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Full Length Article

Fungal Diversity in Crude Oil-Contaminated Soils in Basrah Province with a Preliminary Study of its Ability to Biodegrade Crude Oil

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Abstract

A preliminary study was conducted on fungal diversity in crude oil-contaminated soils in Basrah province to determine the ability of isolated fungi to biodegrade crude oil. Ten oil-contaminated soil samples were obtained from several oil fields in Basrah province, southern Iraq. Nine fungal genera were isolated, with *Aspergillus* representing the highest percentage of occurrence (60%). The percentages of occurance of the other genera were between 10–30%, with *Pencillium* accounting for the second highest at 30%. The rest of the genera included *Trichoderma* (20%), *Acremonium* (10%) and others that appeared in one or two samples. Twenty fungal species were isolated as well as sterile mycelia. 95% of the isolated fungi belonged to Ascomycota, followed by Zygomycota with one species (5%). The indicator 2,6-dichlorophenol indophenol was used to study the preliminary ability of the isolated fungi to biodegrade crude oil. Nine fungal species showed different abilities to biodegrade crude oil, with *Aspergillus niger* and *A. terreus* exhibiting the highest biodegradation ability.

Keywords: Crude oil; Contaminated soils; Fungal diversity; Biodegradation

Introduction

Crude oil is one of the most significant sources of energy in the world today (OPIC 2023). It is used as raw material in most productions and its derivatives are used in other chemical processes (Brown *et al.* 2017; Marchand *et al.* 2017). The release of crude oil from exploitation, transportation, storage and utilization processes often occurs because of improper safety measures and sudden accidents, which cause serious soil pollution (Emilio *et al.* 2021).

Crude oil contains high percentages of aromatic, aliphatic, resin and asphaltene products, which are hazardous to animal and human health (Muraza and Galadima 2015; Ramdass and Rampersad 2021). Hydrocarbon-polluted environments contain a wide diversity of microorganisms that are adapted to metabolizing hydrocarbons; these diverse microorganisms include fungi and bacteria and most of them can utilize hydrocarbons as a source of growth and energy to gain the ability to adapt in harsh conditions; thus, scholars should aim at determining how they can be used in bioremediation methods (Mohammadian *et al.* 2017). Seventy or more known genera of microorganisms that can degrade hydrocarbons, such as fungi and bacteria, algae, *etc*., are found in polluted soil and they have the ability to degrade hydrocarbons (Rufino *et al.* 2013). The removal of hydrocarbon oil spills in the soil can be achieved by several effective techniques, including physical, chemical and natural procedures, which can be useful for the remediation of oil contaminants (Ghannam and Chaalal 2003). Natural processes, such as bioremediation, have been the focus of previous research; bioremediation is a technique for the degradation, removal, or transformation of pollutants or contaminants to less harmful products through biological resources (Shakya *et al.* 2021). Bioremediation techniques are cheaper and eco-friendlier than chemical and physical approaches for the biodegradation of oil-contaminated soil; they use the ability of microorganisms to use pollutants as a source of carbon and energy to transform them into less toxic products than their parent compounds (Abdel-Shafy and Mansour 2016).

Microbiological methods have been developed and widely used in recent years (Rahmati *et al.* 2022). Microorganisms, especially bacteria and fungi, have caught the interest of scientists in performing bioremediation (Ghannam and Chaalal 2003). Soil contains a number of fungal genera, such as *Aspergillus* spp., *Fusarium* spp., and *Trichoderma* spp., that have the ability to biodegrade petroleum oil hydrocarbons (Clarkson and Abubakar 2015; Al-Dossary *et al.* 2021).

Traditional bioremediation techniques are not as effective as fungi, which are better degraders of crude oil; thus, increasing concerns have been focused on fungi because of their ability to produce enzymes that are

applied for degradation of different environmental contaminants including hydrocarbons (Al-Nasrawi 2012; Al-Dhabaan 2021).

Fungi can secrete different classes of enzymes, such as intracellular enzymes (cytochrome P450 monooxygenases) and extracellular (laccases and peroxidases) enzymes that can degrade crude oil (Gnanasekaran *et al.* 2019; Daccò *et al.* 2020). The important advantages of using fungi include their mycelial structure with an invasive nature, large surface area, and secretion of enzymes for bioremediation of crude oil-contaminated soil (Benguenab and Chibani 2021). Numerous fungal strains can degrade crude oil and successful bioremediation of contaminated sites has been documented (Essabri *et al.* 2019; Ryszka *et al.* 2019).

