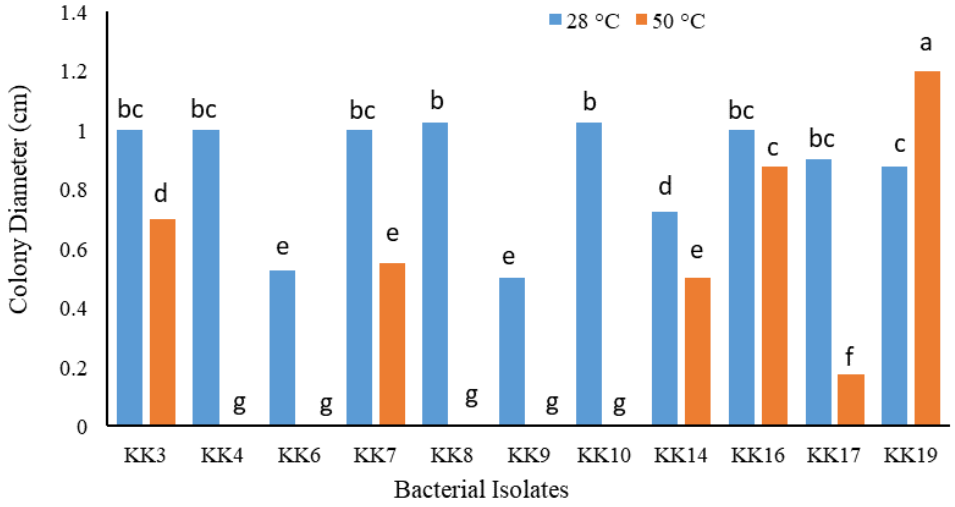

Figure 5. Comparison of bacterial colony growth in spot culture on NA medium at two different incubation temperatures after one week.

Bioremediation treatment

A microcosm experiment was conducted in sterile glass Petri dishes (Figure 6). Each Petri dish contained 20 g of contaminated soil. The experiment followed a completely randomized design with three replicates per treatment and lasted 60 days. The bioremediation protocol was as follows (13):

Surfactant addition: Tween 80 (1% v/v, 5 mL per 20 g soil) was added once at the beginning of the experiment, mixed thoroughly, and left for 24 h before microbial inoculation.

Nutrient supplementation: A nutrient solution containing 1.1 g L^{-1} P, 1.1 g L^{-1} K, and 6.6 g L^{-1} N was prepared by dissolving 5.5 g of 20:20:20 NPK fertilizer and 12 g of urea in 1 L of water.

Microbial inoculation: Bacterial inoculum (OD = 1) was diluted 1:10 with distilled water or NPK solution. Then, 4 mL of the diluted suspension was added to each soil sample and mixed uniformly. Inoculation was performed twice per week (every 3–4 days) throughout the experiment.

The initial inoculum had approximately 1×10^9 CFU/mL; after 10-fold dilution and one week of storage at room temperature, the population was ~0.5×10^8 CFU/mL, still suitable for inoculation. The final bacterial concentration in soil was higher than typical field conditions (often 1:1000 dilution) but was chosen to accelerate degradation detection at the laboratory scale. In the control treatment (contaminated soil without microbial inoculation), surfactant was added, and periodic watering and mixing were performed, but no bacterial inoculation or NPK solution was added.

Maintenance: Aeration was provided by daily manual stirring of the soil. Soil moisture was adjusted every two days by adding 4 mL of distilled water per Petri dish. On days without inoculation, only water was added if needed. All samples were maintained at room temperature (28–30°C) throughout the experiment.

TPH measurement and degradation percentage

TPH concentration was determined according to the UNEP/IOC/IAEA method (22). Briefly, 10 g of air-dried soil was wrapped in extraction thimbles and placed in a Soxhlet apparatus. Extraction was performed with 300 mL of diethyl ether for 4 h. After extraction, the residue was dried in an oven at 70°C for 48 h to constant weight. TPH concentration (as % of dry soil) was calculated as:

(1) TPH (%) = $[(W_i - W\_d) / W\_d] \times 100$

where W_i = initial weight of soil sample before extraction (10 g), and W_d = weight of soil after extraction and drying.

