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Efficiency of Some Egyptian Soil Fungi in Biodegradation of Petroleum Hydrocarbon

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Abstract Discharge pollutants from refinery of crude oil consider one of the critical problems to the environment due to impact of which on the human health and ecosystem. Currently the biological control to remove hazardous from environment is successful process due to it being a safe way to enhance a healthy environment and also with low cost. The data contained in this study shows that all the fungal species were capable of degrading the crude oil in varying degrees. Total of three genera represented by seven species have been reported from soil heavily contaminated by petroleum oil. Out of seven isolates, only *Aspergillus flavus*, *A. niger* and *Absidia corymbifera* were able to biodegraded oil from a higher concentration to below detectable limit changing Czapek's broth color from deep blue to colorless. The higher crude oil biodegradation efficiency was exhibited by *Absidia corymbifera* compared with other species, nevertheless fungal species isolated from contaminated soil can be exploited in the bioremediation of crude oil to remove petroleum hydrocarbon from contaminated environments.

Keywords: *soil fungi, Biodegradation, petroleum hydrocarbon*

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1. Introduction

Worldwide petroleum products consider one of the most environmental pollutants, especially to the soil and the aquatic environment, as a result of inappropriate elimination processes of petroleum or petroleum derived products. Oil spills is the occasional inflow of crude oil into the environment. These spills endanger public health, imperil drinking water and damage natural resources [1]. The regular increase in the number of toxic compounds produced by the oil industry has promoted the significance of the development of new biological tactics to detoxify and degrade these waste products [2]. Diversified types of microorganisms beneficial in the bioremediation of these contaminant compounds are found in nature. Bioremediation process effective, safe and moreover, this technique can be performed at a low cost.

In the past decade, bioremediation techniques have been developed and improved to clean up soils polluted with hazardous chemicals [3,4]. Biological purification of oily pollutant soil be based on microbial activities, either through self-cleaning or by bioremediation technology. While, self-cleaning is a natural process in which pollutants are left to the indigenous microflora to degrade them without human involvement; generally process of self-cleaning occur very slowly. However, bioremediation is the biotechnology in which microorganisms are applied to mineralize and thus remove xenobiotic pollutants [5].

Mycoremediation as a mechanism focuses on the degradation of organic compounds by fungi and this is achieved through the production of extra-cellular and intracellular enzymes which catalyses various reactions [6]. It is practically established that fungi are capable of using their mycelia to bioremediate hydrocarbon products due to their high production of organic acids, chelators, oxidative enzymes and extracellular enzymes that enables them to utilize the hydrocarbon product faster [7]. Also, fungi able to lower the pH of its environment leading to reduce some of these contaminated compounds. Bioremediation includes two different approaches, biostimulation and bioaugmentation. Biostimulation is the stimulation of pollutant-degrading microbes that already exist in the environment by aeration, nutrient addition, or changing the environmental conditions in other ways to optimize degradation. Bioaugmentation is the addition of microbes, fungi, or plants to the contaminated area to enhance degradation of target compounds. Bioaugmentation is used when the organisms required to degrade the contaminant are not present, or when they are too sparse to be effective [8,9]. Culturing and inoculating the contaminated area with microbes that are already naturally present is thought to be more effective than adding non-native cultivars [10] because the native organisms are presumed to be well suited to other components of their environment.

Generally, crude oil composed of four major constituents [11] saturated hydrocarbons, aromatic hydrocarbons, asphaltenes and resins. The toxicity of crude oil or petroleum products varies widely, depending on their composition, concentration, environmental factors and on the biological state of the organisms at the time of the contamination [12]. Many microorganisms are capable of using hydrocarbons as the sole carbon and energy source. Oil (or hydrocarbon) utilization potential is widely distributed among prokaryotic and eukaryotic microorganisms. In case of the high molecular weight hydrocarbons only bacteria and fungi show a capacity to use these compounds as the only carbon and energy source [13].

