Isolation and Characterization of Oil-Degrading *Enterobacter* sp. from Naturally Hydrocarbon-Contaminated Soils and Their Potential Use against the Bioremediation of Crude Oil

Mukkaram Ejaz [1] , Baowei Zhao [1,*] , Xiukang Wang [2,*] , Safdar Bashir [3] , Fasih Ullah Haider [4,5] , Zubair Aslam [4] , Muhammad Imran Khan [3] , Muhammad Shabaan [3] , Muhammad Naveed [3] and Adnan Mustafa [6]

1   School of Environmental and Municipal Engineering, Lanzhou Jiaotong University, Lanzhou 730070, China; mjmukkaram@gmail.com
2   College of Life Sciences, Yan'an University, Yan'an 716000, China
3   Institute of Soil and Environmental Sciences, University of Agriculture Faisalabad, Faisalabad 38000, Pakistan; safdar.bashir@uaf.edu.pk (S.B.); khanimran1173@yahoo.com (M.I.K.); shaban4543@gmail.com (M.S.); mnaveeduaf@gmail.com (M.N.)
4   Department of Agronomy, University of Agriculture Faisalabad, Faisalabad 38000, Pakistan; Fasihullahhaider281@gmail.com (F.U.H.); zauaf@gmail.com (Z.A.)
5   College of Resource and Environmental Sciences, Gansu Agricultural University, Lanzhou 730070, China
6   Biology Centre CAS, SoWa RI, Na Sádkách 7, 37005 České Budějovice, Czech Republic; adnanmustafa780@gmail.com
*   Correspondence: zhbw2001@sina.com (B.Z.); wangxiukang@yau.edu.cn (X.W.); Tel.: +86-13993163262 (B.Z. & X.W.)

Abstract: The contamination of crude oil in soil matrices is a persistent problem with negative repercussions because of the recalcitrant, hazardous, and mutagenic properties of its constituents. To mitigate the effect of crude oil contamination in soil, the use of microorganisms is a cheap and feasible option. In the current study, bacterial species from numerous polluted oil field surfaces were isolated and examined for their ability to degrade crude oil. Random soil samples polluted with hydrocarbons were collected and various bacterial isolates were isolated. Results revealed that 40% of total isolates had potential use for hydrocarbon biodegradation, the synthesis of exopolysaccharides and the solubilization of phosphorous. Following isolation and characterization to degrade crude oil, a pot trial was conducted using maize inoculated with the four best strains—i.e., S_1 (PMEL-63), S_2 (PMEL-67), S_3 (PMEL-80), and S_4 (PMEL-79)—in artificially hydrocarbon-polluted soil with concentrations of crude oil of 0, 1000, and 2000 ppm. Results revealed that S_4 (PMEL-79) had significant potential to degrade hydrocarbon in polluted soils. The root length, shoot length, and fresh biomass of maize were increased by 65%, 45%, and 98%, respectively, in pots inoculated with S_4 (PMEL-79) *Enterobacter cloacae* subsp., whereas the lowest root length was observed where no strain was added and the concentration of crude oil was at maximum. Moreover, S_4 (PMEL-79) *Enterobacter cloacae* subsp. was found to be the most effective strain in degrading crude oil and increasing maize growth under polluted soil conditions. It was concluded that the isolation of microorganisms from oil-contaminated sites should be considered in order to identify the most effective microbial consortium for the biodegradation of naturally hydrocarbon-contaminated soils.

Keywords: biodegradation; crude oil; TPH content; maize; *Enterobacter*

1. Introduction

With the modernization and advancement of industry, the contamination of soil with toxic pollutants has become a major problem for sustainable agriculture worldwide [1–4]. The contamination of soil has increased extensively in the past two decades and is a major reason for the decline in soil productivity and crop production [5–7]. Currently, the major toxic contaminants are pesticide residues, petrol-related products, heavy metals, and

polycylic–aromatic hydrocarbons, and pollution with these contaminates poses severe hazards to the environment and human health [1,8,9]. The contamination of soils with crude oil and its derivatives is a significant environmental issue worldwide [10–12]. Hydrocarbons make their way into the soil during oil extraction, storage, and distribution, refining, and processing; in addition, leaks and discharge of petroleum hydrocarbons sometimes occur as a consequence of blow-out accidents during oil field growth, oil pipelines and storage tank leakages, oil vessels and vessel leakage accidents, the well waxing of oil, and refinery and petroleum chemical manufacturing equipment [13,14]. With the continuous increase in demand for petroleum and oil products, the contamination of soil with petroleum hydrocarbons has also increased exponentially [15]. Thus, attention is required to be paid to marine and terrestrial ecosystems that are being degraded due to contamination with crude oil [16].

In this regard, numerous physico-chemical techniques can be utilized for the remediation of crude oil and other polycylic–aromatic hydrocarbon-contaminated soils [17]. The chemical remediation of soil includes neutralization, oxidation reduction, and solvent extraction [18,19], while the physical remediation of soil includes incineration, soil washing, and vacuum extraction [15,20]. Although these methods to remediate polycylic–aromatic hydrocarbons are effective, these approaches are generally neither cost-effective nor environmentally friendly [10,21]. Furthermore, in situ remediation technologies—i.e., chemical remediation and physical remediation—increase the mobility of contaminants in the soil and may contaminate the underground water [22,23].

