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Isolation of Degraded Bacteria and Fungi of Petroleum Hydrocarbons from Two Polluted Soil under Remediation with Maize Plant

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Abstract: Soil contaminated by petroleum hydrocarbons (PHCs) is a global severe environmental problem, especially if we consider that Iraq has been subjected to several wars and the widespread use of generators to provide electrical energy. In addition to oil extraction sites, gas manufacturing sites, refineries, and ammunition waste sites. Therefore, the need to know the best ways to treat these polluted soils has been increased. For this purpose, this study was conducted to isolate bacteria and fungi from contaminated and non-polluting soils that synergism with plant for remediation of oil-contaminated soil. The study was carried out in the plastic shade scale, and seeds were cultivated in plastic pots filled with 12 kg of soil. The experiment was laid out in Completely Randomized Block Design (CRBD) with three replications. Each replication comprised the following treatments randomly: AL-Daura power station soil planted with maize, AL-Daura oil refinery soil planted with maize, AL-Daura oil refinery soil unplanted (abiotic stress), AL-Daura oil refinery soil sterilized unplanted (treated with $AgNO_3$ (biotic stress)), in addition to control soil planted with maize. The total number of bacteria and fungi and hydrocarbon (HCs)-degrading microorganisms were evaluated during phytoremediation by cultured in a proper media agar. The rhizospheric bacterial and fungal count of the planted soil increased at the end of the experiment. Totally, the study obtained 11 pure bacterial isolates and 7 pure fungal isolates that could grow on Bushnell Hass (BH) agar medium containing crude oil. Current study suggested that the used of maize plants played an important role in stimulation the microorganisms. They were increased the number of microorganisms. Also, the petroleum degraders microbial increased in the oil-contaminated soil and the non-contaminated soil. The microbial degraders are found in the entire treatments.

Keywords: Petroleum-degradation microorganisms, *Zea mays*, Bushnell Hass media.

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Introduction

Total petroleum hydrocarbons (TPHCs) are the term used to describe a large family of heterogeneous compounds found in crude oil and whose main chemical constituents are carbon and hydrogen atoms. Polycyclic aromatic hydrocarbons (PAHs) are formed during incomplete combustion at high temperatures of the organic substances for a long period. The benzene ring is the basic structure of aromatic hydrocarbons, and two or more fused cycles form PAH (1). Their hydrophobic nature leads to increased accumulation and enrichment in soils, which is a cause for remediation of contaminated sites (2). Bioremediation is a useful tool for treating PHCs contaminated terrestrial and marine ecosystems (3). Natural attenuation (NA) represents the single, most important biological process which removes PHCs from the environment. It is a process where microorganisms

present at the site degrade the organic contaminants without the input of external bioremediation enhancers (4). Bioremediation uses algae and microorganisms like bacteria and fungi to remove or neutralize contaminants into less hazardous/ non-hazardous forms with less input of chemicals, energy, and time. It is a spontaneous manner in which microorganisms transform environmental pollutants into final harmless products (5). There are two different paths to bioremediation techniques. The first involves activating indigenous microorganisms in the contaminated area by adding nutrients and forming the best environmental conditions (biostimulation). The second is bioaugmentation, which consists of supplementing oil-utilizing or genetically modified microorganisms (6). A variety of microbes with the ability to metabolize HCs can be isolated from petroleum-contaminated soil (7). The soil with high HCs content contains more HC-utilizers than soil with low HCs content. While most HC biodegradation studies focus on bacteria, fewer investigations deal with fungi (8).

Microorganisms can degrade organic compounds by mineralization or co-metabolism (9). Bacteria oxidize aromatic compounds to acquire their atoms while fungi for their detoxification. The susceptibility of HCs to microbial degradation can be generally classified as follows: linear alkanes > branched alkanes > small aromatics > cyclic alkanes (10). Some compounds, such as the high molecular weight PAHs, may not be degraded (11). Although these microorganisms cannot degrade metals, they can alter their chemical properties via different mechanisms like biosoption, bioleaching, biomineralization, or enzyme-catalyzed transformations. Microorganisms with degradation capabilities may also alter their cell hydrophobicity during growth on PHCs (12). Phytoremediation based on the interactions between plants and microorganisms appears to be especially useful for this purpose. Plant-bacteria association has been considered an effective partnership to cleanup PHCs impacted soils (13). A key element for successful phytoremediation of soil contaminated is the use of plants that can grow in the presence of a high level of contaminants, in combination with beneficial plant-associated rhizospheric and endophytic bacteria capable of degrading organic pollutants (14). It was therefore the aim of the present study to isolate bacteria and fungi from contaminated and non-polluting soils that synergism with plant for remediation of petroleum-contaminated soil.

