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Biodegradation of Oil Contaminated Water by Filament Fungi

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Abstract: Twenty-two fungal isolates were isolated from five samples of oil-polluted water, the ability to grow on a solid mineral salt medium with 2% of crude oil was achieved; only seven isolates (WA2, WB3, WB7, WC2, WD3, WE2, and WE5) give clear-growing fungal colonies with different diameters. These fungal isolates are found to be more predominant in the polluted water samples, then they will be identified as *Aspergillus fumigatus*, *Aspergillus niger*, *Trichoderma viridae*, *Fusarium verticelloides*, *penicillium oxysporum*, *Candida albicans*, *Aspergillus flavus* respectively. In the present study, the primary and secondary screening of the ability of the isolates to degrade the crude oil was showed significant differences between the isolates on growth in the liquid medium containing 2% of crude oil and also in the percentage of crude oil biodegradation, while the isolate *A. niger* showed the highest ability compared with other studied isolates to consume the crude oil as carbon source with the best ratio of biodegradation reached to 95% after 28 days of incubation and the dry weight reached to 3.2 g after 7 days of incubation. Also, the results of GC showed the highest percentage loss of crude oil by *A. niger* which recorded 95% after 28 days of incubation, these results confirm the results of the screening tests for fungal isolates in terms of the similarity of the crude oil biodegradation rate after 7 days of incubation.

Introduction:

Crude oil is a mixture of different types of compounds such as organic materials, heavy metals, hydrocarbons, and others in different amounts, also many persistent volatile organic compounds are found in it. As well, many studies indicated that the aromatic compounds in petroleum oil consider very toxic to all forms of life, therefore, the oil pollutants are the main cause of the chronic pollution of all elements of the environment, whether water, soil, air, and living organisms. [1]. During oil accidents and the attendant cases of oil spills into the environment. So, the aquatic environments are the most affected because of the rapid spread and the difficulty of containing spilled pollutants, because the water environment is more complex due to the difficulty of containing oil spills, whether natural or due to accidents, which makes treatment efforts more complicated. For example, every year about 250 million liters of oil pollutants enter the aquatic environment due to the natural leakage of oil. [2]. As a result of the great and escalating development of the global energy market and thus the increase in demand for petroleum products, where petroleum hydrocarbons are one of the most important raw materials for many industries, and as a result of this expansion and due to a large number of oil pollutants being released to the environment, it is considered one of the most important pollutants that cause environmental pollution, whether at the local level or Global.

Crude oil contains four major different types of compounds includes aliphatic, aromatic, resins, and asphalt. All these hydrocarbon compounds have different physical and chemical properties and also different abilities for the degradation processes. [3]. The release of hydrocarbon pollutants from accidents or that are naturally released into the environment is considered the main cause of water, soil, and air pollution. [4]. The term biodegradation process has been consisting of two parts: the first is bio which refers to living organisms, and the second is degradation which means to process that solves the problem. While the term Biodegradable means the use of living organisms to degrade the pollutants in the environment. [5]. Bioremediation is defined as the process by which living organisms are used to degrade or consume environmental pollutants, or it's a process to remove the effects of the contaminants from the environment and restore the original nature by treating their pollutants. [6].

The biodegradation of oil pollutants by microbial flora found in contaminated sites represents the first step in the primary processes to degrade or remove these pollutants by living microorganisms. [7] and this step considers a cheaper process when compared with the other remediation treatments. [8] The use of bio-

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surfactants that produce by yeasts and filamentous fungi has been recently growing in the process of biodegradation for oil pollutants. [9,10,11] Many researchers have shown that fungi play a major role in facilitating the degradation of persistent hydrocarbon compounds due to their ability to produce extracellular enzymes that break down these compounds and thus convert it into intermediate materials that have less toxicity, which increases the degradation of the oil pollutants by bacteria. [12]. This idea has been confirmed by numerous studies, which are based on the use of mixed fungal cultures to degrade polyaromatic hydrocarbons as a result of the production of degrading enzymes. [13, 14].

