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Extraction of Fungi from Soil Contaminated with Petroleum and Testing Them for Crude Oil Degradation Capability

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Abstract

The excessive and haphazard use of petroleum or its byproducts has resulted in petroleum waste, a serious environmental issue. It's interesting to note that bacteria now dominate the breakdown of petroleum. In order to clean up the environment from petroleum waste, fungi were separated from soils containing petroleum waste and their capacity to break down light and moderate crude oil (MCO and LCO) was assessed. In the north of Baghdad, twenty-one locations with petroleum contamination were investigated. To separate fungus from soil, the serial dilution approach was applied. Fungal community colony forming units (CFU) and the proportion of commonly isolated fungal genera and species per location were computed. Temperature, electrical conductivity (EC), hydrogen ion (pH), and physiochemical properties were all measured. Considering. From the polluted soils, a greater variety of fungus were identified. The most frequently observed genus was Aspergillus species. The greatest number of fungal colonies (115x106 CFU/gm) was found at Site S13. Temperature ranged from 22.4 to 24.9 °C, pH from 7 to 9.3, and EC from 0.1 to 0.8. Crude oil was decomposable by all 10 species of fungi. In terms of growth rate, Paecilomyces variotii was able to break down the two types of oil crude the best (FGR at 7.8), with Fusarium pallidoroseum coming in second (FGR at 6.5 of MCO and LCO at 7.2). Emericella nidulans grew the least on the LCO medium (FGR at 4.5), while Aspergillus flavus grew the least on the MCO medium (FGR at 4.8).

Keywords: Biodegradation; Aspergillus; Physiochemical properties

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1. Introduction

Pollution is a central threat to ecosystems, human health, and other organisms [1]. Hydrocarbon pollutants are one of the most common environmental problems causing water and land pollution [2]. Petroleum is a complex mixture of hydrocarbons and other organic compounds [3]. Indeed, petroleum contamination changes some physicochemical characteristics of the surrounding environment, such as pH, EC, and temperature [4]. Large concentrations are also highly toxic to many organisms, including humans [5].

The interest in the biodegradation or bioremediation of pollutants has grown significantly due to the need to find sustainable means for environmental cleanup [6]. Bioremediation attempts to utilize the endurance features of microorganisms and their significant ability to eliminate many pollutants, including petroleum and other toxic pollutants [7]. Recently, the main mechanism used to clean up environments polluted by petroleum products is microbial degradation [8].

Existing research mentions the critical role of fungi in the bioremediation of crude oil-polluted soils or water [9–11]. Fungi are important organisms with

high capability for hydrocarbon degradation, particularly in soils. The main objective of this study was to evaluate the ability of fungi isolated from petroleum-contaminated soil to biodegrade petroleum hydrocarbons. Subsequently, these fungi could be used for environmental cleanup from petroleum waste.

2. Materials and Methods

2.1 Samples Collection

Twenty-one petroleum-contaminated sites in Al-Taji north of Baghdad were sampled. At each site, six to seven small samples (approximately 150 g each) were randomly collected from the soil surface (0-30 cm depth) and combined in sterile bags. At each site and 200 meters away from pollution point, uncontaminated soil was also collected and combined together at one sample. These were transported immediately to the laboratory.

2.2 Isolation and Identification of Fungi

For fungal isolation, the serial-dilution method was used. 1ml dilutions of 10^{-6} and 10^{-3} were plated onto 10 cm diameter Petri-plates containing potato dextrose agar (PDA) prepared according to the manufacturer's (MilliporeSigma) instructions (39g in 1 liter). The plates were incubated in the dark at 25°C for 7 days and the number of colony-forming units was recorded. Fungal isolates were identified according to microscopic observations and cultural characteristics, and were classified according to taxonomic keys [12-23].

2.3 Primary Screening procedure

The enrichment procedure was used to estimate the biodegradability of isolated fungi using the modified Hanson technique [24]. A 0.5 cm diameter disc of these fungi (7-days-old) was transferred to Bushnell Haas agar medium (Magnesium sulphate 0.2, Calcium chloride anhydrous 0.02, Potassium dihydrogen phosphate 1, Dipotassium hydrogen phosphate 1, Ammonium nitrate 1, Ferric chloride 0.05, Agar 20) in Petri dishes supplemented with 0.1% (v/v) of Tween 80, 1% MCO, or LCO. MCO and LCO were obtained from the Basrah refinery, and their properties were analyzed by GC-MS. All Petri dishes (3 replicates of each species) were incubated for 20 days at 30±2°C. Fungal growth rates (FGR) were calculated by measuring the diameter of growth after 20 days.