Materials and Methods

Collection of Soil Samples

Two sites were chosen to collect polluted soil with oily residues: AL-Daura power station and AL-Daura oil refinery in Baghdad province. The soil was subjected to chronic pollution for many years (15, 16). In addition, one unpolluted site was chosen for comparison as control around 4 Km upwind from AL-Daura thermal power station. All samples were collected during February 2020. The soil samples were collected at a depth of 0 to 30 cm from the soil's top surface for each site. The samples were collected in big labeled sacks, large stones and plant root debris were removed, homogenized, and transported to the plastic shaded area.

Plant Materials

Maize plant was chosen for the conducting of this study as it is tolerant of crude oil pollutants in soil (17) and has the ability to accumulate HMs (18). The seeds of maize Furat variety were obtained from Monarch Seed Company, South Africa.

Treatments and Experimental Design

The experiment was laid out in Completely Randomized Block Design (CRBD) with three replications. Each replication comprised the following treatments randomly: Al-Daura power station soil planted, AL-Daura oil refinery soil planted, AL-Daura oil refinery soil unplanted (abiotic stress), AL-Daura oil refinery soil unplanted and treated with silver nitrate ($AgNO_3$) (biotic stress), in addition, control soil planted. Maize crop was managed according to the recommended conventional agronomical practices.

Tests of Soil Samples

The soil sampling procedure involved taking five individual samples at equidistant points in each pot to obtain a representative sample. Individual samples of 50 g of soil each were mixed to get a single sample of 250 g. For each test, the sampling procedure was performed in triplicate. The physical and chemical properties of the soil samples, Petroleum hydrocarbons, and polyclinic aromatic hydrocarbons in soils were analyzed

Isolation and Enumeration of Petroleum Utilizing Bacterial (PUB) and Petroleum Utilizing Fungi (PUF) in the soil

Enumeration of heterotrophic bacteria (HB) and fungi (HF) and petroleum-utilizing bacteria (PUB) and petroleum-utilizing fungi (PUF) from soil samples were performed by dilution plate method before sowing with maize seeds and after harvesting (after 100 days from sowing). Nutrient agar (NG) medium was used to enumerate the HB, Potato Dextrose Agar (PDA) medium to enumerate the HF, and Bushnell-Haas medium (BH) was used to enumerate and isolate the PUB and PUF, respectively (11). Nutrient agar and PDA mediums were prepared according to the manufacture's procedure, while BH medium was prepared from the following components: K_2HPO4 (1 g/L), KH_2PO4 (1 g/L), $MgSO_4$ (0.2 g/L), NH_4NO_3 (1 g/L), $CaCl_2$ (0.02 g/L), FeCl2 (0.05 g/L), and agar 20% with 1% crude oil as carbon energy source melted in 1 L distilled water, autoclaved at 121 °C, 15 psi for 15 min (19).

Serial dilutions were made, in which a 10 g soil sample was taken from the pot at the desired time and was mixed with a 90 mL sterile normal saline (0.9% w/v of sodium chloride, NaCl) in sterile flasks. After 1 h, each 100 µl of supernatant from sample dilutions was taken and spread on the related medium. The plates were swirled, allowed to solidify, and incubated at 37 °C for 24 h for HB and 5-7 d for PUB 28 °C for 5 d for HF and at 7-10 d for PUF, and. The bacterial and fungal colonies were enumerated and recorded as colony forming units (CFU) 1 g^{-1} soil samples. Pure cultures were obtained by repeated subculturing on fresh NG and PDA media (20). Results were observed in terms of a colony-forming unit per gram of soil. The population of microorganisms

present in 1 gm of soil sample = Average no. of colonies X plate detection factors described by (21).The identification of bacteria was done using the Diagnostics NP 24 and Diagnostics GP 24 kits, which based on 24 miniaturized biochemical tests and an internet database that has been carried out. Isolation of fungal isolates was performed based on their morphology, e.g., shape, size, spore color, and texture. Fungal mycelia of the isolated strains were observed under light (BLE, Italy) (21).