TPH degradation percentage was calculated as:

(2) Degradation (%) = $[(W_i - W\_f) / W_i] \times 100$

where W_i = initial TPH in control soil (time zero), and W_f = final TPH in treated soil after 60 days.

Note: The Soxhlet gravimetric method is relatively old and may have limitations (it cannot distinguish between residual oil and metabolites or native soil organic matter). However, it remains a simple, widely used screening method.

Experimental Design and Statistical Analysis

The experiment was arranged as a completely randomized design (CRD) with three replicates. Treatments included 13 bacterial isolates (11 novel + 2 reference) and one uninoculated control. Data were analyzed using SPSS software. Means were compared using Duncan's multiple range test at $p \leq 0.05$. Figures were prepared using Microsoft Excel.

Results and Discussion

Bacterial Screening

Given the target climate for bioremediation applications (hot regions of southern Iran and the Persian Gulf coast), the screening process was designed to select strains adapted to high temperatures. Isolation yielded several bacterial isolates capable of growing in crude oil-containing medium. Preliminary phenotypic observations confirmed that these isolates belonged to the indigenous microbial community of the contaminated soil and, due to long-term selective pressure from hydrocarbons, possessed inherent potential for hydrocarbon degradation. Such indigenous strains, having adapted to similar climatic conditions, are promising for field-scale bioremediation in warm regions (2).

According to Figures 4 and 5, only five isolates (KK3, KK7, KK14, KK16, KK19) could grow at 50°C. Among these, some (e.g., KK19) not only survived but grew well at this temperature, while others (e.g., KK17) showed weak growth. This suggests an inverse relationship between degradation efficiency at moderate temperature and thermotolerance, possibly due to metabolic costs associated with maintaining degrading enzymes against thermal denaturation. Given the importance of high-temperature tolerance (especially 50°C) for selecting strains suitable for field applications in hot climates, this test served as an initial screening step. Thermophilic or thermotolerant characteristics are important criteria for selecting bacteria for use in warm environments such as the study area (25, 24).

In this study, from naturally crude oil-contaminated soils of Kuwait, 11 bacterial isolates (KK3, KK4, KK6, KK7, KK8, KK9, KK10, KK14, KK16, KK17, KK19) were isolated. Enrichment was performed in minimal medium containing crude oil as the sole carbon source. The diversity in colony morphology (size, color, margin, texture) indicated a diverse microbial community shaped by long-term oil contamination (2). Gram staining and biochemical test results (catalase, oxidase, spore formation) divided the isolates into two main phenotypic groups: Gram-positive spore-formers (KK3, KK14, KK16, KK19) and Gram-negative non-spore-formers (KK4, KK6, KK7, KK8, KK9, KK10, KK17). This pattern is consistent with known oil-degrading genera such as *Bacillus* (spore-former) and *Pseudomonas* (non-spore-former) (11).

Numerous studies in contaminated areas of Iran have aimed to identify effective oil-degrading microorganisms. Nadalian et al. (26) isolated *Pseudomonas aeruginosa* BN2 from sediments of the Arvandkenar region and reported >80% crude oil degradation within five days after optimization (0.25 g L^{-1} NH_4 Cl, 0.024 g L^{-1} K_2 HPO_4). Hosseini-Boldaji et al. (27) sampled around the Tehran refinery and identified three bacterial isolates (*Nocardia*, *Bacillus subtilis*,

and *Acinetobacter baumannii*) capable of reducing or eliminating aromatic compounds in culture medium containing 2% kerosene. Heydaritabar and Moghimi (28) isolated a fungal strain *Gliomastix* sp. (ADH-02) from contaminated soils of the Shazand refinery, which degraded 75% of TPH and 91% of aliphatic compounds and reduced anthracene by 67% within 14 days. Ebrahimpour et al. (29) isolated a highly halophilic biosurfactant-producing bacterium from the salt lake of Qeshm Island that showed excellent crude oil emulsification and growth in the presence of glycerol.

