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# Identification of Oil Degrading Bacteria from Oil-Contaminated Soil in the Northeastern Part of Jordan

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## ABSTRACT

Bioremediation aspects of crude oil-polluted fields can be achieved by isolating and identifying bacterial species from oil-contaminated soil. This allows for the selection of the most active isolates and the enhancement of the effectiveness of other bacteria. This project will be a base to use green technology for clean the oil contaminated soil in Jordan. This study involved the isolation and identification of oil-degrading microbes from soil samples contaminated with oil in the northeastern region of Jordan. The morphological and biochemical tests were used to characterize twenty-five bacterial isolates. Molecular identification of a universal primer 16S rDNA gene was used to identify bacterial isolates. Total petroleum hydrocarbons were analyzed using gas chromatography for soil samples. All soil samples were analyzed for heavy metal contamination (Cu, Cd, Mn, Zn, and Pb). The bacterial growth count (CFU/g) was between $1.06\times10^{5}$ and $2.80\times10^{17}$. The identified bacterial genera included: Staphylococcus, Citrobacter, Lactobacillus, Alcaligin's, Pseudomonas, Micrococcus, Serratia, Enterobacter, Bacillus, Salmonella, Mycobacterium, Corynebacterium, and, Microbacterium. The most species showed high growth rates on different types of hydrocarbons such as toluene, naphthalene, and hexane were Lactobacillus casei, Staphylococcus intermedius, Micrococcus luteus, Pseudomonas putida, Mycobacterium phlei, Corynebacterium xerosis. Soil sample M1A contains the highest levels of Fe, Cd , and Pb and Cu,. While M1C contains the highest levels of Fe and Mn. On the other hand, M2A, and M2C have the least levels of Mn and Fe. While M3C has the least level of Zn and Pb. our study conclude the bacterial isolates could be used for in situ and ex situ cleanup of oil-contaminated desert soil in northeastern part of Jordan.

**Keywords:** hydrocarbons, bioremediation, 16S rDNA gene, oil degrading bacteria.

## INTRODUCTION

Crude oil spills during oil production, storage, and transportation as well as due to pipeline leakage and tank failure have made crude oil the most common organic pollutant in all environments. This pollutant is categorized as a hazardous waste due to its cytotoxic, mutagenic, and carcinogenic effects on organisms and life components. These contaminants that are present in contaminated desert soils can leach into the surrounding subsurface and ground water, posing a threat to the environment and to human health (Jamrah et al., 2007). Many hydrocarbons are insoluble in water, thus remain partitioned in the non-aqueous-phase liquid (Cubitto et al., 2004). The contamination of hydrocarbons has significant harmful impacts on plants, such as diminished seed germination and reduced plant growth. This is due to the ability of hydrocarbons to form a coating on plant roots, which in turn reduces the absorption of water and nutrients. (Kuhn et al., 1998). Hydrocarbon molecules have the ability to deeply penetrate plant tissues and harm the cell membrane, resulting in the release of cell contents and obstruction of the spaces between cells. This ultimately leads to a decrease in the transit of metabolites and the rate of respiration. (Xu and Johnson, 1995). Analyses of desert soil samples revealed that the level of lead (total or bioavailability) was three-fold greater in crude oil-contaminated soils than in uncontaminated soils (AL-Saleh and Obuekwe, 2005). Poor microbial proliferation and diversity are typical for desert soils with sandy texture and low organic carbon, which are also characterized by lower degradation rates as compared to

clay loam soils. The lower abundance of degraders in the sand fraction of contaminated soils is correlated to a higher C:N ratio and lower internal surface. The fertility of desert soils has typically been determined by analyzing a number of chemical and physical parameters, such as water holding capacity, aggregate stability, loss of soil, nutrient content and carbon fractions. The biological parameters, including basal respiration and microbial biomass, and the biochemical properties, such as hydrolases of the carbon, nitrogen, and phosphorus cycles, are more closely associated with the microorganisms in desert soil, making them more sensitive and better indicators of the actual degradation state. (Ros et al., 2003).

Currently, the employed decontamination procedures include both mechanical and chemical treatments. However, these methods are inefficient and expensive (Rajasulochana et al., 2016), necessitating the development of various treatment methods that can reduce risks to workers and be considered environmentally safe during oil spill cleanup (*in situ* and *ex situ*). Azubuike et al., (2016) describe multiple processes of utilizing microorganisms, particularly bacteria, to degrade hazardous waste components, such as crude oil, from the environment. Microorganisms play a crucial role in bioremediation, but their presence and activity are influenced by various environmental factors. These factors include temperature, moisture, pH, and the composition of the microbial population, which includes both bacteria and fungi. (Emmanuel et al., 2017). Microorganisms use organic pollutants as a source of energy, carbon, and nutrients (Azubuike et al., 2016). Certain bacterial genera, including *Pseudomonas*, *Alcaligenes*, *Bacillus*, *Micrococcus*, and add more genera *Serratia, staph., Salmonella, lacto. citrobacter, mycobacterium* are particularly efficient for the initiation of bioremediation (Ojewumi et al., 2018; Fadhil et al., 2017; Godambe et al., 2017; Wu et al., 2016; Phulpoto et al., 2016; Kawo et al., 2016; Emmanuel et al., 2017; Obi et al., 2016). In addition, microorganisms can degrade large amounts of organic pollutants associated with oil, such as aliphatic compounds, n-alkanes, monoaromatic compounds, toluene, and polycyclic aromatic hydrocarbons (PAHs). However, few genera can degrade high-molecular-weight PAHs (Khanafer et al., 2017). One promising new technique to analyze environmental samples for petroleum hydrocarbons is comprehensive gas chromatography (GC). The gas chromatography plot displays the compound peaks in a way that is based on the chemical class (y-axis) and carbon number (x-axis). In the case of petroleum, this leads to distinct chemical groups including cycloalkanes, alkanes, and aromatics with one, two, or more rings; further categorization reveals homologous series within each of these classes of chemicals. A number of chemicals and classes of compounds in crude oil and refined petroleum products have been identified and quantified using gas chromatography. In addition, Some biomarkers detected in crude oil by gas chromatography include sulfur-containing aromatic hydrocarbons (PASHs), alkylated polycyclic aromatic hydrocarbons (PAHs), sterane, and hopane. Determining the chemical composition of degraded petroleum can be quite challenging. This is because severe degradation typically eliminates aromatics and abundant n-alkanes, leaving behind an unresolved complex mixture (UCM) of petroleum hydrocarbons that contains numerous branched and cyclic alkanes. Many of these saturated chemicals can be resolved using volatility-by-shape or volatility-by-polarity selectivity in gas chromatography. (Nelson et al., 2006).

The present study focused on crude oil transportation highways between Jordan and Iraq, which are in the northeastern part of Jordan and contain desert soil. Desert soil samples were collected from three sites contaminated due to oil tanker accidents and oil spills from old pipelines in that area between Jordan and Iraq, which can be affected by the life flora there (Fig. 1). The collected samples represent desert (dry) soil contaminated by crude oil many years ago. The aim of this study was to isolate and identify bacteria from oil-contaminated desert soil in northeastern Jordan, assess their ability to degrade different types of hydrocarbons (crude oil, toluene, naphthalene, and hexane) (Fig. 2), In the further step, we can use these bacterial isolates to clean up the area and remove the spills of crude oil then let the plant to grow up again, as known as the *In situ* Bioremediation step.