The physical, chemical, and biological treatments were used as techniques to treat the pollutants and restore the initial habitats before pollution. [15]. Many studies showed fewer effects when using the physical and chemical processes to treat the pollutants, while, the biological approach seems more effective, low-cost, and appears as environmentally friendly compared with other approaches. [16] Fungi have great importance in biological treatment processes as one of the groups of microorganisms, where they exist or spread in large numbers in environments, especially in the aquatic environment. [17].

Fungi have a higher ability than bacteria to resist the toxicity of hydrocarbon compounds, so they have better advantages than bacteria when used in the biological treatment of petroleum pollutants. [18]. Filamentous Fungi have a good ability to produce several extracellular enzymes such as lignin peroxidase, manganese- peroxidase, and laccase, these enzymes help the fungi to degrade or consume these pollutants by increasing their bioavailability in the ecosystem. [19]. Two genera of fungi *Cladosporium* and *Aspergillus* have confirmed their ability to degrade the aliphatic pollutants, while, other genera of fungi such as *Cunninghamella*, *Penicillium*, *Fusarium*, *Aspergillus*, and *Mucor* appeared good ability to degrade the more resistant aromatic hydrocarbons [20].

Materials and methods:

1. Samples of oil-contaminated water:

Five samples of water polluted with oil were taken from the pipes of Iraq's AI-Dora refinery that supplied the petroleum in this study. It was transported to the laboratory in a black glass bottle that was firmly sealed, dated, marked, and kept cold and dark until it was needed.

2. Culturing of fungal isolates:

0.1 ml from the suspension of water samples contaminated with oil were placed on plates containing PDA medium and spread on the plates, the plates were incubated for 5 days at 27 ˚C, then the isolates were recovered and purified till ensuring the purity of fungal isolates. The pure isolates were subsequently cultured on PDA slants and left for 7 days to grow and then stored at 4 °C as stock cultures (to keep them for months)**.**

3. Fungi growth on crude oil medium:

The ability of fungal isolates to utilize the crude oil was tested, which was done according to the method of [21] by growing the isolates in a solid mineral salts medium, which consists of (1.2 g K2HPO4, 1.8 g K2PO4, 4.0 g NH4Cl, 0.2 g MgSO4.7H2O, 0.1g NaCl, 0.01g FeSO4.7H2O, 2% agar and 2% of crude oil, all components dissolved in 1000 ml distilled water), the plates were incubated at 27 °C and the diameter of growth was measured after 4 and 7 days of incubation.

4. Identification of fungal isolates

The fungal isolates which showed the ability to grow on plates containing crude oil were selected and identified them using microscopic examination by Lacto Phenol Cotton Blue (LPCB) to employ the microscopic observations. The results from this step were compared to the standard description of "A Manual of Soil Fungi [22].

5. Screening of fungal isolates:

5.1 Inoculums preparation.

The liquid inoculum of the selected fungal isolates was prepared from seven-day-old cultures grown on PDA slants; then 5 ml of (5%) Tween 20 was added to fungal colonies in slants and then the rub fungal colonies by the sterile loop. After that, the slants were shaken vigorously for 15 min using a vortex, and the suspension was transferred to a new sterile tube. To remove the hyphae from the inoculum, the suspension was transferred to a sterile syringe attached to a sterile filter Millipore 0.22µm and then collected in a new sterile tube, this inoculum contains only spores [23].

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5.2 Primary screening:

The dry weight of fungal isolates was determined by taking 25 ml from 7 days old fungal cultures which were grown in liquid mineral salts medium supplemented with 2% of crude oil as carbon source and inoculated with 1% fungal inoculum; then these cultures were centrifuged at 5000 rpm for 5 minutes using a cooling centrifuge (4 °C), the supernatant discarded and the precipitate biomass was washed 2-3 times with distilled water, then the fungal biomass placed in sterile weighted plates and then dried at 80 °C for 24 hours using the oven. The dry weight of fungal isolates was estimated [24].