Oil bioremediation is accepted internationally as a cost-effective and environmentally sustainable solution [13,24]. Bioremediation involves two separate processes: bio-stimulation and bio-augmentation (inoculation, seeding) [25]. Bio-stimulation is based on current (native) microbes that can be improved by the careful control of their activities [26]. To improve the remediation of crude oil by microbes in crude oil-contaminated soil, the optimal management of nitrogen and phosphorous fertilizers is important for microbial survival [27]. Bio-augmentation requires the inoculation into the contaminated environment of exogenous microorganisms [28]. In other terms, this strategy ensures that more active microbial strains are introduced to the crude oil-polluted sites [29]. Bioremediation is also characterized as "self-cleaning" and relies on oil remediation by the already existing microbes in contaminated soil without any precise management [10,30]. Recent studies have established more than 79 genera of bacteria that are able to degrade petroleum hydrocarbons [31], and indeed, several experiments have shown that there are a significant number of hydrocarbon-remediating bacteria in oil-rich areas such as oil spills and oil reservoirs, and that their frequency and amount are directly linked to the forms of petroleum hydrocarbons and the environmental factors affecting them [14,32]. From the previous literature, it was revealed that various microbial species such as *Achromobacter xylosoxidans* [33], *Aeribacillus pallidus* [34], *Geobacillus thermodenitrifican* [35], *Gordonia sihwensis* [36], and *Pseudomonas aeruginosa* [37] play vital roles in the degradation/remediation of petroleum hydrocarbons [38].

Recently, very low amounts of crude oil—i.e., 1–6%—have been used in most bacterial remediation studies [14,39]. Nevertheless, after the war of 1991, Kuwait's desert areas became contaminated with oil and have a much higher concentration of crude oil; i.e., 20% or more [10]. The crude oil that poured out of the destroyed wells allegedly filled some 50 "shell-lakes" of differing sizes [21,40]. The existence of higher and lower species at such high oil concentrations becomes difficult due to the disruption of water preservation and soil aeration, both due to the toxicity of oil contamination [1]. The objective of the current study is to screen successful microbial species that can biodegrade crude oil in contaminated soils and determine whether mixing oil-saturated soils with oil-free soil can improve the oil-bioremediation process by diluting the oil concentration in contaminated soil. It is hypothesized that the isolates would acclimatize to polycylic–aromatic hydrocarbons (PAHs) and be more effective in the process of bioremediation, while their effects on maize growth may vary depending on the type of bacteria and inoculation conditions.

Therefore, the specific objectives of current study are to (i) isolate the bacterial strains from crude oil-contaminated soil that are helpful for the effective remediation of crude oil and (ii) evaluate the efficacy of isolated bacterial strains on the performance of maize (*Zea mays* L.) cultivated in crude oil-polluted soil.

2. Materials and Methods

2.1. Reagents and Other Materials

Inorganic basal salt substrate, Luria Bertani broth (LB) [41], nutrient broth/agar (NA), and Bushnell and Haas medium (BH) [42] were used for the isolation of microbial strains from soil samples. Phosphate-solubilizing medium was used to check the phosphate solubilizing capacity of the strains, RDH-CCl$_4$, anthracene, flourene, and phenanthrene (the chemicals used for assaying bioremediation had a purity >97% and were obtained from Fluka, Japan). KH$_2$PO$_4$, NH$_4$Cl, MgSO$_4$.7H$_2$O, FeSO$_4$.7H$_2$O, NaCl, Tryptone, yeast extract, peptone, and agar chemicals were acquired from Sigma-Aldrich.

2.2. Soil Sampling

The soil samples were collected from various regions of the Punjab province of Pakistan (Figure 1). The random sampling was carried out in the districts of Jhang, Faisalabad, and Multan from hydrocarbon-contaminated soils. Composite samples of 1 kg were prepared by sampling at different locations. Sampling sites were areas used for oily chemical sludge drainage and processing. Soil samples were collected using tube augar. The soil sampling thickness was up to 90 cm from the ground layer. The four soil samples obtained were used to extract 10 microbial isolates, and composite samples were used to test the total hydrocarbon content of petroleum (TPH). The collected soil samples were stored at 4 °C in the dark in zipped plastic bags prior to use for bacterial isolation and sample TPH contamination assessment.
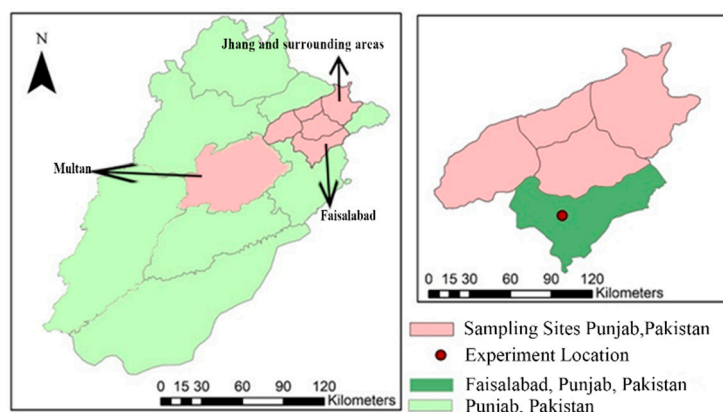
Figure 1. Geographical location of experiment site.
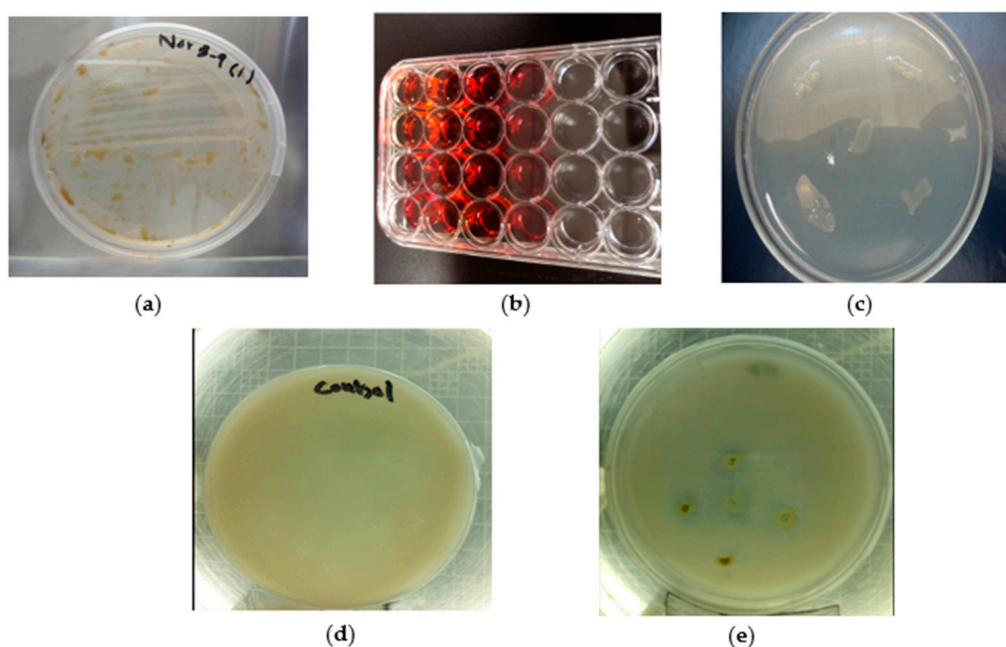
2.3. Bacterial Isolation