## MATERIALS AND METHODS

### Soil sampling

Three soil samples, each weighing 250 grams, were obtained from three oil-contaminated sites in the northeastern region of Jordan. The samples were collected at depths of 5, 10, and 15 cm. the s. The soil samples were ground and filtered through a sieve with a pore size of 2 mm, and they were subsequently placed in polyethylene bags and stored at a temperature of 4 ºC until used.

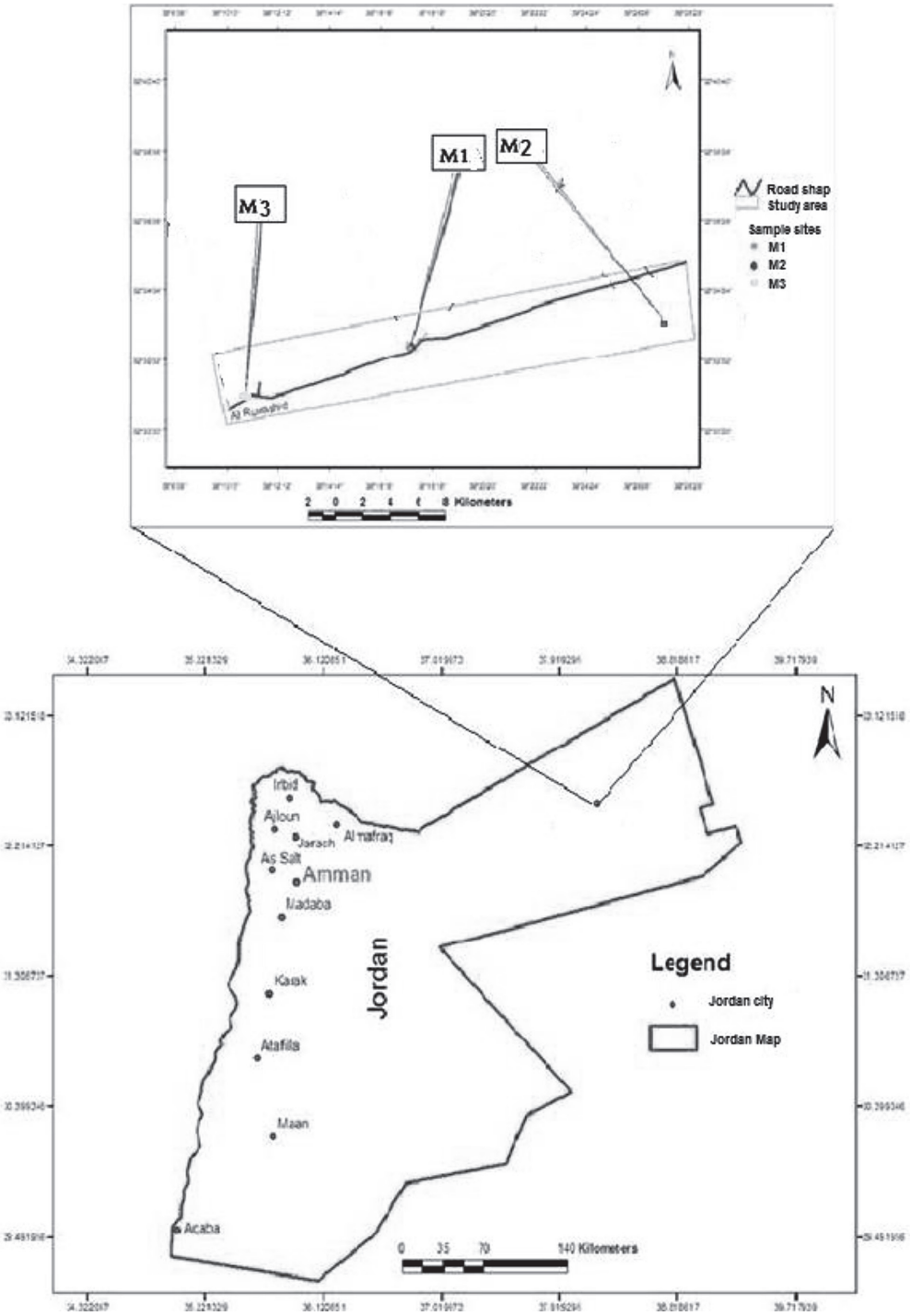


**Figure 1.** The Hashemite Kingdom of Jordan, showed thr northeat part of Jordan area

## Culture methods

Approximately 150 grams of soil samples were mixed with 300 milliliters of minimal-salt medium (Stanier's media) in 500 milliliters Erlenmeyer flasks. The flasks were supplemented with crude petroleum oil at concentrations of 400, 600, 1500, and 2000 parts per million (ppm). The flasks were then incubated at 37 °C along with 160 RPM rotary shaking. As controls, samples devoid of crude petroleum oil were employed.

## Isolation and enumeration of bacteria

To obtain isolated colonies on tryptic soy agar (TSA), and nutrient agar (N.A), a series of dilutions ranging from 10–5 to 10–18 were prepared using Stanier's media as the diluent. Each distinct