Collectively, these studies confirm the high biodiversity and potential of indigenous microorganisms in the region. However, most were conducted in liquid culture media with light or refined oils, whereas the present study used real soil with heavy crude oil under near-field conditions.

Distribution of Thermotolerant vs. Mesophilic Isolates and Their Ecological Significance

Among the 11 isolates obtained, 5 isolates (45.4%) were capable of growth at 50°C (thermotolerant), while 6 isolates (54.6%) were mesophiles (grew only at 28°C but not at 50°C). Therefore, mesophiles were more common, but thermotolerant strains still constituted a significant proportion. There will be some reasoning for this distribution: 1) Initial isolation temperature: The primary isolation and enrichment were performed at 28°C, which inherently favors mesophilic bacteria. This temperature was chosen to maximize the diversity of isolates obtained, as thermophiles would still grow at 28°C (though slower), but mesophiles would not grow at 50°C. 2) Natural selection pressure: The soil originated from Kuwait, where surface temperatures can reach 50°C during summer. This has created selective pressure favoring thermotolerant strains; however, soil temperatures fluctuate diurnally and seasonally, providing niches for both

mesophiles and thermotolerants. 3) Crude oil composition: The heavy crude oil (C12+ >40%) may have favored slower-growing, metabolically versatile mesophiles that can degrade complex hydrocarbons more efficiently, whereas thermotolerance may come at a metabolic cost.

Ecological Strategy: We observed an inverse relationship between degradation efficiency at moderate temperature and thermotolerance, suggesting a trade-off mechanism. Specifically, strain KK17, a mesophile, achieved the highest degradation rate (38%) but failed to grow at 50°C, whereas the thermotolerant strains KK16 and KK14 exhibited lower degradation efficiencies (29% and 28%, respectively) while demonstrating robust growth at 50°C. This pattern indicates that thermotolerant bacteria likely invest significant energy in producing heat-shock proteins and stabilizing enzymes to survive extreme heat, thereby reducing the energy available for hydrocarbon degradation systems.

Bioremediation of contaminated soil

As shown in Figure 6, noticeable visual changes occurred in the oil-contaminated soil during bioremediation. The initial darkness gradually decreased, and soil color turned to light brown. Additionally, soil structure improved, becoming more porous and spongy, indicating better aeration. A marked reduction in petroleum odor was also observed in treated samples compared to the control. However, it must be emphasized that these qualitative observations are only supportive and cannot replace quantitative measurements; soil structure was not quantitatively measured, and odor was not assessed with any instrument. Nevertheless, the qualitative observations clearly indicated improved physical characteristics of the soil as a result of bioremediation.

Figure 6. Comparison of bioremediation treatments after bacterial inoculation vs. control. Dark sample (top right) is contaminated soil without microbial inoculation (control).

Figure 7 presents TPH degradation percentages after 60 days. The control treatment (no microbial inoculation) showed the lowest degradation, which was significantly different from bacterial treatments (Duncan's test, $p \leq 0.05$). The highest degradation (38%) was observed for isolate KK17, while the reference strain S21-1 achieved approximately 21.5% degradation. Notably, isolate KK19 showed low degradation despite being highly thermotolerant (Figures 4 and 5). This significant difference indicates that high-temperature tolerance alone is insufficient for successful bioremediation; an efficient hydrocarbon-degrading enzyme system is also essential.