The present study aimed to determine fungal diversity in crude oil-contaminated soils in Basrah province and investigate the preliminary ability of some isolated strains of fungi to biodegrade crude oil.

Materials and Methods

Samples collection

Ten oil-contaminated soil samples (100 g) for each sample were obtained from the surface layer (5–10 cm) from seven oil fields in Basrah province, southern Iraq from September 2021 until November 2022 (Fig. 1). The samples were preserved in sterilized plastic bags and stored at 4°C until use (Latha and Kalaivani 2012).

Chemicals

Crude oil: Southern Oil Company (Basrah, Iraq) supplied regular Basrah crude oil. The oil was kept in a cold and dark place until use after being transferred to the laboratory in a tightly closed sterilized dark bottle.

Culture media

All chemicals and media were obtained from Hi-Media (Mumbai, India). DNA extraction kit was purchased from Geneaid Biotech.

Fungal isolation

Fungal isolation from oil-contaminated soil was performed using the dilution method described by Wicklow and Wittingham (1974); 10 g of each soil sample was dissolved in 90 mL distilled water to attain a dilution of 10^{-1}, and the dilutions were shaken well for 10 min. A sterile pipette was used to transfer 1 mL of each dilution to sterile Petri dishes. For the primary isolation of fungi, one type of media used potato dextrose agar (PDA). PDA medium was prepared in accordance with the direction of the manufacturing company (HiMedia, India). The antibiotic chloramphenicol (250 mg/L) was supplemented to the medium to inhibit bacterial growth. Approximately 15 mL of each sterile medium was added separately to the Petri dish containing 1 mL diluted sample. The Petri dishes were stirred to mix the ingredients well before solidification. Then, the Petri dishes were incubated at 25°C for 7–14 days. Each colony that appeared after incubation was sub-cultured separately on a PDA medium. The pure cultures were preserved on PDA medium at 4°C for further identification.

Phenotypic and genetic identification of isolated fungi

Fungal species were first examined under a dissecting light microscope and colony features, sporulation rates and colors were recorded. Then, glass slides were prepared from each fungal colony and a compound light microscope was used to study the morphological features of each fungus (Samson *et al.* 2010). The fungi were identified in accordance with the taxonomical keys from the following works: Raper and Fennell (1973), Klich and Pitt (1988), Watanabe (2002) and Guarro *et al*. (2012). In addition, some fungal isolates whose diagnosis was suspected were identified by molecular method. Pure colony from each fungal isolate was sub-cultured on the PDA medium and incubated to grow for 7 days. The technique of Mirhendi *et al*. (2006) was used for DNA extraction from fungal colonies and polymerase chain reaction (PCR) amplification. The forward primer internal transcribed spacer (ITS) 1 (5′-TCCGTAGGTGAACCTGCGG-3′) and reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (19 and 20 base pairs [bp], respectively) were used for the amplification of the ITS regions of ribosomal DNA. The first stage of amplification denaturation was performed at 95°C for 1 min, followed by another 35 cycles of denaturation at the same temperature for 1 min. Annealing was conducted for 45 s at 55°C, extension for 1 min at 72°C, followed by a final extension for 10 min at 72°C. Distinctive products approximately 400–850 bp in length were produced. Purification of PCR products for the recovery of isolates was carried out at Macrogen (Seoul, South Korea). Basic local alignment search tool program was used for the identification and alignment of the resulting products.

Percentage of occurance of fungal isolates

The percentage of occurance of fungal isolates were calculated using the following equation:

$$\text{Percentage of occurance} = \frac{\text{No.of samples in which the genus or species appeared}}{\text{The total No.of samples}} \times 100$$