The enrichment culture system was used to isolate the bacteria. Bacterial isolates were developed on an inorganic basal salt substrate (0.64 g K$_2$HPO$_4$, 0.31 g KH$_2$PO$_4$, 0.5 g NH$_4$Cl, 0.2 g MgSO$_4$.7H$_2$O, 0.005 g FeSO$_4$.7H$_2$O) with 1% crude oil as a single source of carbon. Briefly, 10 g of each specimen was added to a sterile 250 mL Erlenmeyer flask containing 95 mL of inorganic basal media. In a flatbed end-to-end shaker, the flasks were shaken vigorously to homogenize the soil suspension. Following this, a 1/10 serial dilution of this suspension was prepared from the above culture by taking 1 mL and adding it to 9 mL of sterile media to 10^{-5}, which was used to inoculate basal salt medium agar plates. Crude oil extracted through a membrane of 0.2 μm was atomized as a sole source of carbon on solidified agar plates. To check the growth of bacteria, incubation was conducted in Petri plates at 28 ± 1 °C for 72 h. On the basis of the color, shape, and size of the colony, further purification was performed by stretching on the media (Table 1).

Table 1. Composition of the nutritional medium used in the experiment.

Medium Name	Ingredients (g/L)	Persistence
Luria Bertani broth (LB) (Bertani, 2004)	NaCl 10; Tryptone 10; pH 7.0 yeast extract 5	Cultivation of bacterial cultures
Nutrient broth/agar (NA)	Peptone 10; NaCl 5; yeast extract 3; pH 7.0 Agar 20	Maintenance of bacterial cultures
Bushnell and Haas medium (BH) (Bushnell and Haas, 1941)	KH_2PO_4,1.0; K_2HPO_4, 1.0; NH_4NO_3, 1.0; $MgSO_4.7H_2O$, 0.2; $FeCl_3$, 0.05; $CaCl_2.2H_2O$, 0.02; pH 7.0 Agar 20	Preparation of inoculum, screening, and isolation of crude oil degraders
Phosphate solubilizing medium	Glucose 10, $Ca_3(PO_4)_2$ 2.5, $MgCl_2.6H_2O$ 5, KCl 0.2, $(NH_4)_2SO_4$ 0.1, $MgSO_4$ 0.25,	Phosphate solubilizing

2.4. Bioremediation Assay

A PAH degrading capacity bioremediation assay was performed to check the degradation of polyaromatic hydrocarbons using bacterial isolates (N_1, L_1, M_1, H_1, H_2, N + O, H + O, L + O, M + O, and O_1), as shown in (Figure 2). For this purpose, 24 microtiter plates were used. The test compounds used in the current study were a combination of PAHs. Only 40 µL of polyaromatic hydrocarbon mixtures (1 g anthracene, 10 g flourene and 1 g phenanthrene dissolved per liter of pentane) were used in the primary two lanes to be used as a control. There were no Bushnell and Hass media in these wells, and there was no bacterial inoculation. In total, 720 µL of Bushnell and Hass and 80 µL of bacteriological broths were poured in the following two lanes as well as 40 µL of PAHs mixture. In the second control, using five and six lanes, each well of these lanes was supplemented with Bushnell and Hass medium and a PAHs mixture, but without bacterial inoculation. In each plate, a single bacterial isolate was added to each row. These plates were incubated at $28 \pm 1\,^{\circ}\text{C}$ for three weeks. In each well, after the incubation period (21 days), 200 microliters of p-Ido-nitro-tetraazolium (p-INT) indicator was added.

Figure 2. Screening of bacterial isolates for the bioremediation assay. (**a**) The isolation of bacterial isolates, (**b**) bioremediation assay using 24 micro-titer plates, (**c**) exopolysaccharides production, (**d**) no phosphate solubilization, (**e**) phosphate solubilization.

2.5. Exoplysaccharide Production Assay

Enterobacter sp. strains were grown at 30 °C in TSB/10 (Difco Laboratories, Detroit, USA) or an RCV medium modified by Weaver and colleagues (1975). The amounts of chemicals used were as follows: $MgSO_4.7H_2O$—0.1 g L^{-1}; $CaCl_2.2H_2O$—0.1 g L^{-1}; $FeSO_4.7H_2O$—

0.022 g L^{-1}; EDTA—0.02 g L^{-1}; $ZnSO_4.7H_2O$—0.43 mg L^{-1}; $MnSO_4.H_2O$—1.30 mg L^{-1}; $Na_2MoO_4.2H_2O$—0.75 mg L^{-1}; H_3BO_3—2.80 mg L^{-1}; $CuSO_4.5H_2O$—26 mg L^{-1}; $CoSO_4.7H_2O$—70 mg L^{-1}; K_2HPO_4—5.2 mM; KH_2PO_4—4.4 mM, pH 6.8; yeast extract—0.1 mg L^{-1}; glucose—2.0 g L^{-1}.

