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Biodegradation of Kerosene by *Aspergillus niger* and *Rhizopus stolinifer*

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Abstract This study investigated the abiliy of two fungi to utilize Kerosene. The fungal isolates obtained in this study were *Aspergillus niger* and *Rhizopus stolinifer*. In the present study, a significant differences in the percent of Kerosene degrading fungi were evident among the time of biodegradation. The growth profiles were determined by monitoring growth ability in (potato dextrose agar PDA) medium containing 0.0, 5%, 10%, 15%, 20% v/v Kerosene, dry weights and pH of utilizing Kerosene as carbon and energy source were determined. There was no significant in dry weights of fungi at the 7 days of incubation. *A.niger* had the highest dry weight value of 0.530 gm in 10% concentration while *R.stolinifer* had the low dry weight value of 0.522 gm. The pH values decreased in a fungal cells metabolized after 28 days of incubation. *R.stolinifer* had the highest pH value of 6.3 after 28 days incubation, but *A.niger* had the lowest PH of 4.6 on Kerosene and there was no significant. The ability of fungi to degrade Kerosene was measured directly by determination the residual Kerosene by FTIR Spectroscopy and indirectly by gravimetric estimation of residual Kerosene left after biodegradation was made by weighing the quantity of Kerosene in a tared flask. The highest percentage loss of Kerosene concentration by the cultures of fungi was 93% by *A.niger* after 28 day of biodegradation, but the loss of Kerosene concentration in the culture of *R.stolinifer* reached to 88% after 28 day. Both strains *A.niger* and *R.stolinifer* were capable of consuming kerosene as a sole carbon. The data obtained in the present investigation advance our knowledge of kerosene resistance in *Aspergillus niger* isolated from Iraqi marshes and may make this promising candidates for further investigations regarding their ability to remove kerosene from contaminated environment.

Keywords: *environment, pollution, biodegradation, kerosene, fungi*

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1. Introduction

Contamination of the environment is frequently associated with hydrocarbon pollution because of increasing global demand for petroleum hydrocarbons and its products. It is known that the main microorganisms that consume petroleum hydrocarbons are bacteria and fungi. In the literature, the potential of microorganisms pointed out as degrading agents of several compounds indicates biological treatment as being the most promising alternative for reducing the environmental impact of oil spills [1,2]. Filamentous fungi play an important role in degrading diesel and kerosene by producing capable enzymes, because of their aggressive growth, greater biomass production and extensive hyphal growth in soil, fungi offer potential for biodegradation technology [3,4]. The bacterial degradation of aromatics normally involves the formation of a diol, followed by ring cleavage and formation of dicarboxylic acid. Fungi and other eukaryotes normally oxidize aromatic compounds using mono-oxygenase, forming a trans-diol [5].

The major constituents of kerosene are alkanes and cycloalkanes (65-70%), benzene and substituted benzene (10-15%), naphthalene and substituted naphthalene with carbon numbers predominantly in the C9 to C16 range [6]. Kerosene is a component of crude oil, and it is used as a source of energy. Kerosene is used as fuels in some jets, illuminating oil for wicks and lamp [7,8], observed that kerosene-polluted soil has reduced numbers of microbes when compared to non polluted soil. Kerosene exhibit moderate to highly acute toxicity to biota, with products specific toxicity related to the type and concentration of aromatic compounds [9]. Kerosene spills have the potential for causing acute toxicity in some forms of aquatic life.

This work is a laboratory study to investigate the efficiency of kerosene utilization by two fungus in mineral salts medium, to be used in biodegradation of kerosene.

2. Material and Methods

2.1. Organisms and Culture Conditions

A.niger and *R.stolinifer* were obtained from Marches Researches Center, Thi-qar University, Environment Laboratory. Iraq. These fungi isolated by Dr. AI-Jawhary

from the upper surface of sediments in marshes of AI-Nasiriya-Iraq. Stock cultures were maintained on the potato dextrose agar slant subcultured periodically and stored at 4°C. Mineral salts medium containing (g l^{-1}): K_2HPO_4, 1.71; KH_2PO_4, 1.32; $NaNO_3$, 0.42; $MgSO_4$. $7H_2O$, 0.42; $Cacl_2$, 0.02 was used for the induction experiments. All media were autoclaved at 120°C for 20 min. Kerosene at 10% was used as carbon source for the biodegradation.

2.2. Chemicals

All chemicals used in the present study produced by (BDH) company.

2.3. Kerosene

The sample of kerosene was obtained from AI-Nasiriya petrol station.