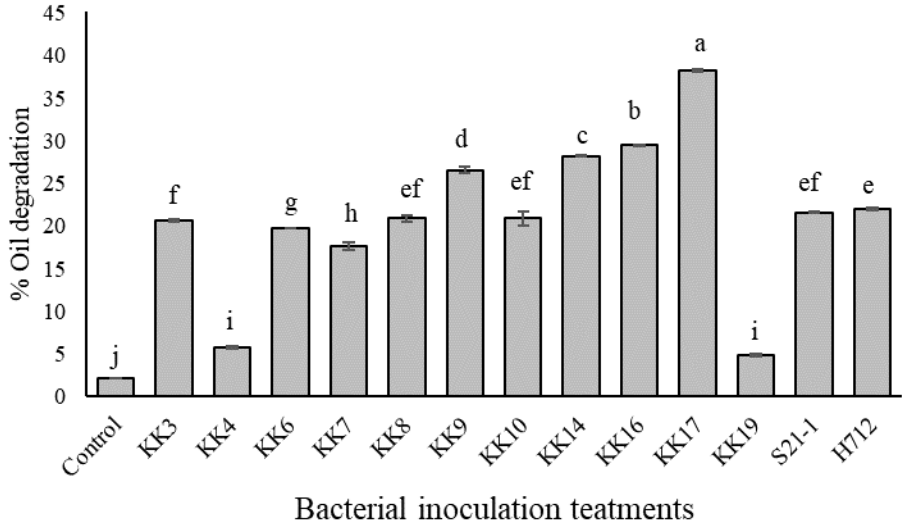

Figure 7. Percentage of oil (TPH) degradation in bacterial treatments after 60 days.

Although direct measurement of hydrocarbon-degrading enzymes (e.g., laccase, peroxidase, monooxygenase) or microbial surfactants was not performed in this study, the significant differences in oil degradation percentages among isolates can be reasonably attributed to differential expression or efficiency of these factors, based on growth patterns, physicochemical changes, and literature evidence. For example, the higher efficiency of KK17 (38% degradation) compared to reference strains (21.5%) likely results from greater production of extracellular enzymes effective against long-chain and aromatic hydrocarbons. The faster reduction in viscosity and earlier color change from dark to light brown in KK17-treated soil (Figure 6) may indirectly indicate biosurfactant production, which enhances oil bioavailability. Conversely, KK16 and KK14, despite lower degradation (29% and 28%, respectively), exhibited good growth at 50°C, possibly due to thermal stability of their enzyme systems or production of heat-resistant biosurfactants – a trait in which KK17 was deficient. Even though direct measurement of biosurfactant production was beyond the scope of this initial screening study, several qualitative observations suggest their involvement. The faster reduction in oil viscosity and earlier soil color lightening in treatments with higher degradation efficiency may indicate biosurfactant-mediated oil emulsification. Moreover, the presence of >40% C12+ hydrocarbons in the crude oil, which are highly hydrophobic and require biosurfactants for bioavailability, suggests that the observed degradation likely involved biosurfactant production. However, definitive confirmation requires future studies focusing on biosurfactant characterization, including surface tension measurement, emulsification index determination, and molecular screening for biosurfactant biosynthesis genes.

Since previous studies have shown that indigenous bacteria in Persian Gulf contaminated soils often act through combined mechanisms including biosurfactant production and oxidative enzymes, it is reasonable to assume that differences in degradation percentages in the present

isolates are largely due to variations in expression or efficiency of these factors. Although confirming these hypotheses requires additional experiments (e.g., enzyme activity assays, surface tension measurement, GC-MS), the indirect evidence from this study provides a clear pattern linking bioremediation efficiency to the metabolic potential of isolates. In other words, KK17 likely possesses a stronger enzyme system for hydrocarbon degradation, while KK16 and KK14, due to their better thermal adaptation, are more practical candidates for bioremediation in Kuwait's hot climate.

Complete removal of petroleum compounds likely requires more time. In field studies of heavily contaminated sites, the goal is often to reduce TPH to below 1%. Bioremediation progress depends not only on initial pollutant concentration but also on pollutant type (aliphatic vs. aromatic ratio), microbial type, environmental conditions (temperature, moisture, aeration, nutrients), and other factors. The relatively low degradation rate (<40%) of KK17 can be attributed to: (1) Crude oil type: GC-MS analysis of the initial sample showed it was heavy crude with a predominance of C12+ hydrocarbons (>40%), which are inherently more resistant to biodegradation due to their complex molecular structure and lower bioavailability. (2) Experimental duration: 60 days is short for complete mineralization of heavy crude oil in soil; meaningful TPH reduction in field studies often requires 6–12 months. (3) Realistic conditions: Unlike studies in liquid culture with fresh (spiked) oil that achieve >80% degradation, the present study used naturally contaminated, aged soil, which is more challenging and realistic.