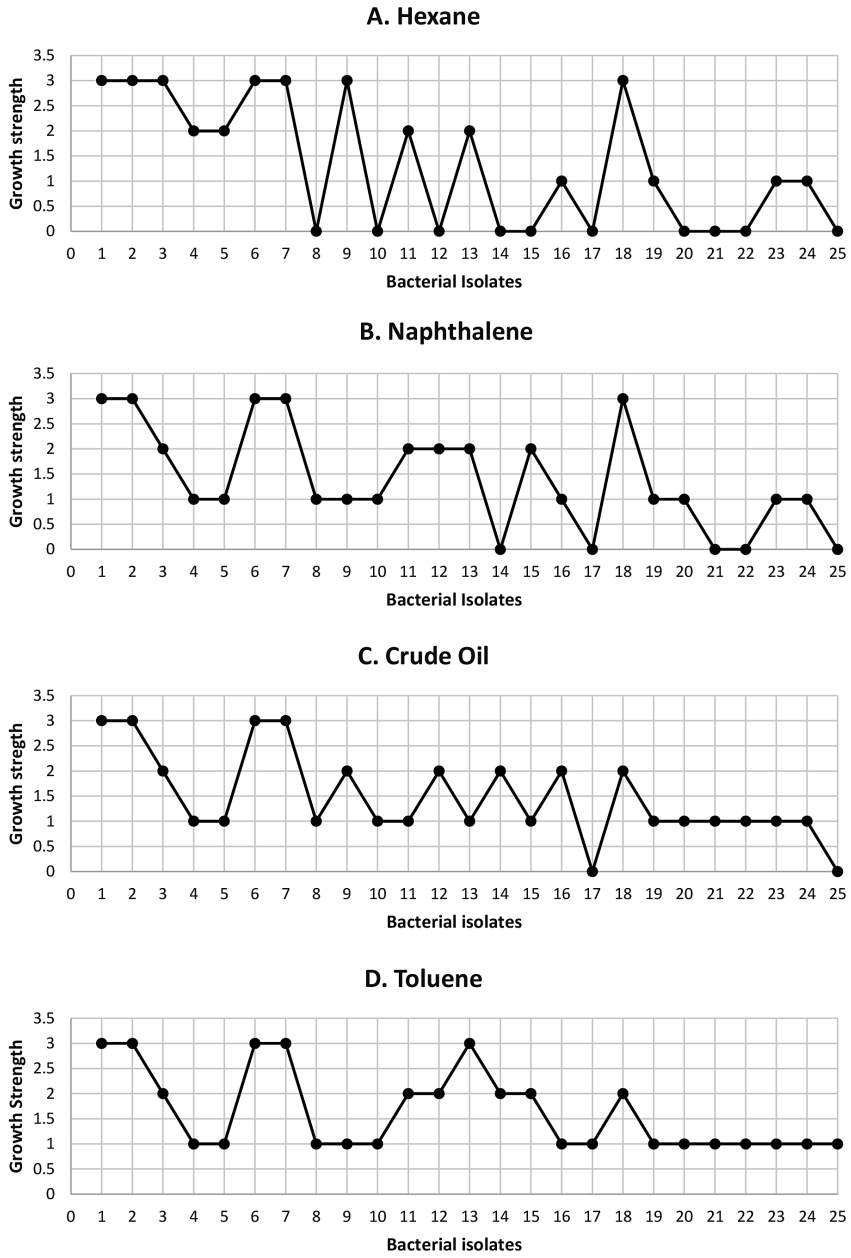


**Figure 2.** Growth rate of oil degrading bacteria on minimal salt media that was supplemented by four kinds of hydrocarbons, (a) hexane, (b) naphthalene, (c) crude oil, (d) toluene. II - +1: very low no. of scattered colonies, +2: Large No. of medium size colonies, +3: bacterial growth all one the plate. III- these results apearance after triplicate number of this isolates

colony type was carefully selected and isolated on individual culture plates.

## Isolation of oil-degrading bacteria from soil samples by using different sources of hydrocarbons

Bacterial colonies were cultured on minimal salt media (staneir's agar), supplemented with 400 ppm of hydrocarbon compounds (crude oil, toluene, naphthalene, and hexane), and then incubated at 37 °C for 10 days.

## Morphological features and biochemical and physiological characterizations

The bacterial isolates were morphologically characterized based on their color, size, colony characteristics (shape and elevation), and gram staining. The identification of bacterial isolates was conducted through the utilization of biochemical and physiological tests (Holt et al., 1994). The bacterial colonies were classified based on their shape, which included circular, punctiform, filamentous, and rhizoid shapes.

Additionally, the colonies were further classified based on their color, which ranged from yellow, brown, and creamy white to green.

## Molecular characterization

### *Extraction of genomic DNA*

The extraction of bacterial DNA was performed as described by the standard protocol of phenol chloroform-based extraction method (Chachaty and Saulnier (2000)). Each individual bacterial isolate was inoculated into 10 ml of nutrient broth, and then incubated for 18 h at 37 °C. Two ml of the broth culture were centrifuged at 14,000 RPM for 15 min. The pellet was suspended in 567 µl TE buffer, 3 µl Proteinase K (20 mg/ml), and 30 µl 25% w/v SDS. Then it was incubated for 30 min at 65 °C. The mixture was incubated for 10 minutes at 65 °C with 100 microliters of 5 M NaCl and 80 ul of CTAB/NaCl. Following the addition of Phenol, Chloroform, and Isoamyl Alcohol in equal amounts (25:24:1), the mixture was agitated and centrifuged at 14000 RPM for 5 minutes. Following the removal and transfer of the solution's supernatant to a new tube, chloroform: isoamyl alcohol in equal volumes (24:1) was incorporated.The mixture was centrifuged again at 14000 RPM for 5 min, and the supernatant of the solution was transferred to a new tube. A RNase enzyme (1.4 mg/ml) was added to the mixture for 35 minutes at 37 $^{o}$C, then the solution was cooled with ice, and an equal volume of ice-cold isopropanol was added to precipitate the DNA. The DNA pellets were washed three times with 70% ethanol and rehydrated in 100 µl TE buffer (pH 7.5).

## Polymerase chain reaction (PCR)

### *Identification of bacterial isolates through sequencing of 16S rDNA*

The target sequence of 16S rDNA was amplified according to the procedure described by Daane and his co-workers (2001). The PCR mixture (50 µl) contains 25 µl master mix (10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, a 200 µM deoxynucleoside triphosphates and 2.5 U of Taq DNA polymerase), 2 µl from reverse and 2 µl from forward 16S rDNA universal primer, 5 µl DNA template and 16 µl D.$H_2O$. Reaction mixtures were incubated in a DNA thermal cycler (Xp cycler, USA, 2008) at 96 °C heat shock for 1 min and 35 cycles of PCR reaction (denaturation at 94 °C for 30 sec, annealing at 45 °C for 30 sec, and extension at 72 °C for 1.5 min), according to Daane et al., 2001. All reaction mixtures were stored at 4˚C. The forward primer sequence (FD1) was '5-AGAGTTTGATCCTGGCTCAG-3' and the reverse primer sequence (RD1) was '5-GGTTACCTTGTTACGACTT-3'. Finally, 16S rDNA samples were sent for sequencing at (Macrogen, Inc., Koria) as described by Edward et al. (1989).

## Gel electrophoresis and photography

An aliquot (5 µl) of each amplification reaction was analyzed on 1.5% w/v agarose gels. Gels were stained with ethidium bromide (0.5µg/ml) and analyzed using BioDocAnalyze (Biometra, Germany). A 1000 base pair marker was included in every gel.

## Petroleum hydrocarbon determination

The EPA method 3510 was utilized to extract soil samples for GC analysis. Ten grams of soil were added to a 40 mL VOA vial. This was followed by the addition of 5 g of anhydrous sodium sulfate, 15 mL of surrogate working solution (phenylacetylene) and 10 mL of methylene chloride to the VOA vial. The caped vial was placed in a sonic bath (Grant, Inc., Germany) for 5 min. After shaking the vial, it was returned to the sonic bath for an additional five minutes. The hydrocarbon components were quantified using gas chromatography (Calruse 500 auto sampler, Perkin Elmer, USA) with a flame ionization detector (FID) and a capillary column Rtx-1 (30m × 53mm; 0.1 um film thickness; Silica fused, Philadelphia, Pa., USA). The temperatures of the injection port and detector were maintained at 290 °C and 320 °C, respectively. Following five minutes at 50 °C, the oven temperature was progressively raised at a rate of 10 °C per minute until it reached 320 °C. The air and hydrogen gas flow rates were both adjusted to 2 ml/min for the flame ionization detector. The carrier gas employed was helium. The temperature progression rate and initial temperature were determined in accordance with the retention period of the spiked compounds.

## Heavy metal detection

Heavy metals such as cadmium, copper, ferrous, manganese, zinc, and lead were extracted

from soils using a diluted HCl acid extraction method that was described by Sutherland (2001). Two hundred milligrams (dry weight) of soil samples were placed in a 100 mL plastic bottle, then 4 mL of 25% HCl, 4 mL of 25% $HNO_3$ and 2 mL of HF were added. The samples were shaken for 2 minutes before being incubated in a water bath at 70 °C for 2 hours. Fifty mL of boric acid were added, and the sample was placed back in the water bath for 15 min. Each sample filtrate was increased in volume up to 100 mL with $D.H_2O$ and analyzed by an atomic absorption spectrophotometer (Analytic Jena, Inc., AG).