Results and Discussion

According to the results, both sites (refinery and the power station) were contaminated with different petroleum compositions. There were differences between the three soils in their TPH, PAH, HMs, and EC contents (Table 1). Roberts (22) reported that a pH range of 6.5 to 8.5 is optimum for hydrocarbon degradation. In this study pH was in the optimum range for hydrocarbon degradation (7.39-7.89). Salinity plays a major role on the acceleration of biodegradation process of crude oil (23), but high salinity decreased the bacteria growth rate (24). Total Petroleum Hydrocarbons contents are normally used as a contamination indicator for oil contaminated soils. TPH directly reflects the real contamination posed by external contaminants because it excludes the polar hydrocarbons which can be accumulated during bioremediation processes (25).

Table (1): TPH, PAH, HMs, and EC contents of the soil samples from three sites.

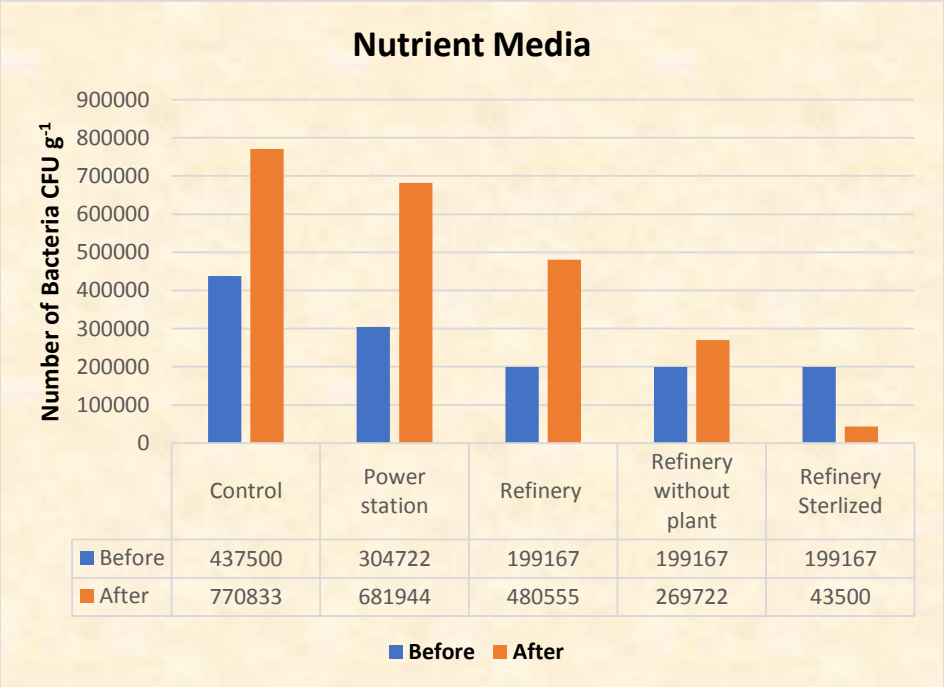
Characteristics and Unit	Value		
	Control	Al-Daura power station	Al-Daura oil refinery
pH	7.39	7.46	7.89
EC ($ds.m^{-1}$)	3.06	7.15	13.40
Total petroleum hydro-carbons $mg.kg^{-1}$	126.7	916.7	1340
polyclinic aromatic hydro-carbons $mg.kg^{-1}$	17.93	63.23	42.18
Heavy Metals $mg.kg^{-1}$			
Pb	6.46	20.02	15.37
Cu	14.56	35.87	44.31
Zn	51.00	67.78	71.78
Cr	29.48	40.19	46.22
Ni	37.82	180.95	153.06

Microorganisms Calculation and Identification

In rhizospheric soil, plant roots, bacteria, and fungi form tripartite associations ranging from beneficial to harmful interactions based on exchange of complex signaling dialogs and nutrient compounds by which each copartner influences the other to bypass the different biotic and/or abiotic stresses able to disrupt their life cycle. Therefore, the microbial communities living in soil or in association with roots are intimately linked to the different exudates released in the rhizosphere (root and microbial exudates), to soil composition, and to climatic conditions (26).