Nalidixic acid, kanamycin and tetracycline were, respectively, used at amounts of 50, 25, and 15 mg mL^{-1} for the appropriate antibiotic selection of rhizobial strains [43].

2.6. Phosphate Solubilization Assay

1. Petriplates were prepared with the following media composition: (g/L) glucose—10 g: $Ca_3(PO_4)_2$—5 g; $MgCl_2.6H_2O$—5 g; $MgSO_4.7H_2O$—0.25 g; KCl—0.2 g; and $(NH_4)_2SO_4$—0.1 g.
2. Each bacterial strain was represented by a loop on the plates (three per plate at different places).
3. Plates were incubated for 2–3 days at 28 ± 1 °C [44].

2.7. Measurement of Total Petroleum Hydrocarbons by Infrared Spectroscopy

In obtained soil samples, a Horiba-350 oil content analyzer (Horiba Ltd., Kyoto, Japan), was used to test TPH. The TPH-oil content analyzer utilizes infrared light to determine the TPH. In a china dish, 5 g of soil was taken and 5 g of sodium sulfate was added to absorb moisture in the soil. These were thoroughly mixed, and a mixture of soil and sodium sulfate was added to 40 mL of $RDH-CCl_4$. This mixture was shaken for 30 min and filtered using Whatman filter paper No.40 (11 cm). In order to absorb moisture and biogenic hydrocarbons, 100 mesh size silica was placed in the funnel during filtration. The filtrate was filled in a cell of 1.5 cm and put in a device for reading in mg/kg. Dilutions were produced using $RDH-CCl_4$ to bring the concentration within the scope of the requirements and equipment. The instrument was calibrated before measuring TPH.

2.8. Experimental Setup and Observations

A pottrial was conducted in the growth room to further check the ability of bacterial isolates to degrade hydrocarbons and to improve maize growth. The pots measurements used in the study were 13 cm × 6 cm, with a vessel size of 13 cm and an internal diameter of 6 cm. These pots were filled with soil that was sieved to separate gravel and debris by using a 2 mm sieve. The seeds were inoculated with peat combined with a 10% sugar solution for inoculation. In total, 15 regular treatments with three repeats were used, as shown in Table 2. All the soil samples were contaminated with a known quantity of crude oil purchased from a local oil company. The levels of crude oil were 0, 1000, and 2000 ppm. The Pioneer-30Y87 variety of maize was used. The seeds were inoculated with the different isolates S_4 (PMEL-79), S_3 (PMEL-80), S_2 (PMEL-67) and S_1 (PMEL-63). The pots were watered regularly. The dose of fertilizer prescribed—i.e., N:P:K 150:100:50 kg ha^{-1}, respectively—was added using urea, di-ammonium phosphate, and sulfate of potash as a fertilizer source. After 30 days of sowing, the root and shoot length of maize was determined using a meter rod. The dried and fresh biomass was determined after harvesting the maize to observe the remediation effect of bacterial isolates on maize plants. The residual TPH was measured using the Horiba oil content analyzer OCMA-350.

Table 2. Experimental treatments.

Factor 1 (Bacterial Isolates)	Factor 2 (Crude Oil Contamination)
No strain (S0)	Control (L0) 0 ppm
PMEL-63 (S1)	L2 1000 ppm
PMEL-67 (S2)	L4 2000 ppm
PMEL-80 (S3)	
PMEL-79 (S4)	

2.9. Bacterial 16S rRNA Sequencing

16S rRNA sequencing was used to identify the most effective and efficient bacterial isolates. This approach included extracting DNA from bacterial isolates, conducting 16S rRNA amplification, and comparing sequenced genes to Gene Bank to find a match [43,44].

2.10. Statistical Analysis

The recorded observations were statistically analyzed using Fisher's analysis of variance, and the means of treatments were compared with a 5% probability level according to the least significant difference test [45]. A principal component analysis (PCA) was carried out to group the strains according to their performances for physico-chemical parameters such as TPH concentration, biomass production, shoot length, root length, and chlorophyll content, and we investigated possible correlations between studied attributes. The principal component analysis was performed using XLSTAT software (Addinsoft).

3. Results

3.1. Bioremediation Assay, Exopolysaccharides, and Phosphate Solubilizing Ability

The color brightness was due to the higher, medium, and lower range. The most extreme color wells are marked as (+ + +), demonstrating the capacity for further bacterial biodegradation (Table 3). The test showed that four of the bacterial strains—i.e., N_1, L_1, H_2, and M + O—showed an intense red color compared to the control and other strains, proving that these strains have the capacity to degrade crude oil (Figure 2b). Due to PAH oxidation, bacterial strain-inoculated wells turned red, showing that bacteria can biodegrade PAH, as shown in Figure 2b. The greater the intensity of the color, the higher the amount of degradation and vice-versa. These strains were later identified to be PMEL-63, PMEL-67, PMEL-79, and PMEL-80 from the genus *Enterobacter* sp. The test for exopolysaccharides production (EPS) was carried on these same strains—i.e., N_1, L_1, H_2, and M + O—and showed that all these strains had the ability to produce exopolysaccharides as shown in Figure 2c. A clearing zone around the colonies indicated that the bacteria could solubilize phosphate. By subtracting the bacterial colony zone from the total diameter of the zone, the diameter of the clear zone could be determined. Tests with every bacterial strain were repeated three times in the experiment. The same strains—i.e., N_1, L_1, H_2, and M + O—showed the ability to solubilize phosphate, as shown in Figure 2e.

Table 3. Results of the bacterial isolate screening for the bioremediation assay, exopolysaccharides production, and phosphate solubilization.