## RESULTS AND DISCUSSION

### Enumeration of bacterial colonies

The findings presented in Table 1 illustrate the number of bacterial colonies (CFU) identified on N.A in each soil sample. The biodegradation ability of the examined bacterial isolates was improved by exposing them to different concentrations of crude oil (400, 600, 800, 1000, 1500, and 2000 ppm) for a duration of 50 to 300 days. The results showed that M1B produced the greatest CFU when exposed to either the lowest (400 ppm) or the highest (2000 ppm) concentration of crude oil. Specifically, the CFU values obtained were $0.75 \times 10^9$ and $2.80 \times 10^{17}$ CFU at concentrations of 400 ppm and 2000 ppm, respectively. In contrast, M2C sample showed the least $1.06 \times 10^5$ and $2.19 \times 10^{14}$ CFU at a concentration of 400 ppm and 2000 ppm, respectively. While uncontaminated (negative control; $9.8 \times 10^4$ CFU·$g^{-1}$ soil) soil. Variations in colony numbers detected among test samples may be related to collection from recently contaminated and old contaminated sites. Old, contaminated sites showed the highest toxicity levels of contaminants and the most adverse effects on microbial diversity. Furthermore, the addition of different concentration of crude oil (400 – 2000 ppm) to the bacterial culture of each site can be showed increasing of bacterial growth parallel with crude oil concentration rising Table 1.

### Oil-degrading bacteria growth on minimal salt media with a different source of hydrocarbons

Bacterial isolates could degrade different sources of hydrocarbons. Of the 25 bacterial isolates; 15 could degrade specific n-alkanes, such as hexane; 25 could degrade compounds with a single benzene ring by growth on the media, such as toluene; and 20 isolates could degrade compounds with two benzene rings, such as naphthalene. *Lactobacillus casei, Staphylococcus intermedius, Micrococcus luteus, Pseudomonas putida, Mycobacterium phlei, Corynebacterium xerosis* showed high growth rates on toluene, naphthalene, and hexane (Fig. 2). While other isolates, such as *Micrococcus luteus, Bacillus subtilis, Pseudomonas putida,* and *Salmonella enterica* have shown low levels of growth on all tested hydrocarbons. In addition, *Corynebacterium xerosis, Staphylococcus epidermidis, Bacillus subtilis, Serratia liquefaciens,* and *Citrobacter freundii* were only capable of utilizing toluene as sole energy and carbon sources (Fig 2). Our results indicate that the identified bacterial isolates could utilize the supplemented hydrocarbons as a sole source of energy and carbon.

### Morphological features, biochemical and physiological characterization of oil degrading bacteria

The bacterial colonies were classified based on their shape, which included circular, punctiform, filamentous, and rhizoide. The isolated

**Table 1.** Growth of bacterial isolates from soil samples at different concentration of crude oil

| Crude oil (ppm) | Soil samples | | | | | | |
|---|---|---|---|---|---|---|---|
| | M1A | M1B | M1C | M2A | M2C | M3B | M3C |
| 400 | 2.28E+06 | 7.50E+08 | 1.60E+07 | 1.86E+06 | 1.06E+05 | 1.56E+07 | 1.04E+07 |
| 600 | 2.39E+08 | 1.32E+13 | 1.95E+08 | 2.64E+08 | 1.86E+07 | 1.79E+08 | 2.14E+09 |
| 800 | 1.15E+10 | 1.84E+14 | 1.25E+10 | 2.87E+10 | 2.33E+09 | 1.89E+09 | 2.54E+10 |
| 1000 | 2.23E+12 | 1.57E+15 | 1.72E+12 | 1.57E+13 | 2.76E+11 | 2.35E+10 | 2.31E+11 |
| 1500 | 1.57E+14 | 1.90E+16 | 1.89E+13 | 1.79E+15 | 1.46E+13 | 2.13E+11 | 2.14E+13 |
| 2000 | 1.20E+16 | 2.80E+17 | 1.80E+15 | 2.76E+16 | 2.19E+14 | 2.05E+13 | 1.78E+15 |

colonies exhibited a wide range of colors, such as yellow, brown, creamy white, and green. The gram stain examination revealed that a significant proportion of the bacterial isolates exhibited the characteristic of gram-negative bacilli, while a lesser proportion comprised gram-positive bacilli and gram-positive cocci. Concerning the response of bacterial isolates to gram stain, most bacterial

**Table 2a.** Morphological features, biochemical and physiological characterization of oil degrading bacteria

| Characterisation | No | Tests | Bacterial isolates | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Morphological characterization | 1 | Gram Stain | + B | +C | + C | - B | - B | - B | + B | + B | + B | + B | - B | + C |
| | 2 | Spore stain | - | NA | NA | NA | NA | NA | - | - | - | + | NA | NA |
| | 3 | Acid fast stain | NA | NA | NA | NA | NA | NA | - | + | NA | NA | NA | NA |
| | 4 | Growth media | N.A | N.A | TSA | N.A | N.A | N.A | N.A | TSA | TSA | TSA | TSA | N.A |
| | 5 | Colony color | Yellow | White | White | White | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Yellow | Brown |
| | 6 | Colony Size | Small | Small | Large | Large | Large | Small | Medium | Large | Small | Small | Large | Medium |
| | 7 | Colony Form | Irregular | Circular | Irregular | Circular | Rhizoid | Circular | Circular | Circular | Circular | Irregular | Irregular | Circular |
| | 8 | Colony elevation | Raised | Flat | Convex | Convex | Flat | Raised | Convex | Flat | Flat | Raised | Convex | Raised |
| | 9 | Colony margin | Entire | Undulated | Entire | Entire | Entire | Filamentous | Entire | Undulated | Filamentous | Entire | Entire | Undulated |
| Biochemical and physiological characterization | 1 | Catalase test | + | + | + | + | + | + | + | + | + | - | + | + |
| | 2 | Oxidase test | + | + | + | + | + | + | + | + | + | + | + | + |
| | 3 | Nitrate reduction test | + | + | + | + | - | + | + | + | + | + | + | + |
| | 4 | Methyl red test | + | + | - | - | + | + | + | + | + | + | + | - |
| | 5 | Degradation of starch | - | - | + | - | + | - | + | - | - | - | - | - |
| | 6 | Degradation of urea | + | + | - | + | - | + | + | + | - | - | + | + |
| | 7 | Degradation of casein | + | + | + | - | - | - | + | - | + | + | - | - |
| | 8 | Degradation of Tween-20 | + | + | + | + | - | - | - | - | - | - | - | + |
| | 9 | Degradation of Tween-80 | - | - | - | + | - | - | - | - | - | - | - | - |
| | 10 | Degradation of gelatin | - | - | - | - | - | - | - | + | + | + | - | - |
| | 11 | Gas and acid production from D-lactose | + | + | - | + | - | + | + | + | +G | - | + | - |
| | 12 | Gas and acid production from D-galactose | + | + | - | +G | - | + | +G | + | - | - | + | - |
| | 13 | Gas and acid production from D-sucrose | + | + | - | +G | - | - | + | + | - | - | + | - |
| | 14 | Gas and acid production from D-insitol | + | + | - | + | - | + | + | + | + | + | + | - |
| | 15 | Gas and acid production from D-maltose | + | + | - | +G | - | + | + | + | - | + | + | - |
| | 16 | Gas and acid production from D-fructose | + | + | - | +G | - | +G | +G | +G | +G | +G | +G | +G |
| | 17 | Utilization of citrate and propionate | + | + | + | + | + | + | + | + | + | + | + | + |
| | 18 | Blood hemolytic | β | β | γ | γ | γ | γ | β | β | β | β | γ | γ |
| | 19 | Triple sugar iron test (TSI) | K/A + H | K/A + H | K/NC + H | K/A | K/NC | K/A + H | A/A + H | K/A - H | K/NC | A/A | A/A | A/A + H |
| | 20 | Growth temperature 4 ˚C | - | - | - | - | - | - | - | - | - | - | - | - |
| | 21 | Growth temperature 37˚C | + | + | + | + | + | + | + | + | + | + | + | + |
| | 22 | Growth temperature 50˚C | - | - | - | - | - | - | - | + | - | - | - | - |
| | 23 | Growth in the presence of NaCl 3% | + | + | + | + | + | + | + | + | + | + | + | + |
| | 24 | Growth in the presence of NaCl 10% | + | - | - | - | - | - | - | + | - | + | - | - |
| | 25 | Growth in the presence of NaCl 15% | - | - | - | - | - | - | - | - | - | - | - | - |
| | 26 | Eosin methylene blue (EMB) | + | + | + | + | + | + | + | + | + | + | + | + |
| | 27 | Macconkey agar | + | + | - | + | - | + | + | + | + | - | - | - |
| Bacterial Species | | | *Lactobacillus casei* | *Staphylococcus intermedius* | *Micrococcus luteus* | *Enterobacter cloacae* | *Alcaligenes faecalis* | *Pseudomonas putida* | *Lactobacillus casei* | *Mycobacterium phlei* | *Lactobacillus casei* | *Bacillus subtilis* | *Citrobacter freundii* | *Micrococcus luteus* |