2.4. Determination of the Fungal Growth Ability under Crude Oil Pollution

The growth assay was used to find the resistant fungal species to kerosene contamination of the soil. The assay was conducted by comparing the growth rates of fungal strains, as colony diameter, on the kerosene contaminated and control petri dishes. Test dishes were prepared by adding kerosene to warm PDA solution. In order to have 0.0, 5%, 10%, 15%, 20% concentration of kerosene in all plates, the solution was thoroughly mixed with a magnetic stirrer, right before it was added to the plates. Pure PDA was used in control plates. All dishes were incubated with 5 mm plugs of fungal mycelia taken from agar inoculums plate. The dishes were incubated at 25°C in an incubator. Fungal mycelia extension on the plates (colony diameter) was measured using with measuring tape after 7 days and compared with control plates. Determination of dry weight of mycelia of fungal strains by harvested after 7 days incubation in flasks containing liquid mineral salts media amended with kerosene and compared with other flasks without containing kerosene (control) on filter paper by filtration and dried in the oven with 85°C. pH, was determined with pH meter.

2.5. Biodegradation Studies and TPH (Total Petroleum Hydrocarbons) Extraction

Growth and degradation studies over a time course were carried out using [10] method with some modifications. 10 ml of kerosene (as the sole source of carbon and energy)/190 ml mineral salts media in 250 ml flasks. The liquid mineral salts media then inoculated with 5 mm disk from the mycelia of the old 7 days fungi colony. The control flasks were not inoculated with mycelia of fungi colony. All fasks were covered with none absorbent cotton wool and incubated at 25°C incubator. The flasks were shaken manually at regular intervals to allow adequate mixing and homogeneity of the contents. The experimental setup was monitored for a period of 28 days. After 7 days of time interval, the flask was taken out, 50 ml of culture broth was transfer to a separating funnel and add 10 ml benzene and was shaken vigorously 5 min to get two layers. The upper layer (organic layer) was transfer to tared beaker and the bottom aqueous layer was extracted again with 10 ml benzene and in the same time the upper layer add to the first upper layer in tared beaker. The extracted kerosene was passed through anhydrous sodium sulphate to remove moisture The upper layer collected in tared beaker and evaporated in vaccum rotary evaporator. The gravimetric estimation of residual oil left after biodegradation was made by weighting the quantity of kerosene in tared beaker. The percentage degradation of kerosene was then calculated as described by [11]. The degraded kerosene was characterized by FTIR spectroscopy using Schimadza, Japan, spectrum one equipment in the mid–IR region (500-4000 cm^{-1}) at 16 scan speed.

CR = IC – FC

PR = (CR / IC) x 100

Where CR = Concentration of Remediate kerosene %,

IC = Initial Concentration of Kerosene %,

FC = Final Concentration of kerosene %, PR = Percentage of Remediate kerosene.

2.6. Statistical Analysis

The present study conducted an Anova (analysis of variance) which was performed on all the treatments and done using the SPSS (version 10.0) package to determine whether or not, a significance difference.

Table 1. Effect of Kerosene on colony diameter to fungal strains

	Concentrations %				
	0.0	5	10	15	20
Fungi					
A.niger	7.3	8.5	8.5	8.5	7.5
R.stolinifer	8.5	8.5	8.5	8.5	8.5

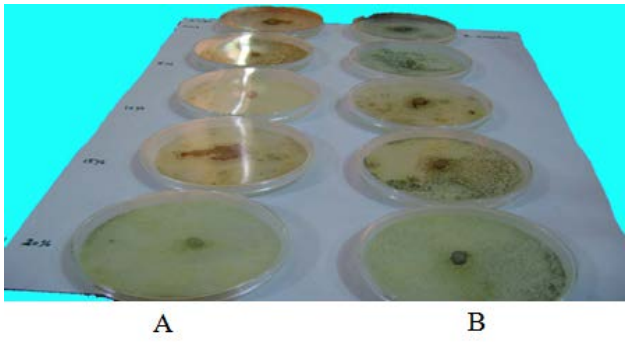

Figure 1. Effect of Kerosene on colony diameter to fungal strains

A: A.niger. B: R.stolinifer

3. Results

3.1. Fungal Growth Ability under Kerosene Pollution

The growth ability of the isolated fungal strains was carried out under 0.0, 5%, 10%, 15%, 20% concentration of kerosene and was expressed as diameter of the colony (Table 1, Figure 1). The results showed that the fungus *A.niger* and *R.stolinifer* are resistant to kerosene pollution. Among the studied fungus, *R.stolinifer* showed the highest resistance to all concentration of kerosene in solid media (with 8.5 cm diameter of colony after 7 days growth), and *A.niger* also resistant. The colony diameters were determined after 7 days in the 0.0, 5%, 10%, 15%, 20% concentration of kerosene polluted PDA media. The same

result was obtained by [11], in their study found that the highest growth diameter of *R.stolinifer* in 5% kerosene contaminated PDA media culture and *A.niger* had the highest growth diameter in 20% kerosene while the *Penicillium sp.* had the lowest growth rate at all the concentrations. The similar observation reported by [12] in which the isolated *Rhizopus* species from the seed of *Detarium senegalense* (J.F.Gmelin) showed the highest ability to degradation of kerosene amongst *Aspergillus flavus*, *Aspergillus niger*, *Mucor* and *Talaromyces.*