2.4 Physicochemical properties of soil

Physical-chemical properties of the contaminated soils were tested in the laboratory [25]. The soil extract was a 1:1 (W/V) soil/water mixture. The pH, temperature, and electrical conductivity (EC) of each soil were measured using a pH and temperature meter (pH meter S400) and EC meter (TDS Meter, BEP-M510). These results were compared with uncontaminated soil collected from the same area.

2.5 Data analysis

The average colony growth of each site was calculated. In addition, the ratio of each genus and species, the number of each isolate of each genus, and species and CFU were calculated according to the equations shown in the Appendix [26]. Multiple regression analysis was used to calculate the interaction between soil physicochemical properties and the presence of fungi, variation in fungal communities and colony forming units.

3. Results

Eight fungal genera and ten species were isolated from the 21 contaminated sites. These were *Aspergillus flavus, A. terreus, A. niger, Paecilomyces variotii, Bipolaris hawaiiensis, E. nidulans, Fusarium pallidoroseum, Cladosporium herbarum, Ulocladium atrum,* and *Penicillium chrysogenum*. In contrast, *Trichoderma harzianum* and *A. niger* were isolated from the uncontaminated soil samples. *Aspergillus* was the most frequently isolated genus at 57%, followed by *Penicillium chrysogenum* at 23.81% (Fig. 1), whereas four genera, *Paecilomyces, Bipolaris, Fusarium* and *Ulocladium* were less (<5%) frequently recorded (Fig. 1). *A. flavus* and *P. chrysogenum* were the most frequently (23.81%) isolated species (Fig. 1).

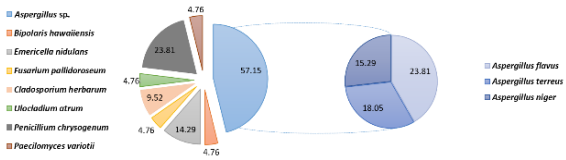

Fig. (1) The percentage of fungal genera and species most frequently isolated from soils contaminated with petroleum waste

A. flavus also had the highest percentage presence at sites S_4 (Fig. 2). However, *A. flavus* was the least capable of degrading the MCO (3±0.5mm), and with the second-lowest ability to degrade the LCO (4.8±0.5mm) in the preliminary screening test (Fig. 3). Out of the *Aspergillus* species, *A. terreus* was the second most frequently isolated at 18.05% and degraded the MCO (6±0.3mm) and the LCO (6.5±0.3mm) (Fig. 3) slightly more efficiently than the other two species. *A. niger* had the highest percentage presence at sites S_{15} and S_{17} (Fig. 2) and grew on the MCO and the LCO media at 6±0.3mm and 6.5±0.4mm, respectively (Fig. 3).

P. variotii was capable of degrading both types of crude oil with growth diameters at 7.8±0.1 (Fig. 3) followed by *F. pallidoroseum*, which degraded the MCO and LCO at rates of 6.5±0.4 mm and 7.2±0.1 mm, respectively.

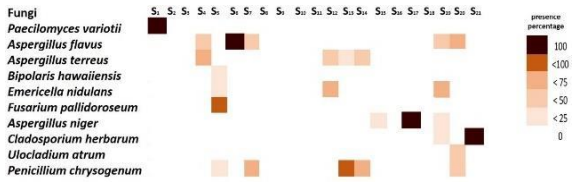

Fig. (2) Heatmap showing the percentage of fungi species present in each soil black oil waste site. White squares represent the absence of fungi, and dark red squares represent the highest rate of presence

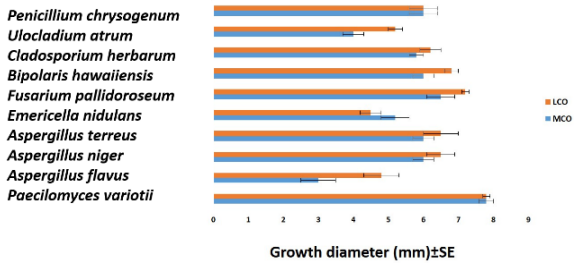

Fig. (3) Mean growth diameter (mm ± SE) for ten fungi species grown on moderate crude oil (MCO) and light crude oil (LCO) media over 20 days at 25°C in the dark

The CFU values varied significantly between sites. The highest number of CFU was recorded at site S at 10^6x10^6 CFU/g soil, followed by site S_{135} at 10x10^6 CFU/g soil. At the same time, no fungi were recorded at eight of the 21 sites.