**Note:** C – cocci, B – bacilli, F – fluorescent, NA: not available. N.A – nutrient agar, TSA – tryptice soya agar, G – gas

isolates were gram negative bacillus, while some were gram positive bacillus and others were gram positive coccus (Table 2). Bacterial isolates were identified using biochemical and physiological tests shown in Table 2. The bacterial genera *were found to be Staphylococcus, Lactobacillus, Micrococcus, Pseudomonas, Bacillus, Microbacterium*, *Serratia, Alcaligenes, Enterobacter, Mycobacterium,*

**Table 2b.** Morphological features, biochemical and physiological characterization of oil degrading bacteria

| Characterisation | No | Tests | Bacterial isolates | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |
| Morphological characterization | 1 | Gram Stain | + B | + B | + C | + C | - B | + B | + B | + B | + B | +C | - B | - B | - B |
| | 2 | Spore stain | - | - | NA | NA | NA | - | + | - | + | NA | NA | NA | - |
| | 3 | Acid Fast stain | + | NA | NA | NA | NA | - | NA | - | NA | NA | NA | NA | - |
| | 4 | growth Media | N.A | N.A | N.A | TSA | N.A | TSA | N.A | TSA | N.A | N.A | N.A | N.A | TSA |
| | 5 | Colony Color | Yellow | White | White | Yellow | Yellow | Yellow | White | Yellow | Yellow | White | Yellow | White | Brown |
| | 6 | Colony Size | Small | Small | Small | Small | Medium | Large | Medium | Medium | Small | Small | Small | Small | Small |
| | 7 | Colony Form | Circular | Circular | Circular | Circular | Irregular | Circular | Circular | Irregular | Circular | Circular | Circular | Circular | Irregular |
| | 8 | Colony elevation | Raised | Convex | Raised | Flat | Raised | Raised | Flat | Raised | Raised | Raised | Raised | Flat | Raised |
| | 9 | Colony margin | Undulated | Entire | Entire | Entire | Entire | Irregular | Entire | Undulated | Entire | Irregular | Entire | Irregular | Entire |
| Biochemical and physiological characterization | 1 | Catalase test | + | + | + | + | + | + | + | + | + | + | + | - | + |
| | 2 | Oxidase test | + | + | - | - | - | - | - | - | + | - | + | - | - |
| | 3 | Nitrate reduction test | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | 4 | Methyl red test | + | - | - | - | + | + | - | - | - | - | - | + | + |
| | 5 | Degradation of starch | - | - | - | - | - | - | - | - | + | - | + | - | - |
| | 6 | Degradation of urea | + | + | - | - | - | + | - | - | - | - | - | - | + |
| | 7 | Degradation of casein | - | - | + | - | + | - | + | - | - | + | - | + | + |
| | 8 | Degradation of Tween-20 | + | + | - | + | - | + | - | + | - | + | - | - | + |
| | 9 | Degradation of Tween-80 | - | - | - | - | - | + | - | - | - | + | - | - | + |
| | 10 | Degradation of gelatin | - | - | - | - | - | - | - | - | + | + | - | + | + |
| | 11 | Gas and acid production from D-lactose | + | + | - | - | - | + | - | - | - | - | - | - | + |
| | 12 | Gas and acid production from D-galactose | + | + | - | - | +G | + | - | - | - | - | - | - | + |
| | 13 | Gas and acid production from D-sucrose | + | + | - | - | +G | +G | - | - | + | + | - | - | - |
| | 14 | Gas and acid production from D-insitol | + | + | - | +G | - | + | - | - | - | - | - | + | + |
| | 15 | Gas and acid production from D-maltose | + | + | - | - | +G | +G | - | - | - | + | - | + | + |
| | 16 | Gas and acid production from D-fructose | +G | +G | +G | +G | +G | + | - | - | - | - | - | + | +G |
| | 17 | Utilization of citrate and propionate | + | + | - | - | + | + | + | + | + | + | + | + | + |
| | 18 | Blood hemolytic | γ | γ | γ | γ | γ | α | γ | γ | γ | β | γ | α | γ |
| | 19 | Triple sugar iron test (TSI) | K/A +G, H | A/A | NC/NC | NC/NC | A/A | K/A G | K/A H | NC/NC | K/A | K/NC | K/NC | K/A | K/A H |
| | 20 | Growth temperature 4 °C | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 21 | Growth temperature 37°C | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | 22 | Growth temperature 50°C | + | - | - | - | - | - | - | - | - | - | - | - | - |
| | 23 | Growth in the presence of NaCl 3% | + | + | + | + | - | - | - | - | - | - | - | - | - |
| | 24 | Growth in the presence of NaCl 10% | - | - | - | - | - | + | - | - | + | - | - | + | - |
| | 25 | Growth in the presence of NaCl 15% | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 26 | Eosin methylene blue (EMB) | + | + | - | - | + | + | + | + | + | + | + | +L | + |
| | 27 | Macconkey agar | + | + | - | - | + | + | + | + | + | + | + | - | + |
| Bacterial Species | | | *Mycobacterium phlei* | *Corynebacterium xerosis* | *Microbacterium steraromaticum strain L20* | *Micrococcus luteus* | *Serratia liquefaciens* | *Corynebacterium xerosis* | *Bacillus subtilis* | *Corynebacterium xerosis* | *Bacillus subtilis* | *Staphylococcus epidermidis* | *Pseudomonas putida* | *Salmonella enterica* | *Citrobacter freundii* |

**Note:** C – cocci, B – bacilli, F – fluorescent, NA – not available, N.A – nutrient agar, TSA – tryptic soya agar, G: gas.