Preliminary testing of fungal isolates in biodegradation of crude oil

The preliminary testing of fungal isolates in the biodegradation of crude oil was performed in accordance with the work of Al-Nasrawi (2012) and depending on the changes in its color, the medium supplemented with the indicator 2, 6-dichlorophenol indophenol (DCPIP) was used

to evaluate isolates with the potential to degrade hydrocarbons. The fungal isolates were activated by culturing on Petri dishes containing PDA culture medium and incubation for 7 days at 25°C. A total of 100 mL conical flasks was prepared, with each containing 50 mL Bushnell–Haas broth medium, which contained the following (g L−1): $MgSO_4$, 0.2; $CaCl_2$, 0.02; KH_2PO_4, 1.0; K_2HPO_4, 1.0; NH_4NO_3, 1.0; $FeCl_2$, 0.05. The medium was supplemented with 0.5 mL crude oil as the carbon source, 0.16 mg DCPIP, and 1 mL Tween 80. A 6 mm cork borer was used to collect two plugs from the edge of 7-day-old colonies and added to each flask. The flasks were incubated in a cooled incubator at 25°C for 7 days. Changes in the medium color from dark blue to colorless were observed and recorded. The medium and crude oil were used to set up the control flasks with DCPIP but without any fungal isolate to ensure that biodegradation did not occur due to contamination.

Results

Identification of fungal genera

In this study, nine fungal genera and sterile mycelia were isolated from ten oil-contaminated soil samples (Table 1). *Aspergillus* represented the highest percentage of appearance at 60%. The percentages of appearance of other isolated genera were between 10–30%, with *Penicillium* accounting for the second highest at 30%. The rest of the genera comprised *Trichoderma* (20%), *Acremonium* (10%) and others that appeared in one or two samples. The sterile mycelia isolated in this study represented a high percentage of appearance at 50%.

Identification of fungal species

Twenty fungal species were isolated from ten oil-contaminated soil samples, 14 species were identified through phenotypic characteristics and 6 species through molecular identification (Table 2). A total of 95% of the isolated fungi belonged to Ascomycota with nineteen species either in their anamorph or telemorph state. Zygomycota had a 5% appearance, with one species observed. The percentage of appearance of species ranged from 10% to 40%; most of the species with high percentages of appearance belonged to *Aspergillus* and *Pencillium* and the rest of the species appeared in low percentages.

Preliminary testing for the ability of isolated fungi to degrade crude oil

A preliminary test was conducted for the fungal species which appeared in this study to determine their initial ability to biodegrade crude oil. The results showed that nine fungal species can change the medium color and use crude oil as a source of carbon and energy with different capabilities. Two fungal species, *Aspergillus niger* and *A. terreus*, had a high ability to biodegrade crude oil and were given a score of (+++). These species can change the color of the medium used from deep blue to colorless. As for the rest of species, their ability ranged from grade (++) for *A. fumigatus*, *Penicillium* spp., and *Rhizopus* spp. to (+), including *A. flavus*, *Trichoderma* spp., and *A. fumigatiaffinise.* Eleven species, including *A. versicolor*, *Cladosporium* spp., *Penicillium rubens*, and *Fusarium oxysporum*, were given a grade of (-) they cannot change the color of the medium and did not show any distinct ability to biodegrade crude oil (Table 3 and Fig. 2).

Table 1: The isolated fungal genera with their occurrence

Fungal genera	No. of samples in which the genera appeared	Occurrence (%)
Aspergillus	6	60
Acremonium	1	10
Cladosporium	1	10
Emericella	1	10
Fusarium	1	10
Penicillium	3	30
Phoma	1	10
Rhizopus	1	10
Trichoderma	2	20
Sterile mycelia	5	50

Table 2: The isolated fungal species with their occurrence%

Fungal species	No. of samples in which the species appeared	Occurrence (%)
Acremonium spp.	1	10
Aspergillus candidaus	1	10
A. flavus	1	10
A. fumigatus	4	40
A. fumigatiaffinise	3	30
A. niger	2	20
A. terreus	3	30
A. versicolar	3	30
Aspergillus spp.	1	10
Cladosporium spp.	1	10
Emericella nidulans	1	10
Fusarium oxysprum	1	10
Fusarium spp.	1	10
Penicillium rubens	1	10
Penicillium spp.1	3	30
Penicillium spp. 2	1	10
Phoma spp.	1	10
Rhizopus spp.	1	10
Trichoderma spp.1	2	20
Trichoderma spp. 2	1	10

*Red color mean this species identified by molecular method

Discussion

In this study fungal diversity showed that *Aspergillus* represented the highest percentage of appearance at 60%. This genus includes a diverse group of species that can adapt to a wide range of environmental conditions and extreme environments, including deserts and soils contaminated with hazardous materials such as oil (Cray *et*