Newly, many investigators studied the role of fungi in biodegradation process of petroleum products and the most common fungi which have been recorded as a biodegrades belongs to: firstly yeasts which include *Candida, Dabaromyces, Endomyces, Leucosporidium, Lodderomyces, Metschnikowia, Pichia, Rhodotorula, Rhodosporidium, Saccharomycopsis, Schwanniomyces, Selenotila, Sporidiobalus, Sporoboloyces, Torulopsis, Trichosporon and Wingea*. Secondly fungal biota such as *Absidia, Aspergillus, Aureobasidium, Beauveria, Botrytis, Cephalosporium, Cladosporium, Corellospora, Cunnninghamella, Dendyphiella, Fusarium, Geotrichum, Gliocladium, Hormodendrum, Lulworthia, Mortierella, Mucor, Paecilomyces, Penicillium, Phiolophora, Phoma, Pleurotus, Polyporus, Rhizopus, Scedosporium, Scoleobasidium, Sporotrichum, Talaromyces, Varicosporina* and *Verticillium* [4,14,15,16].

The main intention of the present study was to isolate, characterize and identify fungal biota from crude oil polluted soil and determine their ability to biodegrade this oil under laboratory conditions (*in vitro*).

2. Materials and Methods

2.1. Source of Crude Oil

Crude oil, which is used to test capability of fungi isolates on biodegradation was obtained from the Burg El Arab (BEA) oil field in western desert that produce crude oil and equipped to refinery for the refining. It was transferred to laboratory in dark glass bottle closed tightly and kept in a cold and dark place until to use.

2.2. Soil Sampling

The soil samples that were used in this study were obtained in October 2017 from grounds of three automobile garages in Port Said city where spillage of motor oil had occurred over a long period. Soil samples were taken randomly just 5 cm below the soil surface by hand trowel. Samples were transported to the laboratory in sterile plastic bags for fungal isolation immediately. Necessary the samples were stored under refrigeration at 4°C until isolation; this was no later than 48 hours after sampling.

2.3. Isolation of Fungi from Oil Contaminated Soils

Collected soil samples were homogeneously mixed with removal of stones and other unwanted soil debris using 2 mm sieve followed by 1 g of soil was transferred to Petri dishes and plated on sterile Czapek's yeast extract agar (CYA) medium (Composition in g/L: sucrose 30 g, sodium nitrate 3 g, potassium dihydrogen phosphate 1 g, magnesium sulphate 0.5 g, potassium chloride 0.5 g, ferrous sulphate 0.01 g, yeast extract 5 g, agar 20 g, distilled water 1000 ml). The medium supplemented by chloramphenicol (250 mg/l) and Rose bengal (1/15000) to suppress bacterial growth. Plates were incubated at 30°C for 3 days or more depending on the rate of growth. The grown cultures were carefully sub-cultured onto fresh Czapek's plates and incubated until the fungus begins to sporulation. A part of the pure culture was then transferred into Czapek's plates and incubated at 30°C for six days and stored as stock cultures at 4°C in the [14,17,18].

2.4. Morphological Characterization Isolated Fungal Taxa

Macro-morphology (colony diameter, colony color, reverse and exudates) and micro-morphology (conidia & phialides if present, mycelium) of isolated fungal species were used as criterions for morphological identification of the fungal biota [19].

2.5. Determination of fungal Growth Ability under Petroleum Hydrocarbon Pollution

Degradation studies were carried out using (CYA) medium without agar and chloramphenicol. The Czapek's broth was supplemented with 5 ml of crude oil as the sole carbon source. Two agars plug 1 cm^2 of the pure cultures of each the fungal isolates were inoculated into CYA (100 mL/250 mL flask) containing sterile crude oil as a sole carbon source without redox indicator. All flasks were incubated at room temperatures with constant shaking about 100 rpm for 4 days at 30 ± 2°C. The oil in the flasks was monitored daily for dispersion and emulsification of oil in synchronous with fungal growth. The control flasks without fungal candidates were incubated at same condition.

2.6. Biodegradation Assay of Crude Oil

0.1 ml of Tween 80 (0.1%) and 0.05 ml of methylene blue solution (redox indicator) were incorporated into the previous broth flasks. The isolates to be assayed were incubated in a shaker (100 rpm, 30°C) for 7 days. After incubation for 7 days, the broth in flasks which changed from deep blue to colorless likely due to oxidized hydrocarbons products was subjected to filtration by filter papers. Pellets were discarded and the supernatant retained. Supernatant thus obtained were measured by spectrophotometer.