While the Soxhlet gravimetric method effectively demonstrated significant TPH reduction across treatments, it cannot distinguish between the degradation of different hydrocarbon fractions or identify degradation intermediates. Although GC-MS analysis of the initial crude oil confirmed its heavy nature (>40% C12+ hydrocarbons), post-experiment GC analysis would have provided

valuable information on the degradation patterns of specific compounds. Future work should employ GC-FID or GC-MS to analyze residual oil fractions, allowing identification of preferential degradation of aliphatic vs. aromatic compounds and detection of metabolic intermediates. Nevertheless, the gravimetric method remains a validated and widely used screening tool for comparing biodegradation efficiencies among multiple treatments (30, 31).

In bioremediation, monitoring physical, chemical, and biological soil indicators is key to evaluating success (13, 32). Although the main focus of this study was quantitative TPH measurement by Soxhlet extraction, complementary qualitative observations (color and odor changes) were recorded as supportive evidence. It is emphasized that these observations do not replace quantitative analyses but are reported as visual status. For example, in the KK17 treatment (highest degradation, 38%), a gradual reduction in oil odor and lightening of soil color compared to the control were observed. This qualitative correlation supports the gravimetric findings but lacks independent analytical value. Although GC-FID analysis for distinguishing aliphatic and aromatic fractions was not performed at this stage, detailed GC-MS analysis of the crude oil sample (see supplementary file) confirmed that it was a heavy, sour crude oil with average molecular weight 156.67 g mol^{-1}, dominated by heavy C12+ hydrocarbons (>40%). The distribution of normal alkanes from C6 to C20+ and the high specific gravity (0.861) clearly indicate the heavy nature of the sample. This specific chemical composition, with high concentrations of high-molecular-weight, long-chain hydrocarbons, is the primary reason for the oil's high recalcitrance and resistance to biodegradation, as heavier compounds are less accessible to microorganisms. Another reason for the relatively low removal efficiency is the experimental duration; it appears that the tested bacteria required more time for significant hydrocarbon

removal. Obtaining efficient, environmentally adapted microbial strains is a key success factor in bioremediation projects, and the main goal of this study was to select and introduce such strains.

Table 2. Gram reaction, endospore staining, catalase, oxidase, and growth at 28°C and 50°C for the isolates used in this experiment.

Isolate	Gram reaction	Catalase	Oxidase	Endospores	Growth at 28°C	Growth at 50°C
KK3	Positive	Positive	Positive	Present	+	+
KK4	Negative	Positive	Positive	Absent	+	–
KK6	Negative	Positive	Positive	Absent	+	–
KK7	Negative	Positive	Positive	Absent	+	+
KK8	Negative	Positive	Positive	Absent	+	–
KK9	Negative	Positive	Positive	Absent	+	–
KK10	Negative	Positive	Positive	Absent	+	–
KK14	Positive	Positive	Negative	Present	+	+
KK16	Positive	Positive	Negative	Present	+	+
KK17	Negative	Positive	Positive	Absent	+	–
KK19	Positive	Positive	Negative	Present	+	+

Note: (+) = growth; (–) = no growth.

A significant limitation of this study is the lack of molecular identification (16S rRNA gene sequencing) of the isolated strains. While phenotypic characterization suggests that the isolates belong to known oil-degrading genera (e.g., *Bacillus*, *Pseudomonas*, *Acinetobacter*), definitive

taxonomic assignment requires molecular analysis. Future studies should employ 16S rRNA gene sequencing to precisely identify these strains and to screen for functional genes involved in hydrocarbon degradation pathways. Despite this limitation, the phenotypic screening approach successfully identified promising candidates for bioremediation based on degradation efficiency and thermotolerance, which were the primary objectives of this study.