Corynebacterium, *Citrobacter* and *Salmonella.* Twenty-five bacterial isolates were detected from culture on a minimal medium supplemented with crude oil, and the isolated bacteria were identified based on their morphological and biochemical characteristics (Table 2). Bacterial diversity among the isolates was apparent. The bacterial isolates showed different colors, such as yellow, brown, and white indicating various pigments produced by these isolates (Table 1).

Similar results were obtained from previous studies have reported that crude oil-contaminated soils are dominated by the genus *Pseudomonas* (Ojewumi et al., 2018; Godambe et al., 2017; Wu et al., 2016; You et al., 2018; Obi et al., 2016). Conversely, other studies have reported that bacterial species belonging to *Micrococcus* (Kawo et al., 2016; Phulpoto et al., 2016), *Alcaligenes* (Godambe et al., 2017; Phulpoto et al., 2016), *Bacillus* (Phulpoto et al., 2016; Godambe et al., 2017; Wu et al., 2016), *Mycobacterium* (Phulpoto et al., 2016; Das et al., 2015), *Lactobacillus* (Adams et al., 2016), *Enterobacter* (Adams et al., 2016; Ejaz et al., 2021), *Corynebacterium* (Mateos et al., 2017; Ezekoye et al., 2017), *Salmonella* (Mateos et al., 2017; Ezekoye et al., 2017), *Serratia* (Azubuike et al., 2016), *Citrobacter* (Oaikhena et al., 2016), *Microbacterium* (Salam et al., 2015), and *Staphylococcus* (Oaikhena et al., 2016).

## Molecular identification of the bacterial isolates

Genomic DNA was isolated from all 25 bacterial isolates. The target sequence of 16S rDNA was amplified using a DNA thermal cycler (Xp cycler, USA, 2008). Therefore, the sequence of 16S rDNA of all isolates was determined (Macrogen, Inc., Koria) and bacterial isolates were identified (Table 3). The identification of the isolated bacterial strains was performed by sequencing the 16S rDNA sequencing as described previously (Katsivela et al., 1999). The 16S rDNA sequences of the newly isolated species and strains have been deposited in the NCBI database

**Table 3.** Bacterial species identification based on 16s rDNA sequencing data

| Isolates No. | Total length | Gene bank accession No. | Similarity % | Identification result |
|---|---|---|---|---|
| 1 | 1100 bp | AP012544.1 | 100 | *Lactobacillus casei* |
| 2 | 1299 bp | MK015768.1 | 99 | *Staphylococcus intermedius* |
| 3 | 870 bp | CP082331.1 | 100 | *Micrococcus luteus* |
| 4 | 1303 bp | MT436392.1 | 99 | *Enterobacter cloacae* |
| 5 | 1260 bp | NR_025357.1 | 97 | *Alcaligenes faecalis* |
| 6 | 1180 bp | MN318320.1 | 100 | *Pseudomonas putida* |
| 7 | 1134 bp | MF168938.1 | 100 | *Lactobacillus casei* |
| 8 | 1095 bp | KF378762.1 | 99 | *Mycobacterium phlei* |
| 9 | 1402 bp | MK774613.1 | 98 | *Lactobacillus casei* |
| 10 | 1470 bp | MT372489.1 | 97 | *Bacillus subtilis* |
| 11 | 975 bp | MH371322.1 | 99 | *Citrobacter freundii* |
| 12 | 1193 bp | MN905159.1 | 98 | *Micrococcus luteus* |
| 13 | 1330 bp | GU142927.1 | 98 | *Mycobacterium phlei* |
| 14 | 1260 bp | FN179319.3 | 97 | *Corynebacterium xerosis* |
| 15 | 953 bp | MK721043.1 | 98 | *Microbacterium esteraromaticum* |
| 16 | 1208 bp | CP026366.1 | 99 | *Micrococcus luteus* |
| 17 | 1151 bp | MN540916.1 | 99 | *Serratia liquefaciens* |
| 18 | 1216 bp | CP046322.1 | 96 | *Corynebacterium xerosis* |
| 19 | 1266 bp | OR144785.1 | 99 | *Bacillus subtilis* |
| 20 | 1026 bp | KU315398.1 | 99 | *Corynebacterium xerosis* |
| 21 | 880 bp | OR394146.1 | 99 | *Bacillus subtilis* |
| 22 | 1130 bp | MN850519.1 | 99 | *Staphylococcus epidermidis* |
| 23 | 1183 bp | MT373557.1 | 97 | *Pseudomonas putida* |
| 24 | 1149 bp | FJ544366.1 | 97 | *Salmonella enterica* |
| 25 | 1148 bp | ON231736.1 | 99 | *Citrobacter freundii* |

as shown in table 3. The phylogenetic tree of oil degrading bacteria that were isolated and identified by 16s rDNA sequencing data is shown in Figure 5.

Molecular identification was performed using PCR-amplified 16S rRNA sequences, which is currently used as a sensitive and specific detection method for microorganisms (Thijs et al., 2017; Obi et al., 2016). A universal primer pair specific to a 16S rRNA gene fragment was used to identify the 25 bacterial isolates, and positive results were recorded for all isolates with an amplification band corresponding to 1500 bp, which confirmed that all isolates were bacterial species (Fig. 5). In this study, all bacterial isolates were identified at the molecular level (Table 3). Bacterial identification using the 16S rDNA universal primer was done. A PCR product of 1500 bp was obtained for all tested isolates with the 16S rDNA universal primer pairs conserved sequences between all bacteria. In details, molecular identification were identified 13 different genus of bacterial isolates (*Micrococcus, Pseudomonas, Lactobacillus, Bacillus, Citrobacter, Corynebacterium, Mycobacterium, staphyloccuous, salmonella, Serratia, Enterobacter, Alcaligenes, Microbsacterium)* and 14 different bacterial species; *Micrococcus luteus, Pseudomonas putida, Lactobacillus casei, Bacillus subtilis, Citrobacter freundii, Corynebacterium xerosis, Mycobacterium phlei*, *Staphylococcus intermedius, Enterobacter cloacae, Alcaligenes faecalis, Microbacterium esteraromaticum, Serratia liquefaciens, Salmonella enterica*, *Staphylococcus epidermidis* (Table 4).

Moreover, bacterial isolates were identified more than one time at different soil sample as fellow; *Micrococcus luteus, Pseudomonas putida, Lactobacillus casei, Bacillus subtilis, Citrobacter freundii, Corynebacterium xerosis, Mycobacterium phlei*. These bacteria isolates known as a common isolate in bioremediation, and they can be survived in the stress condition (Table 4).

**Table 4.** List of the oil degrading bacterial isolates that extracted from different soil samples

| Number of isolates | Bacterial isolates codes | Species |
|---|---|---|
| 1 | M1A(1) | *Lactobacillus casei* |
| 2 | M1A(2) | *Staphylococcus intermedius* |
| 3 | M1A(3) | *Micrococcus luteus* |
| 4 | M1B(4) | *Enterobacter cloacae* |
| 5 | M1B(5) | *Alcaligenes faecalis* |
| 6 | M1B(6) | *Pseudomonas putida* |
| 7 | M1B(7) | *Lactobacillus casei* |
| 8 | M1B(8) | *Mycobacterium phlei* |
| 9 | M1B(9) | *Lactobacillus casei* |
| 10 | M1B(10) | *Bacillus subtilis* |
| 11 | M1B(11) | *Citrobacter freundii* |
| 12 | M1C(12) | *Micrococcus luteus* |
| 13 | M1C(13) | *Mycobacterium phlei* |
| 14 | M1C(14) | *Corynebacterium xerosis* |
| 15 | M1C(15) | *Microbacterium esteraromaticum strain L20* |
| 16 | M1C(16) | *Micrococcus luteus* |
| 17 | M2A(1) | *Serratia liquefaciens* |
| 18 | M2A(2) | *Corynebacterium xerosis* |
| 19 | M2C(3) | *Bacillus subtilis* |
| 20 | M2C(4) | *Corynebacterium xerosis* |
| 21 | M3B(1) | *Bacillus subtilis* |
| 22 | M3B(2) | *Staphylococcus epidermidis* |
| 23 | M3C(3) | *Pseudomonas putida* |
| 24 | M3C(4) | *Salmonella enterica* |
| 25 | M3C(5) | *Citrobacter freundii* |

**Note:** M – name of site, A – 0–5 cm depth of soil samples, B – 5–10 cm depth of soil samples, C – 10–15 cm depth of soil samples, D – 15–20 cm depth of soil samples.