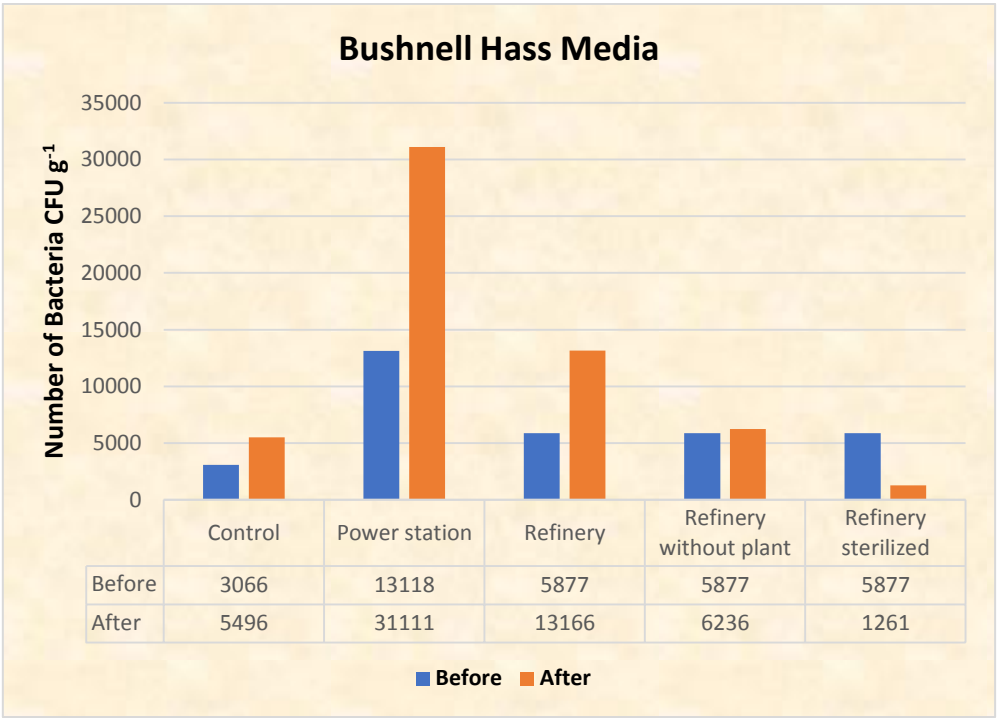
Bacteria

Petroleum hydrocarbons and PAHs in the soil can be used as a carbon source by microorganisms. The natural selection of the native microbiota is probably due to the area contamination history by oily wastes (27). Therefore, the persistence and the action of microorganisms in the plant environment play a critical role in improving petroleum dissipation (28). The results revealed a positive effect of maize on rhizospheric total heterotrophic bacteria and total petroleum degrading bacteria (Figures 1,2). The total number of bacteria and HCs-degrading microorganisms were evaluated during phytoremediation by cultured in a proper media agar. The mean total aerobic bacterial count in planted and unplanted soils ranged between 4.35×10^4 to 7.7×10^5 CFU.g^{-1} cultured on nutrient media agar. No significant difference was observed in the mean total aerobic count among all soils at the beginning of the study. Nevertheless, there is a significant difference at the end of the study, where control soil recorded the highest value with 7.7×10^5 CFU. g^{-1} followed by cultivated power station soil (6.8×10^5 CFU. g^{-1}) then cultivated refinery soil (4.8×10^5 CFU. g^{-1}) (Figure 1).



$LSD_{0.05}$ before planted= N.S; $LSD_{0.05}$ After planted=389386; $LSD_{0.05}$ Before planted vs. After planted, Control=N.S, Power station soil planted =324828, Refinery soil planted=N.S, Refinery soil unplanted=N.S, Refinery soil sterilized= 106807.

Figure (1): Total bacterial account cultured on nutrient media agar.



$LSD_{0.05}$ before planted= 2817.5$LSD_{0.05}$ after planted=7395.8
$LSD_{0.05}$ before planted vs. After planted, Control=824.61, Electricity soil planted=14059, Refinery soil planted=5819.8, Refinery soil unplanted=2368.9, Refinery soil sterilized= 2307.1.

Figure (2): Total bacterial account cultured on Bushnell Hass media agar.