Conclusion

This study aimed to isolate and screen indigenous oil-degrading bacteria from contaminated soils of Kuwait and evaluate their efficiency in reducing TPH. From soil containing 10% TPH, 11 bacterial isolates were obtained, of which five were capable of growth at 50°C. Based on the 60-day bioremediation test at 28–30°C, isolate KK17 showed the highest TPH reduction (38%), followed by KK16 (29%) and KK14 (28%). However, isolates KK16 and KK14, due to their resistance to 50°C, are more suitable candidates for field applications in hot regions (e.g., the Persian Gulf), even though their TPH reduction percentages were lower. This finding emphasizes the necessity of multi-criteria screening, where both maximum degradation and stability under realistic environmental conditions are considered. GC-MS analysis of the initial crude oil revealed it was heavy crude with >40% C12+ hydrocarbons, inherently more resistant to biodegradation – a likely primary reason for the relatively low TPH reduction (<40%) across all treatments.

Although this study confirmed the ability of several indigenous isolates to reduce TPH, the achieved degradation percentages (below 40%) indicate that the search for even more efficient strains for field applications remains essential. The gap between laboratory results and field expectations can only be bridged through deeper molecular and biochemical studies. Therefore, future work should focus on: (1) identifying genes involved in alkane and aromatic compound

degradation pathways; (2) quantitative measurement of key enzyme activities (e.g., oxygenases, dehydrogenases); (3) quantitative assessment of biosurfactant production using standard methods and identification of responsible genes; and (4) designing bacterial consortia with complementary capabilities. These steps will pave the way from initial laboratory observations toward practical, effective bioremediation applications in contaminated areas.

Based on the findings of this study, we recommend the following for field-scale bioremediation in Kuwait and similar hot regions: for strain selection, KK16 and KK14 are recommended over KK17 despite their lower degradation rates, due to their thermotolerance and ability to survive at 50°C; however, a consortium approach combining these thermotolerant strains with the mesophilic but highly degradative KK17 could provide high degradation activity during cooler periods while maintaining viability during heat extremes. Additionally, nutrient supplementation (NPK fertilizer) should be optimized to support bacterial growth, as used in this study, and the use of Tween 80 or other surfactants may be necessary to enhance oil bioavailability, particularly given the heavy nature of the crude oil. Finally, field bioremediation should be planned over longer timeframes (months rather than weeks), with regular monitoring of TPH reduction at regular intervals to assess progress.

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Declaration of competing interest

The authors declare no conflict of interest.

References

(1) Al-Maamary HM, Kazem HA, Chaichan MT. Climate change: the game changer in the Gulf Cooperation Council Region. Renewable and Sustainable Energy Reviews. 2017 Sep 1;76:555-76. https://doi.org/10.1016/j.rser.2017.03.048

(2) Aziz ZS, Jazza SH, Dageem HN, Banoon SR, Balboul BA, Abdelzaher MA. Bacterial biodegradation of oil-contaminated soil for pollutant abatement contributing to achieve sustainable development goals: A comprehensive review. Results in Engineering. 2024 Jun 1;22:102083. https://doi.org/10.1016/j.rineng.2024.102083

(3) Sarikhani MR, Afsharnia M, Zarei M, Lotfollahi A. Comparison of Different Bioremediation Methods in Reducing Petroleum Hydrocarbons of a Contaminated Soil (Case Study: Evaporation Ponds of Tabriz Refinery). Water and Soil Science. 2025a : 23-41. https://doi.org/10.22034/SPS.2025.66736.1004

(4) Afsharnia M, Sarikhani MR, Zarei M. Isolation of oil degrading bacteria from oil contaminated soil around the oil refinery and petrochemical plants of Tabriz and identification of the efficient bacteria. Water and Soil Science. 2022 Dec 22;32(4):91-104. https://doi.org/10.22034/ws.2021.45023.2408