Isolates	Bioremediation Assay	Exo-Polysaccharide Production	Phosphate Solubilization
N_1	+	↑	↑
L_1	++	↑	↑
M_1	–	↓	↓
H_1	–	↓	↓
H_2	+++	↑	↑
N + O	–	↓	↓
L + O	–	↓	↓
M + O	++	↑	↑
H + O	–	↓	↓
O1	–	↓	↓

N_1 = normal soil; L_1 = low contaminated soil; M_1 = medium contaminated soil; H_1 = high contaminated soil; N + O = normal soil + oil; L + O = low contaminated soil + oil; M + O = medium contaminated soil + oil; H + O = high contaminated soil + oil; + represents the intensity of color and amount of degradation; _ represents no degradation of crude oil; ↑ represents the exopolysaccharide production and phosphate solubilization; ↓ represents no exopolysaccharide production and phosphate solubilisation.

3.2. Growth Attributes of Maize

Bacterial inoculation significantly improved the roots of inoculated maize crop. Under higher contamination of crude oil, bacterial strains S_4 (PMEL-79), S_3 (PMEL-80), S_2 (PMEL-67), and S_1 (PMEL-63) significantly enhanced the root length of maize, which was recorded

as 48.08%, 42.18%, 37.27%, and 30.15% higher than control S0L4, respectively. Of all the strains, the bacterial strain S_4 (PMEL-79) increased the root length more significantly then the other strains, as shown in Figure 3a, reaching a level that was 32.11% higher than control S0L0. The lowest root length was observed in treatment SoL4, where no strain was added and 2000 ppm of crude oil was applied. The bacterial inoculation significantly affected the root length in crude oil concentration; i.e., 1000 and 2000 ppm, respectively. The elongation of shoot of maize was significantly enhanced by bacterial strains S_3 (PMEL-80) and S_2 (PMEL-67), by 16.9% and 20.41% more than the control, respectively. The highest increases in shoot length were observed in strain S_4L_2, which was 33.94% higher than control S0L0 (Figure 3b). The results show that, overall, the S_4 (PMEL-79), strain performed very well as it increased the root and shoot length of maize under both lower and higher levels of crude oil contamination in soil.

(a)

(b)
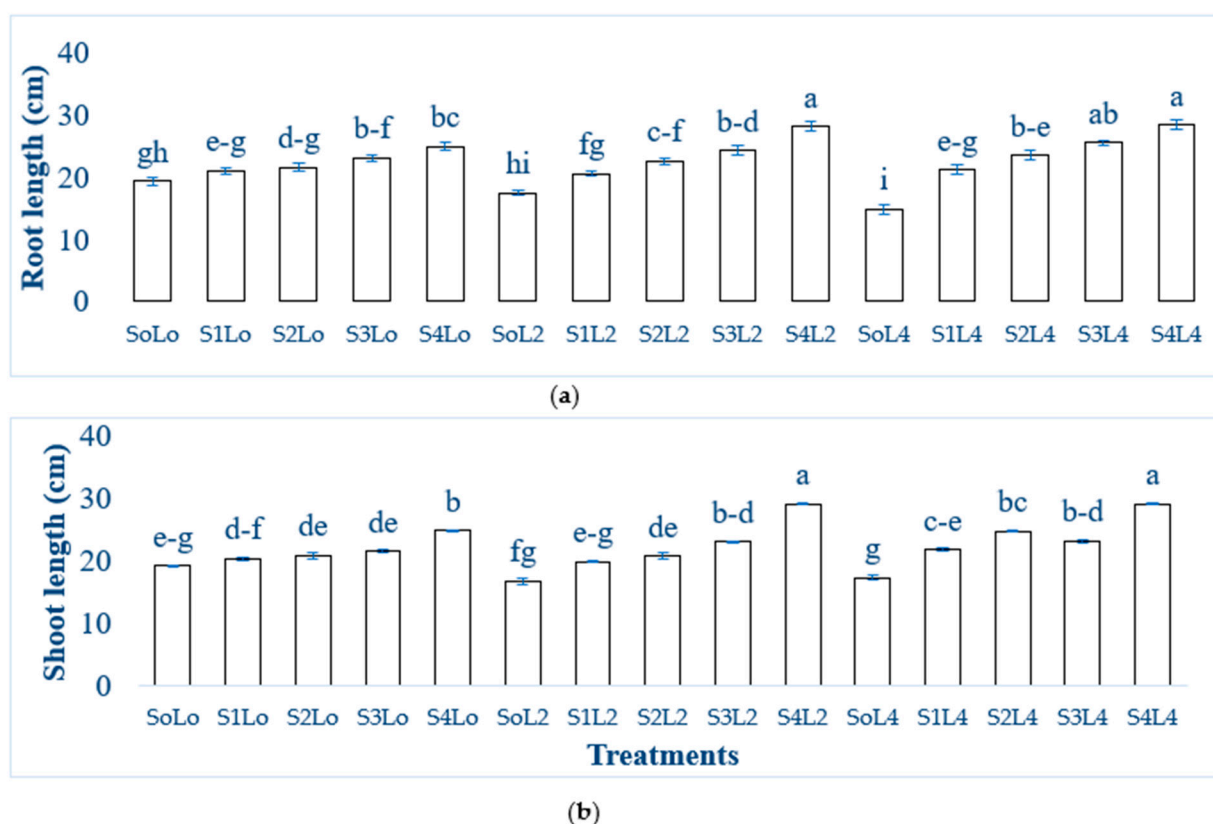
Figure 3. Remediation influences of bacterial isolates on root length (cm) (**a**) and shoot length (cm) (**b**) of maize cultivated in crude oil-contaminated soil. Values are means ± standard deviation of three replicates. Means sharing the same case letters, for interaction and main effects for a parameter, do not differ significantly at $p \leq 0.05$. S0 = no microbial isolation; S1 = PMEL-63; S2 = PMEL-67; S3 = PMEL-80; S4 = PMEL-79, L0 = 0 ppm crude oil; L2 = 1000 ppm crude oil; L4 = 2000 ppm crude oil.