2.7. Screening for Biodegradation Potentials

Two agar plugs (1 cm^2 each) of a pure growth of each isolate were inoculated into Czapek's extract broth (50 ml/250 Erlenmeyer flask) incorporated with sterile crude oil (1% v/v), redox indicator (2 % v/v) and Tween

80 (0.1% v/v). The control flask had no organism incubation was at room temperature (28-30°C) with constant shaking at 150 rev/min for 5 days [20,21]. The aliquots in the flasks were monitored daily for color change (from deep blue to colorless).

3. Results

3.1. Overview

During this part of study, a total of three genera represented by seven species have been reported from soil heavily contaminated by petroleum oil. Isolated fungal biota belong to three taxonomic classes of which Hyphomycetes comes first by being represented by five species followed by Ascomycetes and Zygomycetes by being showing one species each (Table 1).

Table 1. Taxonomic classes of isolated fungal taxa

Class	No. of species isolated	%
Hyphomycetes	5	71.4
Ascomycetes	1	14.3
Zygomycetes	1	14.3
Total No. of species	7	100%

3.2. Cultural Characteristics of Fungi from Oil Contaminated Soils

A total of seven fungal isolates were obtained from oil contaminated soil using Czapek's yeast extract agar defined as Aspergillus niger, Absidia sp, Emericella sp, Aspergillus flavus, Aspergillus fumigatus, Aspergillus terreus and Aspergillus sydowii (Table 2 & Figure 1). These isolates were able to grow within 4-7 days of incubation at 30°C.

Table 2. Mean count and frequency of fungal biota isolated from petroleum polluted soil

No.	Species	Count	Frequency
1	*Aspergillus flavus*	833	100%
2	*Aspergillus niger*	733	100%
3	*Absidia corymbifera*	500	100%
4	*Aspergillus terreus*	222	66%
5	*Aspergillus fumigates*	100	66%
6	*Emericella nidulans*	133	66%
7	*Aspergillus sydowii*	133	66%

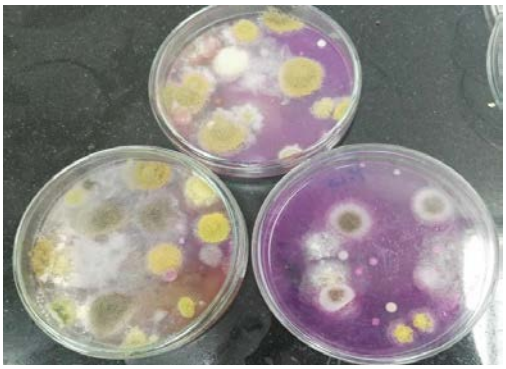

Figure 1. Agar plates showing isolated fungal species (macro-morphology)

3.3. Microscopic Characterization of Fungal Isolates

Different characteristics for the isolates were noted when observed under light microscope at a magnification of x 400. *Aspergillus flavus* had double walled vesicles with conidia spreading from the strigmata, vesicles being flask shaped (Figure 2). *Aspergillus niger* had conidiophores terminates in vesicles, smooth walled, colorless with brownish shade. *Absidia* had sporangiophores are hyaline, simple or sometimes branched and sporangia are globose and supported by a characteristic funnel-shaped apophysis (Figure 2). *Aspergillus terreus* had globose vesicles and had biseriate conidial head.