At the same time, the mean total aerobic bacterial count ranged from 1.2 × 10^3 to 3.1 × 10^4 CFU. g^{-1} cultured on BH media agar, and there are significant differences between the soils at the beginning and the end of the study, with the superiority of the cultivated power station soil (1.3× 10^4 and 3.1×10^4 CFU. g^{-1}), followed by the cultivated refinery soil (5.8×10^3 and 1.3×10^4 CFU. g^{-1}) at the beginning and the end of the study, respectively (Figure 2). It may be due to the presence of maize plants stimulated microbial number and activity in the rhizosphere, as demonstrated by the higher values obtained in planted soils relative to the unplanted soils. Milic *et al*. (29) obtained the total number of bacteria in the soil during bioremediation in the range 10^7–10^8 CFU. g^{-1} dry soil and the number of HCs-degrading bacteria was 10^6–10^7 CFU g^{-1}. Furthermore, they observed that the microorganisms which decompose HCs were the dominant microbial population at the end of the process, with a share of more than 80 % (range 10^7 CFU. g^{-1}).

At the beginning of the study, the bacterial count culture on nutrient agar in control treatment was more than that obtained with the other contaminated treatments. The low number of bacteria in the contaminated soil may cause by the poor soil conditions which may be due to sub-optimal soil aeration and increased demand for oxygen caused by the increased population of decomposing oil microbes attracted to the soil due to the contamination. Also, high levels of HMs can impede successful bioremediation due to their toxicity to microorganisms. In addition to electrical conductivity, which is one of the several environmental factors that affect the biodegradation process as

reported by other authors (30, 31). The rhizospheric total aerobic bacterial counts for vegetated and non-vegetated soils cultured on NA and BH agar increased at 100 days of culture except for the refinery sterilized soil which decreased. Increasing the number of bacteria for the vegetated soils indicates that the plant stimulates the growth of bacteria, and the high microbial count on BH indirectly shows the ability of the bacterial culture to utilize crude oil as an energy and carbon source by the organism.

Plants produce allopathic compounds analogous to organic contaminants that stimulate microbial defenses against toxic compounds and supply microorganisms with nutrients (28). In addition, roots offer mechanical support for the attachment of microorganisms and an improvement of soil physicochemical properties (e.g., aeration), which further benefit the development of microorganisms in the rhizosphere (28). Furthermore, the moisture content of soil plays a vital role in microbial activities (32), and eventually, the populations of pollutant-degrading microbes are increased in the rhizosphere and endosphere (33, 34). Similarly, the increase in the number of bacteria on both refinery unplanted and sterilized soil responds to environmental conditions and the humidity and temperatures that were more suitable for the growth of bacteria because the climate has changed in May, which in line with previously reported (35). Microbial communities in planted soils are more significant and more active than unplanted ones. The current study indicated this positive influence of maize plant roots on microbial population and activity.

The bacteria were isolated from three soil sites on BH medium agar. Bushnell Hass medium is a proper medium for isolating oil or HCs-degrading bacteria. This media contains all the nutrients except HCs, which are necessary for the growth of bacteria. Thus, the bacteria can decompose various HCs such as kerosene, mineral oil, paraffin wax, and gasoline (36). After 72 h of incubation in BH agar medium, then on NA, MacConkey, blood media and diagnosed by identification kit (microtitration plate), successfully obtained bacterial isolates from those soils. Totally, 11 pure bacterial isolates could grow on BH agar medium containing crude oil from these contaminated soils (Table 2).

Table (2): Bacterial strain isolated from the three soil sites.