(5) Ebrahimi M, Fallah A, Sarikhani MR. Isolation and identification of oil-degrading bacteria from oil-polluted soils and assessment of their growth in the presence of gas oil. Water and soil science. 2013 May 5;23(1):109-21. https://water-soil.tabrizu.ac.ir/article_201.html?lang=en

(6) Zeynali K, Shariati S, Pourbabaei AA, Shorafa M. The application of biosurfactant producing bacterial consortium as a petroleum degrader in increasing the hydraulic conductivity coefficient of TPH-contaminated soil. Iranian Journal of Soil and Water Research. 2024b Nov 21;55(9):1585-99. https://doi.org/10.22059/ijswr.2024.376847.669716

(7) Moradi S, Sarikhani MR, Ale-Agha AB, Hasanpur K, Malboobi MA, Semenov M. Effect of Over a Century of Oil Contamination on Total and Cultivable Bacterial Communities in Arid Desert Soils, Iran. Water, Air, & Soil Pollution. 2026 Jan;237(1):31. https://doi.org/10.1007/s11270-025-08744-y

(8) Moradi S, Sarikhani MR, Ale-Agha AB, Hasanpur K, Shiri J. Effects of natural and prolonged crude oil pollution on soil enzyme activities. Geoderma Regional. 2024 Mar 1;36:e00742. https://doi.org/10.1016/j.geodrs.2023.e00742

(9) Baniasadi M, Mousavi SM. A comprehensive review on the bioremediation of oil spills. Microbial action on hydrocarbons. 2019 Feb 9:223-54. https://doi.org/10.1007/978-981-13-1840-5_10

(10) Auffret MD, Yergeau E, Labbé D, Fayolle-Guichard F, Greer CW. Importance of *Rhodococcus* strains in a bacterial consortium degrading a mixture of hydrocarbons, gasoline, and diesel oil additives revealed by metatranscriptomic analysis. Applied microbiology and biotechnology. 2015 Mar;99(5):2419-30. https://doi.org/10.1007/s00253-014-6159-8

(11) Zeynali K, Shariati S, Pourbabaee AA. The role of effective oil-eating bacteria in the remediation of oil-contaminated soils (Case study: *Bacillus* genus). Journal of Sol Biology. 2024a Sep 22;12(1):105-39. https://doi.org/10.22092/sbj.2024.366140.266

(12) Sadighbayan K, Farazmand A, Mazaheri-Asadi M, Monadi Sefidan A, Aliasgharzad N. A Comparative Study on Four Strains of Petroleum Hydrocarbon-degrading Bacteria for the Bioremediation of Petroleum-contaminated Soils. Journal of Microbial Biology. 2021 Mar 21;10(37):51-65. https://doi.org/10.22108/bjm.2020.122322.1290

(13) Sarikhani MR, Lotfollahi A, Hashemzadeh S. Comparative Analysis of Bacterial Isolates for Bioremediation of an Oil-Contaminated Soil. Journal of Soil and Plant Science. 2025b Oct 23;35(3):79-94. https://doi.org/10.22034/sps.2025.67844.1014

(14) Ghavidel A, Najirad S, Alikhani HA. Isolation and Study of Indigenous Bacteria of the Contaminated Soils in Southern of Tehran Oil Refinery plant for Bioremediation of Oil ontaminations. Water and Soil Science. 2016 Nov 21;26(3):175-85. https://water-soil.tabrizu.ac.ir/article_5543.html?lang=en

(15) Bouyoucos GJ. A recalibration of the hydrometer method for making mechanical analysis of soils. 1951, 434–438. https://doi.org/10.2134/agronj1951.00021962004300090005x

(16) Rich CI. Soil chemical analysis. Soil Science Society of America Journal. 1958 May;22(3):272-272. https://doi.org/10.2136/sssaj1958.03615995002200030025x

(17) Richards LA, editor. Diagnosis and improvement of saline and alkali soils. US Government Printing Office; 1954. http://dx.doi.org/10.1097/00010694-195408000-00012

(18) Martin AE, Reeve R. A rapid manometeic method for determining soil carbonate. Soil Science. 1955 Mar 1;79(3):187-98.