Regarding soil physicochemical properties, the pH of all soil sites ranged from 7.0 to 9.2. In contrast, the pH of uncontaminated soil was 6.5. EC of the sites ranged from 0.12 to 0.8., compared to 0.16 in the uncontaminated soil. The soil temperatures were calculated immediately after they were collected and ranged from 22.4–24.9°C. This compared to 23.1°C for the uncontaminated soil. The correlation data showed that the relationship between pH and the presence of fungi was poor ($p<0.1$) with a high impact ($R2 = 0.41$).

In contrast, there was no effect of either temperature or EC on the presence of fungi. Concerning variations in fungal communities and the effect of soil physicochemical properties on them, the results showed a small relationship ($p<0.1$) with a high impact ($R_2 = 0.66$) between EC and variation in the fungal communities. In comparison, there was no effect of pH and temperature on the variation of the fungal communities. Finally, the relationship between soil physicochemical properties and CFU showed that pH significantly affected the fungal community ($R_2=0.57$, $p=0.0003$). At the same time, EC and temperature had no effect. The GC-MS results showed differences between LCO and MCO concerning the aromatic and saturated compounds.

4. Discussion

Bioremediation is an important branch of biotechnology that uses different organisms, including microorganisms and plants, to clean up environments and degrade contaminants to levels with minimal toxicity [27]. Compared to traditional bioremediation techniques, fungi are the most efficient petroleum degraders [28].

The preliminary step for using fungi in bioremediation and biodegradation of petroleum is isolating and identifying fungi associated with petroleum-polluted areas, and conducting primary screening to evaluate their ability for biodegradation. This study isolated 10 species belonging to eight fungal genera from 21 sites with petroleum-contaminated soil north of Baghdad. They have previously been isolated from petroleum-contaminated soils [29-31]. *Aspergillus* was the most frequently isolated genus among the eight fungal genera in this study. Filamentous fungi play a significant role in biodegrading petroleum and its derivatives [32]. *Aspergillus* and *Penicillium* are the filamentous fungi most frequently isolated from petroleum-contaminated soils worldwide [33]. *Aspergillus* species have been documented as possible candidates for biodegradation of a broad range of petroleum hydrocarbons in the environment [34,35]. The main reason for the ability of *Aspergillus* to biodegrade petroleum could be that this genus can degrade both aromatic and saturated hydrocarbons [33].

In the present study, *Aspergillus* was represented by three species, *A. flavus*, *A. terreus* and *A. niger*. *A. flavus* was the dominant species at site S_6 and was the most frequently isolated species. This species was previously isolated from different petroleum polluted sites [29,36]. However, in the present study, *A. flavus* was the least capable of degrading the MCO and the second less capable of degrading the LCO. This finding is somewhat counterintuitive and contrary to previous studies, which have demonstrated that *A. flavus* has the highest ability to degrade crude oil at several concentrations of crude oil derivatives [29]. Considerably more work will need to be done to determine the ability of *A. flavus* for degrading different petroleum kinds.

A. terreus was isolated from a petroleum-contaminated site in a previous study [37], and has a high capacity to degrade heavy oil [38], and other aromatic hydrocarbons [39]. In the current study, *A. terreus* was the third most frequently isolated species and degraded both the MCO and the LCO.

A. niger recorded the highest percentage presence at sites S_{15} and S_{17} and was capable of degrading the MCO and the LCO. These results reflect those of Gesinde et al. [40], who also found that *A. niger* had the highest capability of degrading four kinds of crude oil, Arabian light, Durb oil, Bonny light, and Escravos light.