5.3 Secondary screening:

The fungal isolates were inoculated in a liquid mineral salt medium with 2% of crude oil and then incubated at 27 °C for 7 and 14 days in a shaker incubator at 120 rpm. The broth of fungal cultures after 4 and 7 days of incubation was taken and the residual crude oil was extracted using the same volume of Dichloromethane; the mixture of fungal cultures and organic solvent were transferred to a separation funnel and shaken well, then the layer of solvent was transferred and passed through anhydrous sodium sulfate to discard the moisture, this step repeated three times. The solvent collected from the extraction process was transferred to the clean, sterile, and pre-weighted plate and the solvent was vaporized for 24 hours. The control was achieved under the same steps but without fungal inoculum, then the gravimetric method was used to estimate the crude oil biodegradation as a percentage according to the equation below [25].

$$\%\ of\ biodegradation = \frac{\text{Weight of residual oil in control} - \text{Weight of residual oil in test}}{\text{Weight of residual oil in control}} \text{x } 100$$

6. Crude oil biodegradation by best isolate using GC analysis:

The more efficient fungal isolate WB3 was used to determine the percentage of crude oil biodegradation after 28 days of incubation using GC analysis. Flasks containing 100 ml of mineral salt medium with 2% of crude oil were inoculated with 1% of WP5 inoculum; then the flasks were incubated at 27 °C in a shaker incubator. After each period of incubation, the residual crude oil was extracted as described above. After that, 5 ml of n-hexane was added to the crude oil residual and mixed well; then 2 µl from it was injected in Gas chromatography with a capillary column (length: 30 m, and id: 0.24 mm) to determine the total concentrations of n-alkanes and aromatic compounds using helium as carrier gas with a flow rate of 1.5 ml/ min under 100 KPa of column pressure. The interface temperature of 280 °C, while the programmed temperature was started at 60 °C and increased 10 °C /min gradually to reach 280 °C at the final of the program, this temperature program was maintained for 10-20 min to elute all compounds from the column. to exit from the column. Results from GC- analysis were used to estimate the percentage of biodegradation according to the areas of sample and control [26].

7. Statistical Analysis

The analysis of variance (ANOVA) using the SPSS program (version 10.0) was performed in the current study to determine whether or not, significant differences in all the oil degradation results.

Results and Discussion

1. Fungal isolation:

Twenty-two fungal isolates were isolated from five water samples contaminated with oil as in table (1), the results indicate that there is a difference in the number of isolates obtained from each contaminated water sample, and the sample WB gave the highest number of fungal isolates compared with other water samples, which amounted to 7 isolates.

Table (1): The number of oil-polluted water samples and the number of fungal isolates collected from each sample.

Sample symbol	Number of isolates
WA	3
WB	7
WC	2
WD	4

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WE	6

Several results indicated that fungal isolates have a good ability to dismantle a wide range of hydrocarbon compounds, and this may be due to the presence of adaptation of these fungal isolates from the environment from which they are isolated. [27] Soil contamination with petroleum pollutants will affect the fungal community at that site, but it does not prevent the growth and diversity of fungal isolates due to the ability of many fungal species to use petroleum compounds as a carbon source and thus increasing the growth of other fungal species.

At the same time, it was noted by many researchers that the presence of organic compounds in the water led to an increase in the number and types of fungal isolates due to the ability of these isolates to produce and secrete different types of extracellular enzymes, which increases the degradation of oil pollutants and thus causes a decrease in pH of water. [28] Other research reported that the isolates *A. flavus* and *P. notatum* have a good ability to consume crude oil as a carbon source compared to other studied fungal isolates. [29]

2. Fungal ability to grow on crude oil:

The growth ability of all fungal isolates was carried out in a mineral salt medium with 2% of crude oil, the diameter in cm of the fungal colonies was estimated after 4 and 7 days of incubation, and the results indicated that only seven isolates showed the ability to grow on the culture medium containing crude oil as a carbon source, while the other fungal isolates were unable to grow. Also, the results showed differences between the seven isolates in terms of the diameter of the growing colonies after 4 and 7 days of incubation and that the isolate WB3 gave the largest growth diameter reached to 8.5 and 8.7 after 4 and 7 days of incubation respectively, as in Table (2).