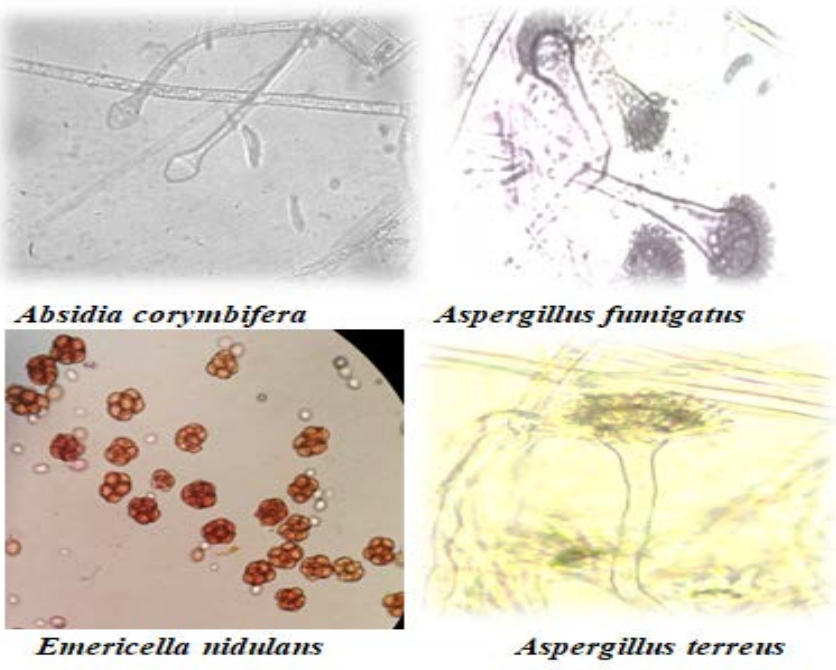

Figure 2. Micro-morphology of some isolated fungal biota

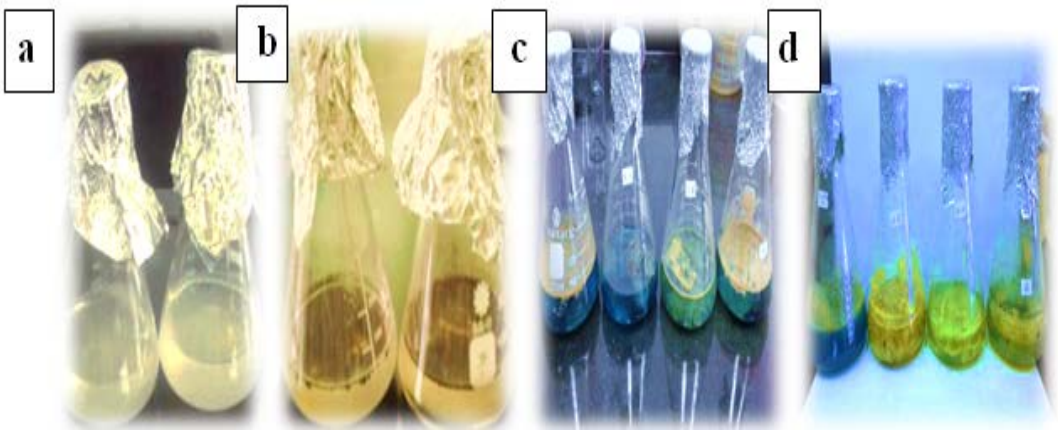

Figure 3. Ability of some selected fungal isolates in biodegradation of crude oil**: a-** control without fungal candidates, **b-** control without fungal candidates but with crude oil**, c & d-** rate of biodegrade by tested isolates

3.4. Biodegradation Potentials of *Aspergillus flavus, Aspergillus niger* and *Absidia corymbifera*

Czapek's broth changed color from blue to colorless meaning that the isolates responsible might be potential hydrocarbon oxidizers. Out of seven isolates, only *Aspergillus flavus***,** *A. niger* and *Absidia corymbifera* were able to biodegraded oil from a higher concentration to below detectable limit changing Czapek's broth color from deep blue to colorless.

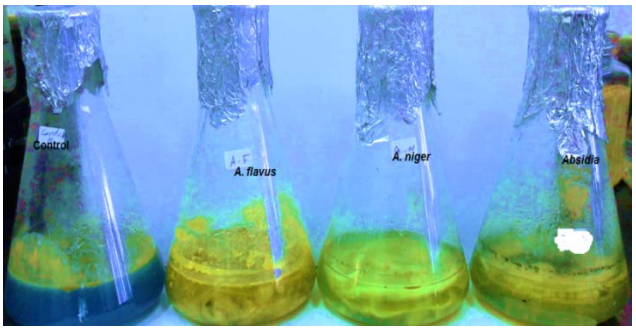

Figure 4. The end of fungal biodegradation

3.5. Spectrophotometer Analysis

Spectrophotometer apparatus was used to determine the residual hydrocarbon present in the media after 14 days of incubation**.** Control was first obtained by injection 3 µl into the cuvette and a reading is taken as a standard. After taking the standard, 3 µl of each sample was injected to get the reading. Each sample measuring 3 µl was injected into the cuvettes in a capillary column inside spectrophotometer and measured at wavelength 609 nm.