In the pot trial, the highest maize fresh biomass was produced in response to inoculation with bacterial strain S_4 (PMEL-79). This extreme rise in the fresh biomass of the maize was 98% compared to the uninoculated control, as shown in Figure 4a. Further bacterial strains that efficiently amplified the maize fresh biomass were S_3 (PMEL-80) and S_2 (PMEL-67), at 43.13% and 28.9% more than the control, respectively. The S_4 (PMEL-79) bacterial strain upheld its consistency in promoting plant growth with maize as it caused the maximum increase in the fresh biomass of maize. Of the bacterial isolates, the lowest increases in maize fresh biomass were observed in treatments S_1L_2 and S_1L_4, at 11% and 13%, respectively, produced by the inoculation of strain S_1 (PMEL-63) as compared to the control. The greatest increase in the oven-dried biomass of maize was detected in response to inoculation with S_4 (PMEL-79) and S_1 (PMEL-63) bacterial strains, showing

improvements of 52.89% and 51.33%, respectively, compared to the control. This increase was double that of the uninoculated control. Other bacterial strains performed efficiently except the bacterial strain S_3 (PMEL-80), where the maize oven-dried biomass was less by 9.39% than the control, as shown in (Figure 4a). In brief, the bacterial strains S_4 (PMEL-79) and S_1 (PMEL-63) were both consistent with maize in increasing the oven-dried biomass relative to the uninoculated control; nevertheless, the percentage of improvement with these steady bacterial strains over the uninoculated control diverged from parameter to parameter. Strains S_4 (PMEL-79) and S_1 (PMEL-63) were found to perform best in the hydrocarbon-contaminated soils, affecting all the crop parameters and significantly enhancing the growth as compared to uninoculated contaminated soil.
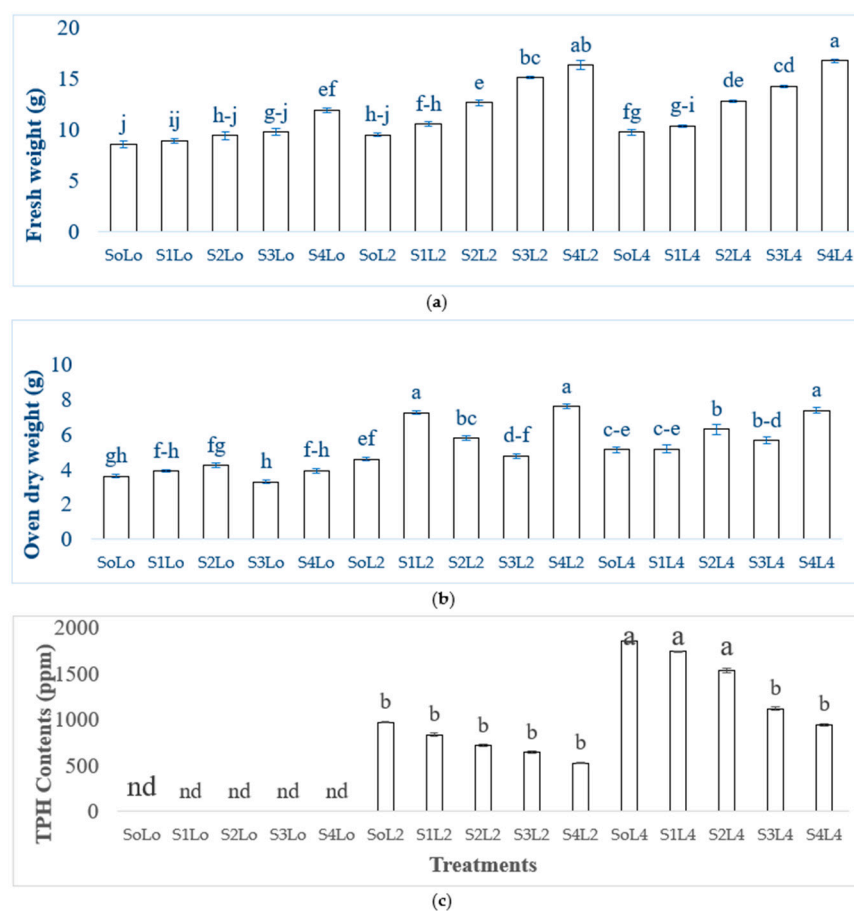
Figure 4. Remediation influences of bacterial isolates on fresh and dried weight (g) (**a**,**b**) of maize and soil total petroleum hydrocarbon (TPH) content (**c**) in crude oil-contaminated soil. Values are means ± standard deviation of three replicates. Means sharing the same case letters, for interaction and main effects for a parameter, do not differ significantly at $p \leq 0.05$. S0 = no microbial isolation; S1 = PMEL-63; S2 = PMEL-67; S3 = PMEL-80; S4 = PMEL-79, L0 = 0 ppm crude oil; L2 = 1000 ppm crude oil; L4 = 2000 ppm crude oil.

3.3. TPH Removal in Association with Maize

Maize simplified the petroleum hydrocarbon degradation under the controlled conditions. A removal rate of up to 54% of TPH was observed with respect to the control of the combined effect of the S_4 (PMEL-79) bacterial strain and maize. The efficiency of maize alone was not notable because it triggered the elimination of 10% TPH compared to the control. This can also be seen by comparing only the combined maize effect and the isolation of S_4 (PMEL-79), which caused a removal rate of TPH of 44% more than that above (Figure 4c). S_4 (PMEL-79) bacterial isolation was the most effective; however, the efficacy of other bacterial strains such as S_3 (PMEL-80), S_2 (PMEL-67), and S_1 (PMEL-63) cannot

be overlooked as these bacterial strains in association with hydrocarbons bio-remediated maize by 40%, 36%, and 39%, respectively, compared with un inoculated contaminated soil (Figure 4c).