### Petroleum hydrocarbon determination

The total petroleum hydrocarbons (TPH) were measured by GC/FID analyses of n-alkanes ($C_4$-$C_{30}$), monoaromatic hydrocarbons, and polyaromatic hydrocarbons (PAHs). The total petroleum hydrocarbons (TPHs) content of the eight oil-contaminated soils ranged from 3,629 to 43,298 ppm (Figure 3). Total petroleum hydrocarbons (TPH) were analyzed by gas chromatography, M1A soil sample contains the highest levels of total petroleum hydrocarbons (43,298 ppm), o-xylene, and naphthalene. In contrast, the M3B soil sample contains the lowest levels of total petroleum hydrocarbons (3,629 ppm).

The gas chromatography instrument was used to determine the concentrations and type of total petroleum hydrocarbons (ppm) in the analyzed soil samples, based on retention time. The results show that soil samples M1A, M1C, and M2C had the highest levels of total petroleum hydrocarbons (ppm) (43,298, 19,281, and 16,586 ppm, respectively), while samples M2A and M3B had the lowest (Figure 3).

### Heavy metals determination

Bacterial isolates were positively affected by some heavy metals such as iron and manganese, which were considered important constituents of trace elements and reflected low toxicity. However, other metals such as copper and zinc showed toxicity at high concentrations. Other heavy metals, including lead and cadmium, are considered important trace elements but reflect high toxicity and show reduced biological activities. The concentration (ppm) of heavy metals (i.e., Cu, Cd, Zn, Pb, and Mn) present in contaminated soil samples were expressed as mg/kg dry weight of soil. The type and concentration (ppm) of heavy metals detected at 10 soil samples at different locations within polluted sites is shown in Figure 4. However, Figure 4 presented results indicate that soil sample M1A contains the highest levels of Fe, Cu, Cd and Pb. While M1C contains the highest levels of Fe and Mn. On the other hand, M2A, and M2C have the least levels of Fe and Mn. While M3C has the least level of Zn and Pb.

Heavy metal contamination has negatively influenced the viability of crude oil biodegrades in oil contaminated soil. Some heavy metals have toxic and inhibitory effects on microorganisms. Copper toxicity is mainly due to its interaction with nucleic acids, which alter the enzyme active sites and lead to the oxidation of membrane components, and processes that can be related to the ability of copper to generate toxic hydroxyl free radicals (Cervantes and Corona, 1994).

Heavy metals were analyzed by a flame atomic absorption device. They affected bacterial growth and, because of degradation of crude oil and CFU measurement were affected. M1A contains the highest levels of Cu, Mn, Cd and

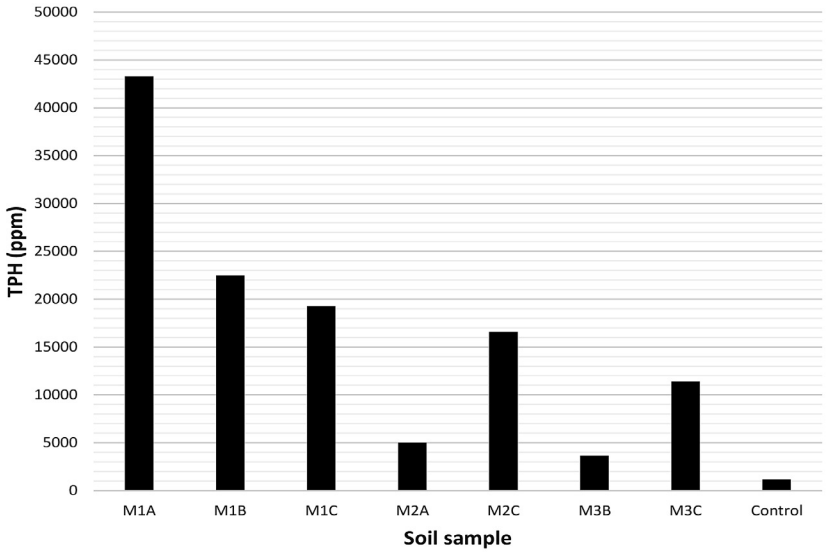


**Figure 3.** I – total petroleum hydrocarbones in 8 soil samples and showing the time of oil contamination for each site. II – M: soil of contaminated by oil, C: soil uncontaminated by crude oil, A: 0–5 cm of depth, C: 10–15 cm of depth and D: 15–20 cm of depth. III – www.restek.com (retention time of hydrocarbones in a same condition from this website)