(19) Walkley A, Black IA. An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. Soil science. 1934 Jan 1;37(1):29-38. http://dx.doi.org/10.1097/00010694-193401000-00003

(20) Olsen SR, Sommer LE. Phosphorus. Pp. 403–430. In: Page, A.L, Page AL, Miller RH, Keeney DR. Methods of soil analysis, part 2. Chemical and microbiological properties. 1982;2:643-98.

(21) Jones Jr JB. Laboratory guide for conducting soil tests and plant analysis. CRC press; 2001 Jun 28. USA.

(22) Anonymous. United States. Environmental Protection Agency. Office of Solid Waste, Emergency Response. Test methods for evaluating solid waste: Physical/chemical methods. US Environmental Protection Agency. Office of Solid Waste and Emergency Response; 1986.

(23) Reddy CA, Beveridge TJ, Breznak JA, Marzluf G, editors. Methods for general and molecular microbiology. American Society for Microbiology Press; 2007 Aug 17.

(24) Meintanis C, Chalkou KI, Kormas KA, Karagouni AD. Biodegradation of crude oil by thermophilic bacteria isolated from a volcano island. Biodegradation. 2006 Mar;17(2):3-9. https://doi.org/10.1007/s10532-005-6495-6

(25) Al-Maghrabi IM, Aqil AB, Islam MR, Chaalal O. Use of thermophilic bacteria for bioremediation of petroleum contaminants. Energy Sources. 1999 Jan 1;21(1-2):17-29. Energy Sources, 21(1-2):17-29. https://doi.org/10.1080/00908319950014920

(26) Nadalian B, Ebrahimipour G, Shahriari Mogadam M. Biodegradation of crude oil contamination by a bacterium strain isolated from sediment of Arvandkenar area. Journal of Health. 2016 Jul 10;7(3):263-75. http://healthjournal.arums.ac.ir/article-1-989-fa.html

(27) Hosseini-Boldaji SA, Soltani H, Teimouri M. Isolation and purification of petroleum-decomposing bacteria from contaminated soils and identification the resulted compounds of degradation of such compounds. Applied Biology. 2023 Aug 23;36(1):109-34. https://doi.org/10.22051/jab.2022.40410.1486

(28) Heydaritabar R, Moghimi H. Evaluation of anthracene biodegradation by *Gliomastix* sp. isolated from contaminated soil of Shazand oil refinery, Iran. Nova Biologica Reperta. 2026 Jun 8;4(3):246-54. https://www.sid.ir/paper/250530/fa

(29) Ebrahimpour GH, Fouladi J, Ferdousi A. Isolation and characterisation of crude oil biodegrading extreme halophilic biosurfactant-producing bacterium and an examination of the effect of salt concentrations on crude oil biodegradation by this strain. 2005; 9-19. https://envs.sbu.ac.ir/article_96971.html?lang=fa

(30) Sun Y, Ma J, Yue G, Liu S, Liu H, Song Q, Wu B. Comparisons of four methods for measuring total petroleum hydrocarbons and short-term weathering effect in soils contaminated by crude oil and fuel oils. Water, Air, & Soil Pollution. 2021 Sep;232(9):381. https://doi.org/10.1007/s11270-021-05341-7

(31) Ezeani EU, Ngobiri NC, Agbagwa IO. A review of techniques for the determination of total petroleum hydrocarbons in soils. Asian Journal of Applied Chemistry Research. 2022 Jun 14;11(2):38-43. https://doi.org/10.9734/AJACR/2022/v11i230254

(32) Tayyeb SR, Kazemi Pour N, Hassanshahian M, Rokhbakhsh-Zamin F, Khoshro M. A Comparative Study on the Effects of Biostimulation and Bioaugmentation on the Remediation of Contaminated Sediments with Crude Oil in the Persian Gulf. Journal of Microbial Biology. 2023 Jun 22;12(46):99-112. https:// doi.org/10.22108/bjm.2023.134414.1477