Table 3. Rate of absorbance (nm) light of tested fungal taxa in liquid media revealing the ability of these fungi in biodegradation of crude oil

Species	Absorbance (nm)
Absidia corymbifera	0.246
Aspergillus niger	0.176
Aspergillus flavus	0.049

4. Discussion

Fungal analysis of surveyed oil polluted soil samples based on dilution-plate technique using Czapek's-yeast extract agar media revealed that three genera represented by seven fungal species were identified (Table 2). The genus *Aspergillus* showed high occurrence by being represented by 5 species followed by *Absidia* and *Emericella* represented by only one species each. *Aspergillus flavus*; *A. niger*, and *Absidia corymbifera* were the most common species. The remaining fungal genera and species were less frequent. Most of the recorded fungal species in this study had identified before oil contaminated soil.

Generally, different microorganisms (including bacterial & fungal species) have been reported to be capable of biodegrading effectively petroleum hydrocarbons [13]. Although essential biodegradation is best demonstrated in field situations [15,16], the laboratory studies provide greater control and are useful in providing a basis to distinguish between biotic and abiotic processes, and to determine the optimized conditions for the biodegradation.

In our investigation, isolation of fungal biota from oily contaminated soil indicated that, the isolates were able to exist in the contaminated environment while those that could not survive in this environment being eliminated by the unfavorable conditions caused by the oil [14]. Only two genera represented by three species were able to biodegraded crude oil namely; *Absidia corymbifera*, *Aspergillus niger* and *Aspergillus flavus*. The result of the present study shows that *Absidia corymbifera* had higher biodegradation potential then *A. niger* and *A. flavus*. This result was also demonstrated by [21,22,23]. Our result has been also similar to the findings of [24] which showed that *Aspergillus versicolor* and *A. niger* exhibited biodegradation of hydrocarbons higher than 98%. The result was obtained by [25] in their study obtained that the fungus *Penicillium chrysogenum* loss of crude oil concentration percentage in axenic culture to 76% after a month period. [18] study the capabilities of fungi to degrade hydrocarbons, obtained result showed that *A. niger*, *A. fumigatus*, *Fusarium solani* and *Penicillium funiculosum* degraded of up to 75% of the contaminants after 21 days.

However, our data is in agreement with previous studies that have identified numerous fungal species including *Acremonium* sp., *Aspergillus* sp., *Fusarium* sp., *Mucor* sp., *Phanerochaete* sp., *Phialophora* sp. and *Trichoderma* sp. that can degrade Polycyclic Aromatic Hydrocarbons (PAHs) such as naphthalene [26,27], phenanthrene [27,28], pyrene [28,29] and benzo[a]pyrene [30,31,32].

The outstanding mycelia growth of fungal biota used may be due to the higher production of extra-cellular enzymes and organic acids that enabled them to utilize the hydrocarbon faster. This agree with the findings of [7]. that mycelia mats are used for bioremediation because they produce extra-cellular enzymes and acids that break and dismantle the long chains of hydrocarbon, the base structure common to oils, petroleum products and many other pollutants. [33] studied the ability for removal of hydrocarbons was characteristic for fungal strains and probably associated with their high degree of adaptation for living in habitats heavily contaminated with petroleum hydrocarbons. It was observed that petroleum hydrocarbon removal rates depended on fungal proteolytic activity, biomass production, and easily degradable protein content in the medium.

Our data also showed that the use 0.1% of Tween 80 facilitated the transport of the oil to tested fungi and enhanced the metabolism of the hydrocarbon. This result agree with the finding of [24]. The high rate of hydrocarbon degradation by the three fungal candidates could emerge from their massive growth and enzyme production responses during their growth phases. This could be supported by the reports of [34], which showed that extracellular ligninolytic enzymes of white rot fungi are produced in response to their growth phases.

5. Conclusions

Many species of fungi can catabolism hydrocarbons and this study indicates that these catabolic reactions may convert crude oil to intermediates or even mineral elements. The data contained in this study shows that all the fungal species were capable of degrading the crude oil in varying degrees. The higher crude oil biodegradation efficiency was exhibited by *Absidia corymbifera* compared with other species, nevertheless fungal species isolated from contaminated soil can be exploited in the bioremediation of crude oil to remove petroleum hydrocarbon from contaminated environments.

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