Table (2): The ability of fungal isolates to grow on mineral salts medium with 2% crude oil.

Fungal isolates	Colony diameter (cm) after 4th day	Colony diameter (cm) after 7th day
WA2	**3.7**	**3.9**
WB3	**8.5**	**8.7**
WB7	**4.7**	**6.4**
WC2	**6.3**	**7.00**
WD3	**2.4**	**3.00**
WE2	**4.1**	**4.9**
WE5	**4.4**	**5.7**

The results in the above table indicate that all studied fungal isolates possess resistance to oil pollutants, which allowed them to be able to degrade these pollutants.

2. Identification of fungal isolates:

The features of the hyphal cells and morphology of cells and spores were used to determine the microscopic and macroscopic changes to identify the fungal isolates. Therefore, and according to obtained results these isolates (WA2, WB3, WB7, WC2, WD3, WE2, and WE5) were identified as belonging to *Aspergillus fumigatus*, *Aspergillus niger*, *Trichoderma viridae*, *Fusarium verticelloides*, *penicillium oxysporum*, *Candida albicans*, *Aspergillus flavus* respectively.

2. Screening of fungal isolates:

The seven fungal isolates that showed the ability to grow in the culture medium containing 2% crude oil were tested to determine their dry weight after 7 days of incubation when grown in a liquid mineral salts medium containing 2% crude oil, the results indicated, as shown in Table (3), that all fungal isolates were able to grow in the culture medium containing crude oil, and that the fungal isolate *A. niger* gave the highest dry weight of 3.2 g after 7 days of incubation, while the isolate *P. oxysporum* gave the lowest dry weight of 0.5 g and below The same conditions as the experiment.

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Table (3): The biomass (dry weight) of fungal isolates after 7 days of incubation in a liquid medium with 2% crude oil.

Fungal isolate	Dry weight (gm)
A. fumigatus	0.8
A. niger	3.2
Trichoderma virida	1.8
F. verticelloides	2.8
P. oxysporum	0.5
Candida albicans	1.0
Aspergillus flavus	1.4

The same result was observed by [30] when using the changes in the dry weight of selected fungal isolates (*A. niger*, *A. flavus*, *Curvularia lunata*, *Rhizopus* sp., and *Trichoderma* sp.) as a step of screening of oil biodegradation when used different concentrations of crude oil (0.5, 1.0, 2.0 ml) in fungal cultures medium. The results of screening appeared in this study that the dry weight of *Trichoderma* sp. was increased with increasing the crude oil concentrations, and the best dry weight was recorded in *A. niger* reached 2.68 mg, while the lowest dry weight was found in the culture of *Rhizopus* sp. reached 1.3 mg.

The second step of screening was achieved to calculate the percentage of biodegradation after 7 days of incubation at 27 C using a mineral salt medium with 2% crude oil; the residual crude oil in fungal cultures was extracted as the previous method. Table (4) shows that all the studied fungal isolates showed a good ability to consume crude oil, where the rate of biodegradation of isolates varied, as isolate *A. niger* showed the highest ability to degrade the crude oil, as the biodegradation rate reached 75%, while it was found that isolate *P. oxysporum* gave the lowest degradation rate, which reached 26%.

Table (4): The percentage of crude oil biodegradation in the culture medium of fungal isolates after 7 days of incubation.

Fungal isolate	% of biodegradation
A. fumigatus	33
A. niger	75
Trichoderma virida	59
F. verticelloides	62
P. oxysporum	26
Candida albicans	49
Aspergillus flavus	55

3. Detection of crude oil biodegradation using GC analysis:

The GC- analysis was used to estimate the residual crude oil in fungal cultures and control, the ability of biodegradation in the experiments was calculated after 28 days of inoculating the best isolate *A. niger* in the presence of 2% crude oil in a liquid mineral salt medium. The results in figure (1) and figure (2) reveal that many peaks in the sample of the culture medium of isolate A. niger disappeared when compared with the control sample. Also, the results showed that 95% of crude oil was degraded in the culture medium of *A. niger* after 28 days of incubation.