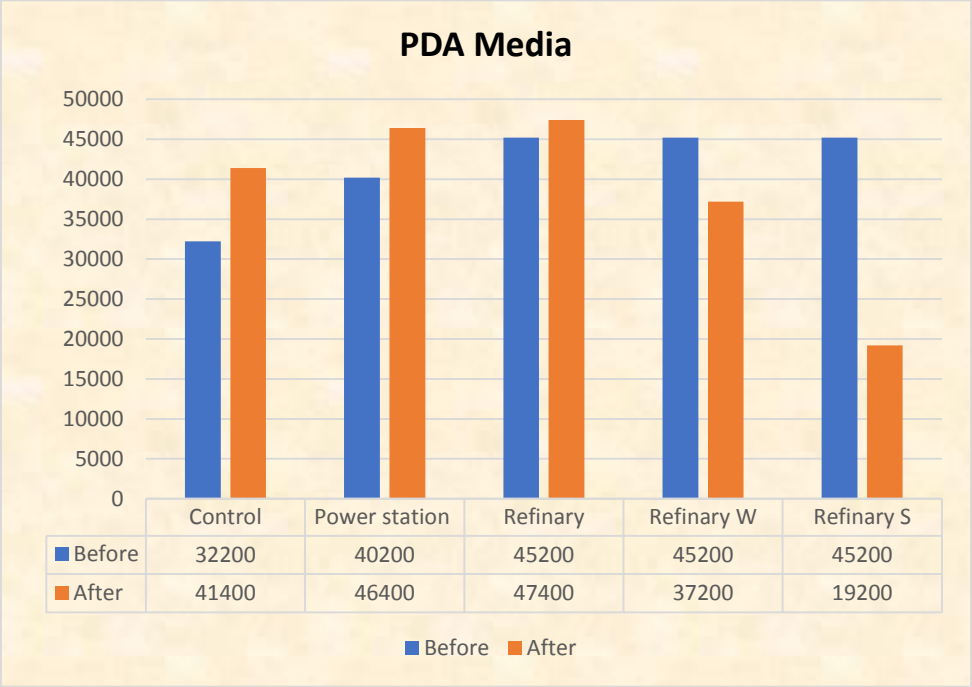
Control soil	Refinery soil	Power station soil
Staphylococcus chromogenes	*Burkholderia cepacia*	*Burkholderia cepacia*
Pseudomonas aeruginosa	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*
Actinomyces radingae	*Staphylococcus piscifermentans*	*Vibrio parahaemolyticus*
Micrococcus lutous	*Bacillus subtitis*	*Erysipelothrix rhusiopathiae*
	Bacillus cereus	*Pseudomonas fluorescens*

Although the control soil showed high microbial growth on NA media, only a few isolates could grow on BH medium. By contrast, power station and refinery soils that presented low growth on the NA media, had a more potential number as oil degrader on BH medium. It is clear that isolated bacterial strains were oil-degrading due to their capability to grow on an oil-containing medium and used crude oil as a carbon source. Therefore, the petroleum-degrading bacterium was able to enhance the biodegradation of oil in the

field significantly. Ra's (37) isolated 16 bacteria from two petroleum-contaminated soils as well as control soil (garden soil), were 16, 5, and 17 bacterial strains grown on BH media, and bacterial plate count (6.0×10^2), (4.0×10^3) and (6.0×10^4) CFU.g^{-1}, respectively. High soil, roots, and shoots endosphere colonization capabilities by bacteria strains and the interaction between inoculated endophytes and autochthonic soil microbiota (38).

Fungi

Fungi play a significant role in eliminating hazardous compounds from soil and water contaminated with oil spills. They inhabit such substrates and utilize hydrocarbons as a source of carbon. Fungi are known to be one of the best oil-degrading organisms (39). Results of fungal analysis of the three soils before and after 100 days of phytoremediation with maize are represented in figure 3.



$LSD_{0.05}$ Before planted= 12235 $LSD_{0.05}$ After planted=10068
$LSD_{0.05}$ Before planted vs. After planted, Control= N.S, Power station soil, planted=N.S, Refinery soil planted=N.S, Refinery soil unplanted=N.S, Refinery soil sterilized= 11157.

Figure (3): Fungal account on Potato dextrose agar media.

The mean total fungal count in planted and unplanted soils ranged between 1.92×10^4 to 4.7×10^4 CFU.g^{-1}. The initial number of fungi cultured on PDA media agar indicates that the contaminated soils were contained a high number of fungi with a significant difference comparing with the control soil. Refinery and power station soils recorded 4.5×10^4CFU. g^{-1} and 4×10^4 CFU. g^{-1}, respectively comparing with 3.2×10^4CFU. g^{-1} for the control soil. There is no significant difference between the power station and refinery soils, which indicates that soil contamination did not affect the number of fungi, on the contrary, the oil content increased their number. Milic *et al.* (29)

obtained the total number of yeast and molds 10^4–10^5 CFU.g^{-1}, and the number of hydrocarbon-degrading fungi 10^5–10^6 CFU. g^{-1}. Organic chemical pollution in soil and water has been demonstrated to affect microbial populations (40). Many studies have reported that the soil fungal population is not significantly affected by contaminants. For example, the number of fungi was relatively higher in HMs polluted soils than in non-polluted soils (41). Hiroki (42) stated that although the number of bacteria decreased with increasing HMs content, there was no significant correlation between the number of fungi and HMs content, the degree of tolerance to HMs appears to be: fungi > bacteria.