3.4. Bacterial Sequencing

16s rRNA sequencing showed that the S_4 bacterial isolate was *Enterobacter cloacae* subsp. dissolvens, with a similarity of 97.31%; S_3 was also *Enterobacter cloacae* subsp. dissolvens, with a similarity of 99.7%; S_2 and S_1 were also *Enterobacter cloacae* subsp. dissolvens, with a similarity of 100% (accession number MT212231.1). A phylogenetic tree of all bacterial strains was constructed using Mega X computer-based software to further explain the relationship of strains with neighboring species based on their evolutionary history (Figure 5).


Figure 5. Neighbor-joining tree illustrating the phylogeny of *Enterobacter cloacae* subsp. branches, which are labeled with a parsimony bootstrap built using Mega X.

3.5. Selection of TPH-Degrading and Growth-Promoting Strains

On the basis of the results of the bioremediation assay, a trial was conducted under crude oil-contaminated conditions; four strains (S_1, S_2, S_3, and S_4) were used to verify the TPH degradation in maize. The principle component analysis was used to test the results. Factor coordinates of bacterial strains based on TPH content, root length, shoot length, biomass production, and chlorophyll content are presented in Table 4. The maximum coordinate value (5.82) was observed in S_4 (PMEL-79) followed by S_3 with an F_1 value of 0.96 (PMEL-80). Factorial planes clearly show their unique behavior in terms of the TPH degradation and growth promotion of maize. The total variability between F_1 and F_2 biplots is 90%. The Pearson's correlation matrix showed that variables other than chlorophyll content were positively correlated. We detected some correlation between the TPH content, biomass production, shoot length, and root length, while there was negative correlation for the chlorophyll content. The active coordinates retained by PCA were used to construct the biplot in Figure 6.

Table 4. Factor coordinates of bacterial strains based on TPH content, root length, shoot length, biomass production, and chlorophyll content.

Strains	F_1	F_2	F_3	F_4
S_0	−5.109	−0.888	−0.655	0.729
S_1	−1.911	1.087	−0.956	−1.004
S_2	0.201	2.148	1.473	0.360
S_3	0.967	−2.219	1.286	−0.456
S_4	5.852	−0.128	−1.148	0.371
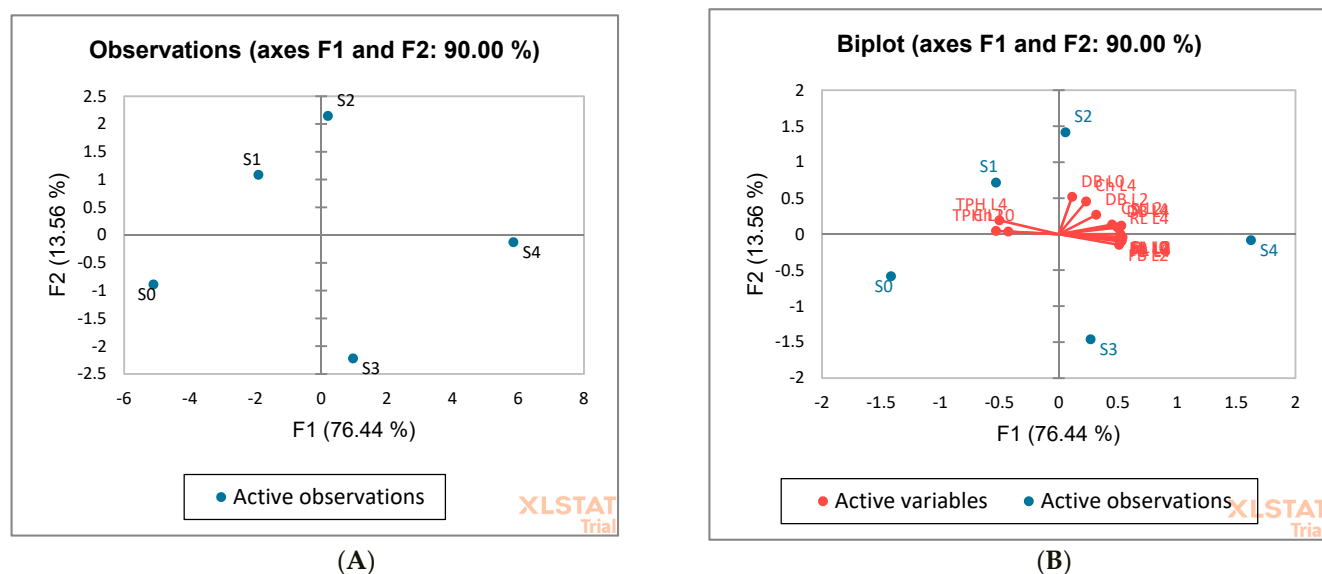
Figure 6. Principal component analysis (PCA) showing score plots (**A**) and loading plots (**B**) of different attributes of maize plants in crude oil-contaminated soil.