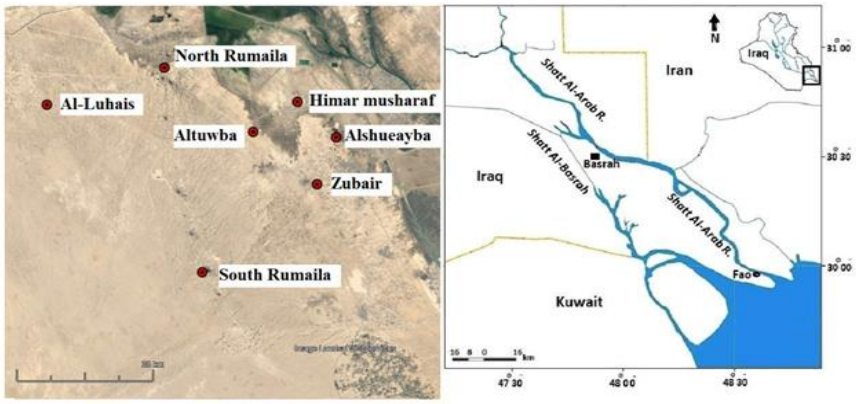

Fig. 1: Study area in Basrah Province, Iraq

Table 3: Preliminary test for the ability of fungal species to degrade crude oil

Fungi	degree of biodegradation
Aspergillus niger	+++
A. terreus	+++
A. fumigatus	++
Penicillium spp. 1	++
Rhizopus spp.	++
A. flavus	+
A. fumigatiaffinise	+
Trichoderma spp.1	+
Trichoderma spp. 2	+
Aspergillus spp.	-
A. versicolor	-
A. candidus	-
Acremonium spp.	-
Cladosporium spp.	-
Emericella spp.	-
Fusarium spp.	-
Fusarium oxysorum	-
Pencillium rubens	-
Penicillium spp. 2	-
Phoma spp.	-

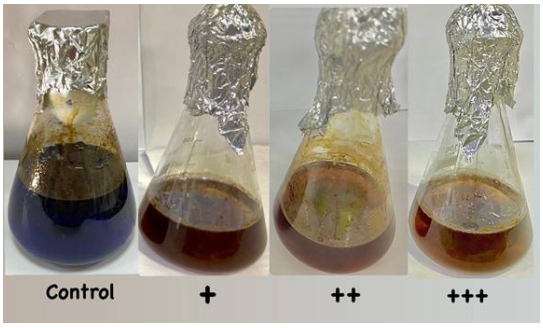

Fig. 2: Preliminary test of the activity of fungal species to biodegrade of crude oil compared to the control set.
+++: High biodegradation, ++: moderate biodegradation,+: low degradation ability, _ : No visible biodegradation

al. 2013). *Aspergillus* produces a large number of asexual conidia, which can tolerate stress and produce sexual ascospores that sustain its widespread dispersal (Krijgsheld *et al.* 2013). In addition, *Aspergillus* can produce enzymes, such as lignin, manganese peroxides and cytochrome P-450 monooxygenase, which can degrade enormous types of organic materials in the environment, including hydrocarbons, for use as a sole source of carbon and energy (Durairaj *et al.* 2016). The genus *Penicillium* appeared in the second percentage with 30%. This genus is also a type of anamorphic fungi, such as *Aspergillus*, which produces a large number of conidia, and can adapt to different types of environments (Tian *et al.* 2017). The rest of the genera appeared in one or two samples possibly because of the harsh environment from which they were isolated and the toxic effects of crude oil (Oliveira *et al.* 2013). The high percentage of appearance was 50% for sterile mycelia, this finding can be explained as follows; given that they may lose their ability for normal growth because of the effect of the harsh environment from where they isolated, fungi may lose their ability to produce a reproduction unit but still grow as sterile mycelia, which may need different culture media to grow (Fan *et al.* 2014; Silva *et al.* 2015; Prathyusha *et al.* 2016; El-Hanafy *et al.* 2017). In general, the differences in the percentages of appearance of fungal genera may be due to their ability to endure and adapt to difficult conditions, their adaptation to a wide range of temperatures and their ability to secrete a group of enzymes that enable them to decompose various materials and exploit them as a source of energy and growth, in addition to their ability to produce a large number of reproductive units that enable them to spread in the environment (Taylor and Sinsabaugh 2015). The results of the current study are consistent with those of (Abdullah *et al.* 2010; Al-Daamy *et al*. 2018; Minati and Mohammed-Ameen 2020) in which *Aspergillus* recorded the highest appearance compared with the rest of the isolated genera during their research.