Interestingly, *P. variotii* had the highest capacity to degrade the two kinds of crude oil. This species also dominated site S_1 in this study. A possible explanation for this might be that *P. variotii* has a high capacity to degrade different hydrocarbons, whether aromatic or saturated. Despite the significant differences between LCO and MCO concerning the aromatic and saturated compounds, shown by GC- MS, *P. variotii* was capable of degrading them both. Consequently, *P. variotii* could be a potential candidate for eliminating oil spills.

decomposed by fungi. Secondly, the density of LCO is less than MCO; thus, LCO has less viscosity. Thirdly, MCO has a large amount of asphaltene that could make it more resist biodegradation [43]. However, the high ability of some of these fungi, such as *P. variotii* and *F. pallidoroseum*, as biodegraders of LCO and MCO is an important issue for future research.

5.1 Fungal community

Petroleum waste degradation through the microbial community of soil depends on many factors, including abundance, the kind and catabolic performance of microorganisms, and environmental conditions and chemical structure of the compounds to be biodegraded [44]. On the other hand, crude oil pollutants are a crucial issue for the abundance of microorganisms in the soil. In this study, the results of CFU showed significant variation between the sites, with the mean total of colony forming units being $6.5x10^6$ CFU/g soil. This finding is consistent with that of Cheraghi [4] who mentioned that the lower populations of fungi and bacteria in petroleum-contaminated soil could be due to changes in soil texture and chemical content resulting from the petroleum wastes. However, the number of fungi in the soil is still in the accepted range 10_3 to 10_8 CFU/g soil [45]. Site S_{13} had the highest number of CFU followed by site S_5. Whereas at eight of the sites, no fungi were recorded. This outcome may be due to the toxicity resulting from the petroleum contaminants [46,47], or because the properties of the soil, such as pH, moisture and organic content, are altered [5, 48].

5.2 Physicochemical properties of soil

Soil physicochemical properties significantly affect microbial degradation of hydrocarbons [49]. One of the most important factors of soil is pH value because it affects numerous chemical processes in soils [50]. The pH is considered a determining factor in soil pollutants destination, their decomposition, and leakage in the soil [4]. Comparison of the findings with those of other studies confirms the pH of the soil was impacted by the wastes in oil crude and its derivatives [4,51-53]. The second factor is electrical conductivity (EC) which represents the concentration of ions in soil [4]. The fluctuations in the EC results in current research indicate that oil crude may influence soil ionic stability. The third factor studied in this study was soil temperature (°C), and

Another species with a strong ability to degrade the oils was *F. pallidoroseum*, with the second- highest capability of degrading the MCO and LCO. This result is not surprising as *Fusarium* spp., *Aspergillus* spp. and *Penicillium* spp. have been listed as species with significant abilities to biodegrade hydrocarbons [41, 42].

All the fungal species except *E. nidulans* could degrade the LCO more efficiently than the MCO. The most likely reasons for this result are that LCO contains many hydrocarbons, which are easy to
the results revealed no effect of oil crude on soil temperature.

The most striking results to emerge from the data are the correlation between soil physicochemical properties and the presence of fungi, variation in the fungal communities, and CFU. The results of relationship between pH and presence fungi revealed a small relationship ($p<0.1$) with a high impact ($R_2=0.41$). This outcome confirms that soil pH values can slightly affect the presence and absence of fungi. The correlation between soil physicochemical properties and variation of the communities showed a small relationship ($p<0.1$) with a high impact ($R_2=0.66$) between EC and variation of the fungal communities. This outcome confirms that the EC value of soil contributes to some variation in the composition of the fungal communities in soil. This finding was not surprising, as EC was reported as one of the factors affecting the microbial community in different types of soil [54,55]. The outcome of pH is contrary to that of Lloret et al. [55] who found that pH had the highest impact on the fungal communities. This could be because fungi can grow in a wide range of pH [56].

What is interesting in these results is that the relationship between pH and the CFU was statistically very highly significant. This result reflects another study that found that microbial biomass was impacted by soil pH [57].

Indeed, physicochemical properties could have an impact on fungi in soil whatever the presence of fungi or variation in the fungal communities or CFU.