4. Discussion

Crude oil contamination in soil has been recognized as a key factor for plant growth reduction. The findings of this study show that crude oil contamination suppressed maize growth, with the effect being more pronounced at higher levels of soil pollution (Figures 3 and 4). This impact may be due to disturbances in water and nutrient absorption caused by oil in the soil, as well as soil nitrogen, and phosphorus depletion [46]. These results are in accordance with previous published studies [1,38,47] reporting that root morphology and plant biomass were reduced in soil contaminated with petroleum hydrocarbons. Plant growth and photosynthesis pigments—i.e., chlorophyll contents—may

have been hindered by the presence of toxic petroleum hydrocarbon compounds [10]. The current study shows that the presence of microorganisms significantly improved the plant height, biomass, and root morphology of maize in crude oil-contaminated soil (Figures 3 and 4). In addition, the results also show that the presence of microbes stimulated the biodegradation of crude oil in the rhizosphere in all treatments except the control (Figure 4c). The higher degradation rate in the crude oil-contaminated soil might be attributed to the increased microbial activity as a result of seed inoculation with microbes [6,48]. The results of the bacterial isolate screening for the bioremediation assay, exopolysaccharides production, and phosphate solubilization can be seen clearly in Table 3. Bio-surfactant production has been observed in many *Bacillus* sp., especially in *Bacillus subtilis* L., which implies good potential for the bioremediation of organic contaminants such as fuel hydrocarbons due to its emulsifying properties by following mechanisms including bioaccumulation (the net accumulation of contaminants in the microorganisms' cells), biomineralization (the transfer of aqueous contaminates into crystalline or amorphous precipitates), biotransformation (the transformation of contaminates from a toxic to less toxic form), and biosorption (the binding of contaminates with cation-binding proteins present on the cell wall of microorganisms) [22,49].

The current experiment demonstrates that the concentration of crude oil and total biomass production are directly related to each other. A lower concentration of crude oil proved to be less toxic as regards biomass production. Contrary to our findings, Gao and Zhu [50] observed no substantial effects of a reduced amount of crude oil on plant biomass, but the inhibition of growth was noticeable at higher concentrations of crude oil. The inherent toxicity of crude oil might be the cause of lower plant biomass production, as was clearly seen with higher concentrations of crude oil [2]. Plants are sensitive to low-molecular-weight volatile hydrocarbons that are soluble in hydrophobic plant materials and can infiltrate cell membranes [10]. Cheema et al. [21] proposed secondary adverse effects of PAHs; PAHs might reduce the capacity of contaminated soil to offer water and nutrients to plants, leading to a reduction in biomass production.

Additionally, in the current experiment, it was observed that plants did not show obvious signs of toxicity stress—i.e., necrosis—suggesting that this species can be grown in crude oil-contaminated soils and as a result is a feasible option for the phytoremediation of petroleum hydrocarbons. Various legumes and cereals species can be effectively used for the phytoremediation of petroleum hydrocarbons [1,30]. The phytoremediation of petroleum hydrocarbons is significantly regulated by various factors such as environmental conditions, agronomic practices, plant species, and soil characteristics, affecting the petroleum hydrocarbon uptake in the rhizosphere [14,31].

The screening of these four strains showed that the all the strains were found to be *Enterobacter cloacae* subsp. The results showed that S_4 (PMEL-79) was recognized to be from the genus *Enterobacter cloacae* subsp. dissolvens with a similarity of >97%. Meanwhile, the other strains S_1 (PMEL-63), S_2 (PMEL-67) and S_3 (PMEL-80) were also from the genus *Enterobacter cloacae* subsp. Dissolvens; the similarity percentage was 100% for S_1 (PMEL-63), but for S_2 (PMEL-67) and S_3 (PMEL-80), this value was 99.72%. These strains are all from the same genus *Enterobacter* sp. Huang et al. [30] conducted an experiment to evaluate the bioremediation potential of *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Acinetobacter lwoffi* individually and in combination to degrade crude oil. Hydrocarbon-contaminated soil and water was used for the inoculation of these bacteria. In addition, greater degradation by the combination of these bacterial strains was observed as compared to individual bacteria; however, at rates of 77.8% and 76.7%, respectively, crude oil degradation was detected in the experimental units receiving a sole inoculation of *Pseudomonas aeruginosa* and *Bacillus subtilis*, respectively [30]. The proposed mechanism for the degradation of crude oil by *Enterobacter cloacae* subsp. may be cell uptake, which is comprised of two parts. The first phase is the immediate exposure of *Enterobacter cloacae* subsp. to massive hydrocarbon particles for absorption. In the second phase, the bacteria interact with small pseudo-soluble, quasi-soluble, or encapsulated hydrocarbons particles for absorption.

Finally, the crude oil is degraded down into CO_2 and H_2O, which are basic hydrocarbons [51–53]. Moreover, soil contaminated with petroleum hydrocarbons should be kept properly aerated and moist to optimize the remediation of crude oil by microbes [10,31]. Ghazali et al. [54–57] also reported the effectiveness of *Bacillus* strains in the remediation of petroleum hydrocarbons. They used different species from dissimilar genera such as *Pseudomonas, Micrococcus,* and *Bacillus* and concluded an important role of species from the genus *Bacillus* sp as they observed a greater degradation of petroleum hydrocarbons (57%) by a combination dominated by *Bacillus* sp. as compared to a combination comprised of *Pseudomonas* and *Micrococcus* species. Furthermore, appropriate agronomic practices—i.e., optimum nutrient management in contaminated soil—significantly improve the microbial biomass and density, further enhancing the biodegradation potential of microbes in petroleum-contaminated soils [29,33,56,58,59].

Findings from the present research expand our awareness of the plant–microorganism interaction in petroleum-contaminated soils and provide new insights on the use of beneficial microorganisms to enhance the remediation of petroleum hydrocarbons and crop productivity under petroleum hydrocarbon stress; furthermore, the results open new areas for researching the significance of biological interactions in petroleum hydrocarbon-contaminated ecosystems.

5. Conclusions

Due to the extreme threat to environmental and human safety, crude oil hydrocarbons are among the most alarming contaminants. The results of the present study showed that S_4 (PMEL-79) was recognized to be from the genus *Enterobacter cloacae* subsp. dissolvens with a similarity of >97%. Furthermore, S_4 (PMEL-79) increased the root length, shoot length, and fresh biomass of maize to a greater extent than the control. All of the strains selected— S_1, S_2, S_3, and S_4—exhibited exopolysaccharides production capability and phosphate solubilization. The current study showed that the potential remediation of hydrocarbons by various isolates of bacteria is an eco-friendly, cheap and alternative option in soils polluted with hydrocarbon.

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