As regards fungal species, Ascomycota represented 95% of the isolated species with nineteen species. The high percentages of certain fungal species isolated from crude oil-contaminated soil were attributed to their adaptation to this area and their ability to degrade a wide range of organic compounds (Al-Jawhari 2014; Burghal *et al.* 2016). Also the appearance of anamorphic state of the ascomycetes

fungi in high percentages may be due to their ability to secrete different enzymes, produce a large number of reproductive units and tolerate harsh environments; these features and others made them one of the largest group of fungi isolated from different environments. These features allow them to use a large number of organic materials, including crude oil, as a source of carbon and energy for growth and reproduction in harsh environments and conditions (Al-Saadoon *et al.* 2014; Alrumman *et al.* 2015; Altaee and Al-Dossary 2021). Zygomycota appeared in low percentages (5%) possibly because they cannot grow in extreme conditions, high temperatures and toxic soil with crude oil, may need isolation methods and special culture media for isolation, or grow slowly and thus need long growth periods especially with Ascomycota (Raja *et al.* 2017; Wu *et al.* 2019). The percentage of appearance of species ranged from 10 to 40%; most of the species with high percentages of appearance belonged to *Aspergillus* and *Pencillium.* These fungal species adapt very well to their environment and can grow and reproduce in large quantities in contaminated soil, which enables them to use crude oil as a sole source of growth and energy (Al-Dossary *et al.* 2019). The rest of the species appeared in low percentages due to the nature of the environment from which the fungi were isolated during this study. This harsh environment only has crude oil as a sole source of carbon and energy, and thus, the fungi that cannot use this source cannot compete in this environment. In addition, crude oil is a toxic material that negatively affects the growth of fungal species and lowers their percentage of appearance. Moreover, the nature of soil, dryness, high temperature and types of media used for isolation affect the percentage of appearance of fungi. In general, the number of species isolated during this study were less compared with those of Al-Jawhari (2014); this result may be due to the nature of the environment from which the fungi were isolated, the number of samples, and isolation methods and media used for isolation.

A preliminary test was conducted to determine the initial ability of the isolated fungi to biodegrade crude oil. This method of detection relies on the ability of fungi to biodegrade DCPIP, which is used as an indicator in the bioanalysis of microorganisms for their ability to biodegrade crude oils by oxidization of the dye, which initially changes the color of the liquid medium from blue to colorless, and the disappearance and dispersion of crude oil from the medium (Al-Nasrawi 2012; Moustafa 2016).

The preliminary test on the isolated fungi showed that about 50% of fungal species had showed good ability to change the color of the medium, and *A. niger* and *A. terreus* exhibited high abilities to biodegrade crude oil and oxidize the dye in the medium. Their good ability to break down crude oil components may be their high adaptation to crude oil and consuming it as a sole source of carbon and energy, which indicates the effectiveness of their enzymatic system (Galitskaya *et al*. 2021). The other fungi showed a weak ability to break down crude oil due to the effect of their enzymes and tolerance to crude oil (Zhang *et al*. 2022). The other species did not show any ability to use crude oil nor change the color of the medium, which indicated that they lack any enzymatic activity that will allow them to degrade crude oil (Al-Hawash *et al*. 2018a). This result is consistent with the findings of Al-Hawash *et al*. (2018b) and Al-Dossary *et al*. (2020) on several fungi with a weak enzymatic ability to biodegrade hydrocarbon compounds.

Conclusion

Numerous fungi had been found in oil contaminated soils and the anamorphic fungi were dominant in this area with the genus *Aspergillus* being the most dominant. The number of isolated fungi were little compared with other area because of the nature of the area and the toxicity of the oil. A good number of the isolated fungi were able to degrade crude oil and they can be used in the bioremediation of contaminated area.

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Author Contributions

All authors have contributed to the work and each one has been seen and approved the content befor submission

Conflicts of Interest

There is no conflict of interest betwwen the authors

Data Availability

The copyright statement has been clearly identified

Ethics Approvals

There is no animals used in this work

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