6. Conclusion

The purpose of the current study was to isolate fungi from soils contaminated with crude oil and evaluate their capability to degrade two kinds of Iraqi crude oil (moderate and light). *A. flavus, A. terreus, A. niger, P. variotii, B. hawaiiensis, E. nidulans, F. pallidoroseum, C. herbarum, U. atrum* and *P. chrysogenum* belonging to eight fungal genera were isolated from different soil sites north of Baghdad that were contaminated with crude oil. This study has shown that all these species can degrade both kinds of crude oil. The research has also shown that *P. variotii* has the highest ability to degrade the two kinds of crude oil. This species requires further study. Related to soil physicochemical properties, pH can influence the presence of fungi, and

has a significant effect on CFU. Whereas EC can influence the composition of fungal communities in soil. These findings need more research in future to determine the best conditions for fungi to effectively eliminate crude oil waste.

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Appendix

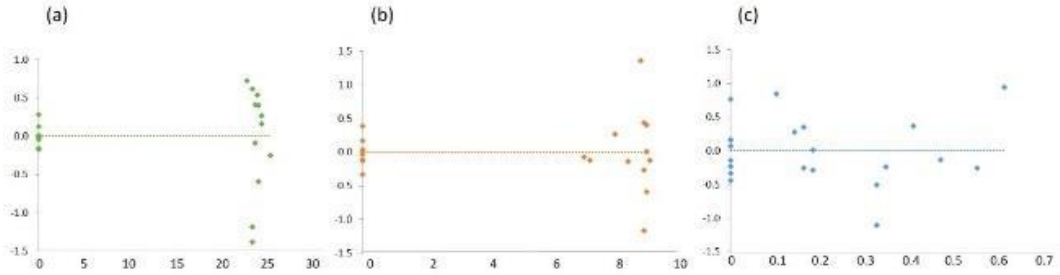
% genus presence= $\frac{\text{no. of a genus in the site}}{\text{no. of all other genera on the site}} \times 100$

% species presence= $\frac{\text{no. of a species in the site}}{\text{no. of all other species on the site}} \times 100$

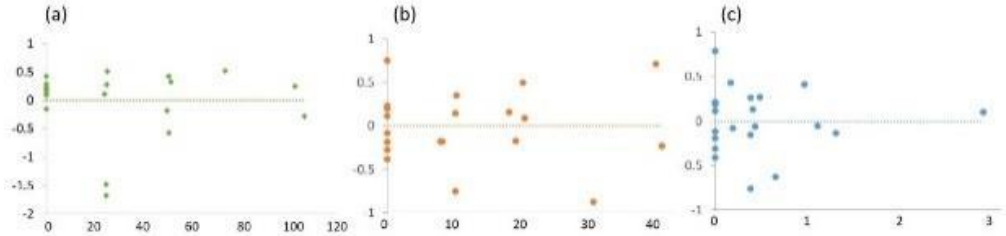
% frequency of a genus = no. of angoe. noufssiintetshe site ×100

% frequency of a species = $\frac{\text{no. of a species in site}}{\text{no. of sites}} \times 100$

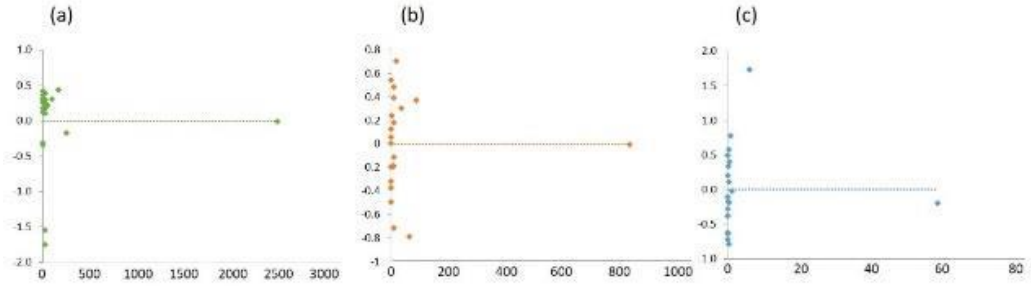
CFU= (no. of colonies x dilution factor) / volume of culture plate



Supplementary (1) Multiple regression analysis showing correlation between three physicochemical variables and fungal species based their presence or absence: (a) correlation between temperature and the presence of fungi, (b) correlation between pH and the presence of fungi, and (c) correlation between EC and the presence of fungi



Supplementary (2) Multiple regression analysis showing correlation between three physicochemical variables (a) temperatures (b) pH and (c) EC and variation of fungal community



Supplementary (3) Multiple regression analysis showing correlation between three physicochemical variables (a) temperature, (b) pH, and (c) EC and rate of fungal community (CFU)