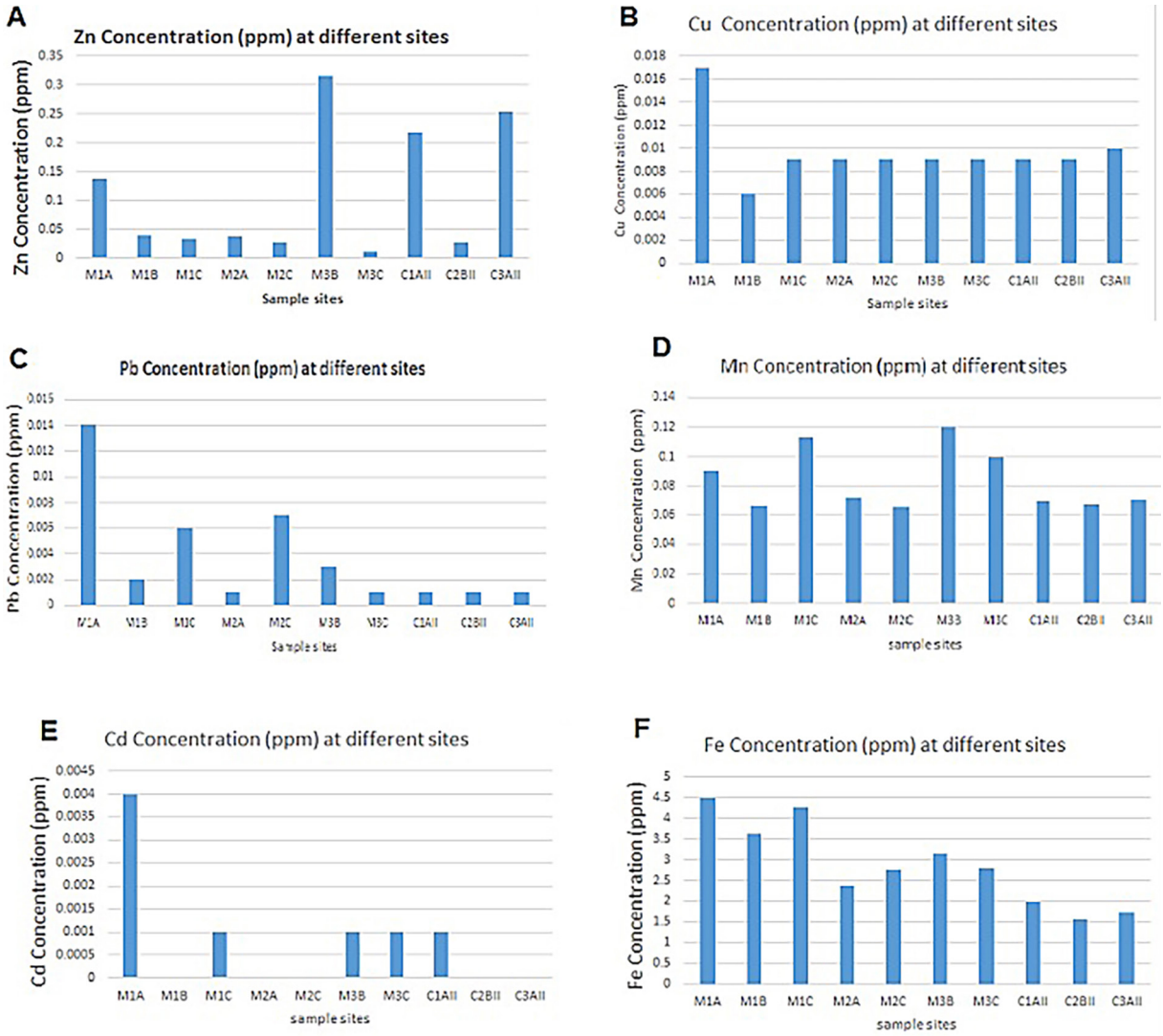


**Figure 4.** I – Showed the concentration of many heavy metals (Zn, Cu, Pb, Mn, Cd, Fe) for different sites (M1A, M1B, M1C, M2A, M2C, M3B, M3C), II – M: soil of contaminated by oil, C: soil uncontaminated by crude oil, A: 0–5 cm of depth, B: 5–10 cm of depth, C: 10–15 cm of depth and D: 15–20 cm of depth. III – references soil, that was taken from uncontaminated soil by crude oil

Pb, while M3B and M3C have the highest levels of Zn and Mn concentrations. So these soil samples gave the lowest number of isolates and low of CFU result which could be due to the inhibition of growth. On the other hand, M1B and M2A have the lowest concentration of all heavy metals which indicated that high number of CFU results and bacterial isolates number. Among the heavy metals that have been detected more frequently than others are cadmium, lead, copper, and zinc, which have shown increased toxicity in the following order: lead < zinc < copper < cadmium (Kavamura and Esposito, 2010).

In conclusion, the present study was conducted to isolate and identify oil-degrading bacteria from contaminated desert soil in the northeastern part of Jordan. Molecular detection and identification of bacterial species revealed 13 different genus (*Micrococcus, Pseudomonas , Lactobacillus, Bacillus, Citrobacter, Corynebacterium, Mycobacterium, staphyloccuous, salmonella, Serratia, Enterobacter, Alcaligenes, Microbacterium)* and 14 different species (*Micrococcus luteus, Pseudomonas putida, Lactobacillus casei, Bacillus subtilis, Citrobacter freundii, Corynebacterium xerosis, Mycobacterium phlei*, *Staphylococcus intermedius, Enterobacter cloacae, Alcaligenes faecalis, Microbacterium esteraromaticum, Serratia liquefaciens, Salmonella enterica*, *Staphylococcus epidermidis).* The isolates of this study shows and exhibit oil degradation potential that could utilize petroleum as a carbon source.

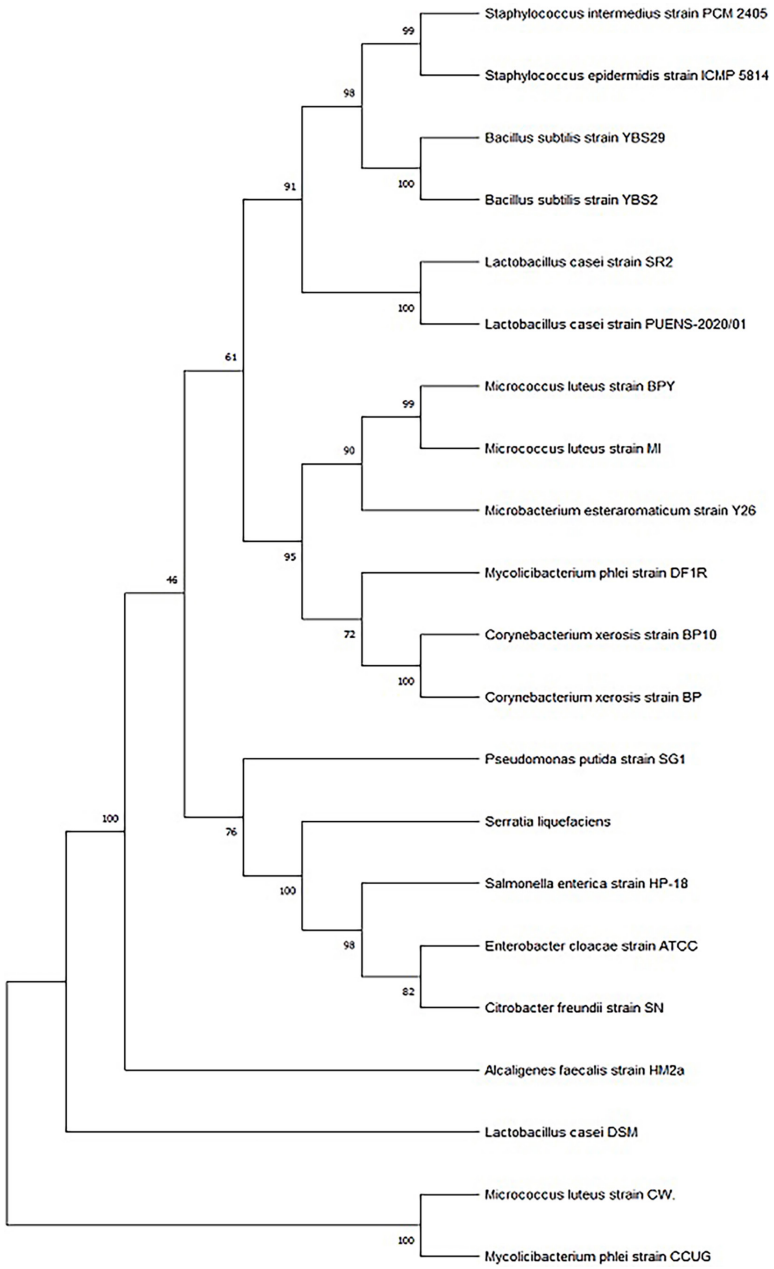


**Figure 5.** Phylogenetic tree of oil degrading bacteria that were isolated and identified by 16s rDNA sequencing data

Further study in our project is needed to determine the strength enzyme activity of oil degradation isolates to shoes the best applicable degrader for oil contaminant. The species were identified in this study could be used for *in situ* and *ex situ* green cleanup of oil-contaminated desert soil in northeastern Jordan.

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