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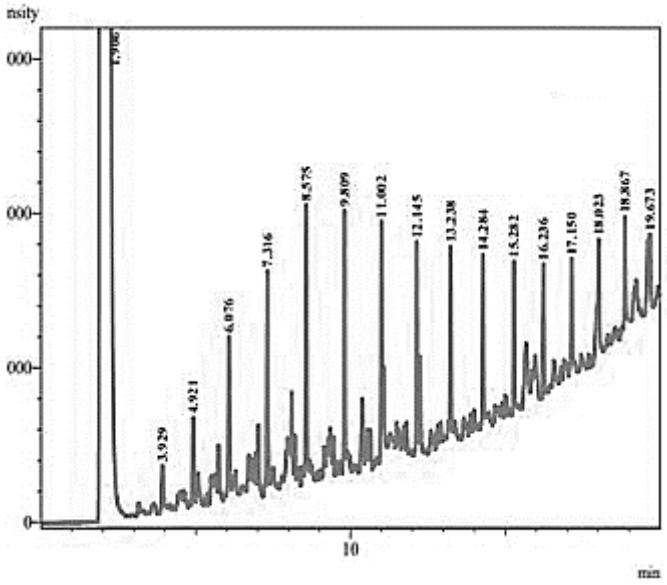

Figure (1): GC chromatogram of residual crude oil extracted from control flask after 28 days of incubation.

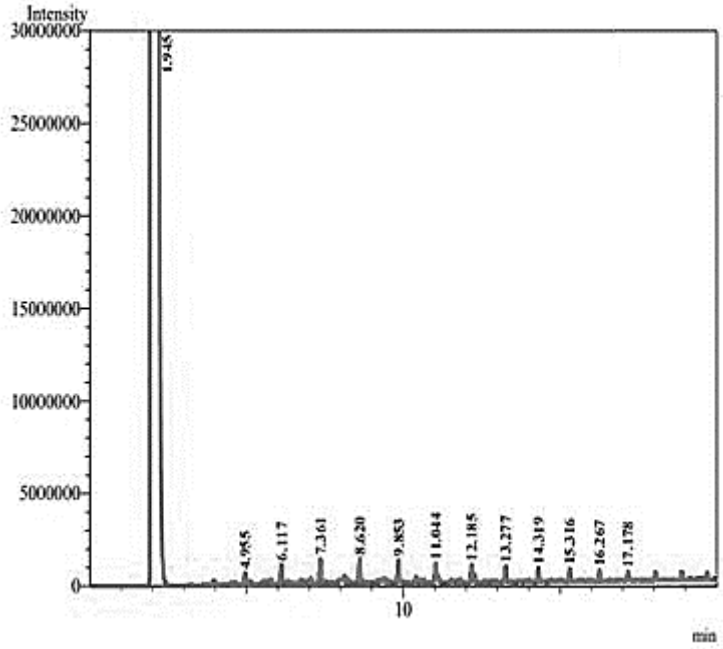

Figure (2): GC chromatogram of residual crude oil extracted from culture of *A. niger* after 28 days of incubation.

Similar results were confirmed by [31] when used two fungal isolates for crude oil biodegradation, these isolates are called *Aspergillus versicolor* and *Aspergillus niger*. Results revealed that more than 98% of the oil was degraded. While another study achieved by [32] showed that the fungal isolate belonging to *Penicillium chrysogenum* can degrade 76% of crude oil after 30 days of incubation. Also, similar research done by [33] used the fungal isolates *Penicillium funiculosum* and *Aspergillus sydowii* in the biodegradation of crude oil, and results showed that 86, and 81% of crude oil were removed by two isolates respectively. While results from another study completed by [34] showed that *A. fumigatus* can degrade 80% of total polyaromatic hydrocarbons after 120 days of incubation.

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