Results of fungal analysis of the three soils after 100 days of phytoremediation with maize are represented in Figure 3. The rhizospheric fungal count of the planted soil increased at the end of the experiment with no significant differences from each other. However, the fungal number of contaminated cultivated soil of refinery and power station was higher than that of the control cultivated soils, which were given 4.7×10^4, 4.6×10^4, and 4.1×10^4, respectively. On the other hand, decreased in unplanted (3.7×10^4) and sterilized (1.9×10^4) refinery soil were significantly from planted refinery soil. Iffis *et al*. (30) speculate that in PHCs contaminated soils, arbuscular mycorrhizal fungal (AMF) may release chemical compounds by which they recruit beneficial microbes to tolerate or degrade the PHCs pollutants present in the soil. Alternatively, Fungi can be related to the release of chemical substances and enzymes by the plant roots, which favor microbial growth (43).

When comparing the number of fungi at the beginning of the experiment and after 100 days for each treatment, a non-significant increase in the number of fungi was observed at the end of the experiment, with the exception of sterile refinery soil, in which a significant decrease of 59.5% occurred. These results indicate that the presence of the maize was beneficial to microbial activity. According to a study published by Atagana *et al.* (44), fungi showed the potential to degrade high-molecular-weight PAHs and other recalcitrant organic compounds due to their complex enzymatic systems with the ability to synthesize and excrete. In addition, Chikere and Azubuike (45) observed higher fungal counts (5.7×10^4 CFU. g^{-1}) and attributed to the site closeness to the petroleum industry; such closeness increased in total PHCs content, which in turn increased PHCs-degradation microbial activities. Diagnosis of fungal strains grown on PHCs was carried out after cultivation for a week on the BH medium and then on PDA; obtained strains are listed in Table (3).

Table (3): Fungal strain isolated from the three soil sites.

Control soil	Refinery soil	Power station soil
Aspergillus spp.	*Aspergillus* spp.	*Aspergillus* spp.
Byssochlamys nivea	*Fusarium verticillioides*	*Aspergilluscandidus*
Emericellanidulans	*Emericellanidulans*	*Emericellanidulans*
Rhizopusoryzae	*Aspergillusterreus*	*Rhizopus oryzae*
		Aspergillusterreus

Different studies have identified numerous fungal genera capable of utilizing crude oil as a source of carbon and energy, including *Cephalosporium, Rhizopus, Paecilomyces, Alternaria, Mucor, Talaromyces, Gliocladium, Aspergillus, Fusarium, Rhodotolura, Cladosporium, Geotrichum, Penicillium, Torulopsis,* and *Pleurotus* (46,47). Chikere and Azubuike (45) used BH medium for the isolation; the genera of fungi isolated were: *Aspergillus, Candida, Penicillium, Rhizopus, Saccharomyces, Cladosporium, Fusarium, and Mucor*. Among the genera of fungi isolated, *Aspergillus* had the highest frequency of occurrence, 36.84%, and 27.59%, while *Rhizopus* had the least frequency of occurrence, 5.26% and 3.45% for contaminated water and sediment samples, respectively. The total heterotrophic fungal counts ranged from 1.9×10^3 to 2.3×10^4 CFU.ml^{-1} and 3.4×10^3 to 3.8×10^4CFU. g^{-1}.

Research results differed about which is more efficient in degrading PHCs, is bacteria or fungi. Ikuesan (6) and Ali (8) indicated that bacteria are more active than fungi; results of Omotayo *et al.* (48). Came on the contrary, they were found that the fungi were more active than bacteria in the PHCs biodegradation. However, most biodegradation studies have shown that the potential for microbial biodegradation isolated from oil-contaminated sites was higher than those obtained from non-contaminated sites (10). This suggested that crude oil contamination of soil caused adaptation to the microbial population for PHCs biodegradation (49).

Conclusions

The used of maize plants played an important role in stimulation the microorganisms. They were increased the number of microorganisms. Also, the petroleum degraders microbial increased in the oil-contaminated soil and the non-contaminated soil. The microbial degraders are found in the entire treatments. The long-term exposure of soil microorganisms to high PHCs concentration decreases the soil total bacterial account.

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