Four isolated strains were capable to grow in polluted PDA media and utilized kerosene as sole carbon source. In the present study the results showed that the above fungi are resistant to kerosene polluted mineral salts media with 10% concentration but the dry weight of these fungi were decreased with 20% concentration. Among the studied fungi, *A.niger* showed the highest resistance to 10% kerosene pollution (with 0.530 gm dry weight of mycelia after 7 days growth), and the dry weight of *R.stolinifer* reached to 0.522 gm (Table 2).

Table 2. Effect of kerosene on mycellial dry weight to fungal strains

	Concentrations %				
	0.0	5	10	15	20
Fungi					
A.niger	0.540	0.323	0.530	0.502	0.215
R.stolinifer	0.753	0.354	0.522	0.511	0.518

Table 3. Biodegradation of kerosene by using Gravimetric method

	Time (days)	weight of Initial Kerosene (gm)	weight of residue Kerosene (gm)	weight of kerosene Degradation (gm)	percentage of 20 biodegradation (gm)
Fungi					
A.niger	7	10	4.0	6.0	60
	14	10	1.8	8.2	82
	21	10	1.4	8.6	86
	28	10	0.7	9.3	93
R.stolinifer	7	10	5.0	5.0	50
	14	10	3.7	6.3	63
	21	10	2.0	8.0	80
	28	10	1.2	8.8	88

Figure 2. Growth of *A.niger* colony on 10% kerosene after 28 day incubation

Figure 3. Growth of *R.stolinifer* colony on 10% kerosene after 28 day incubation

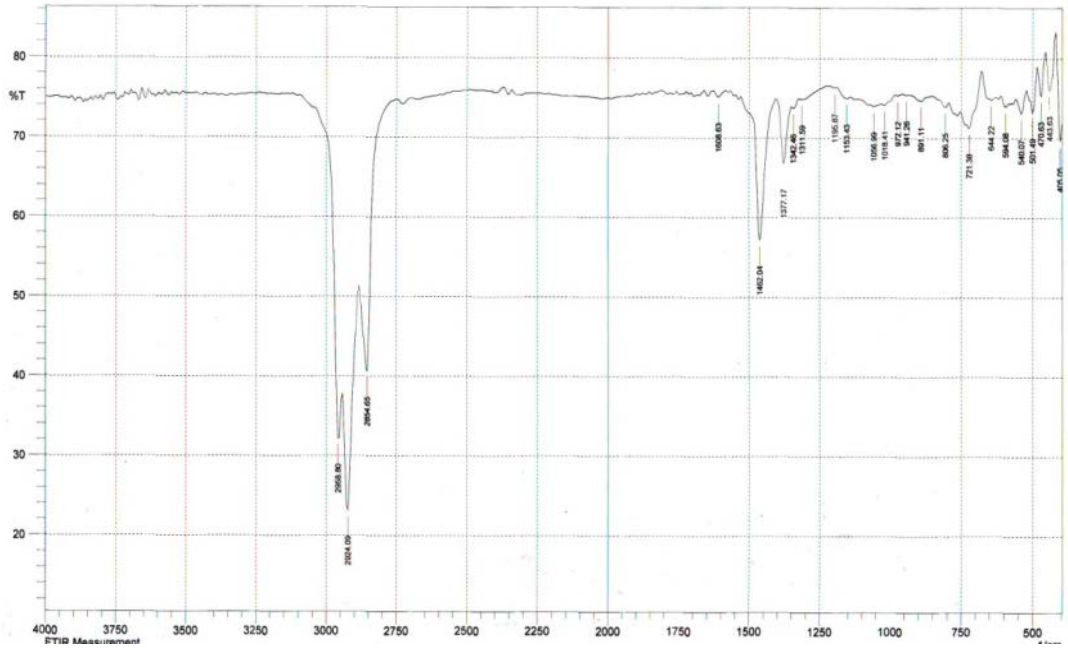

Figure 4. kerosene (standard)-uninoculated

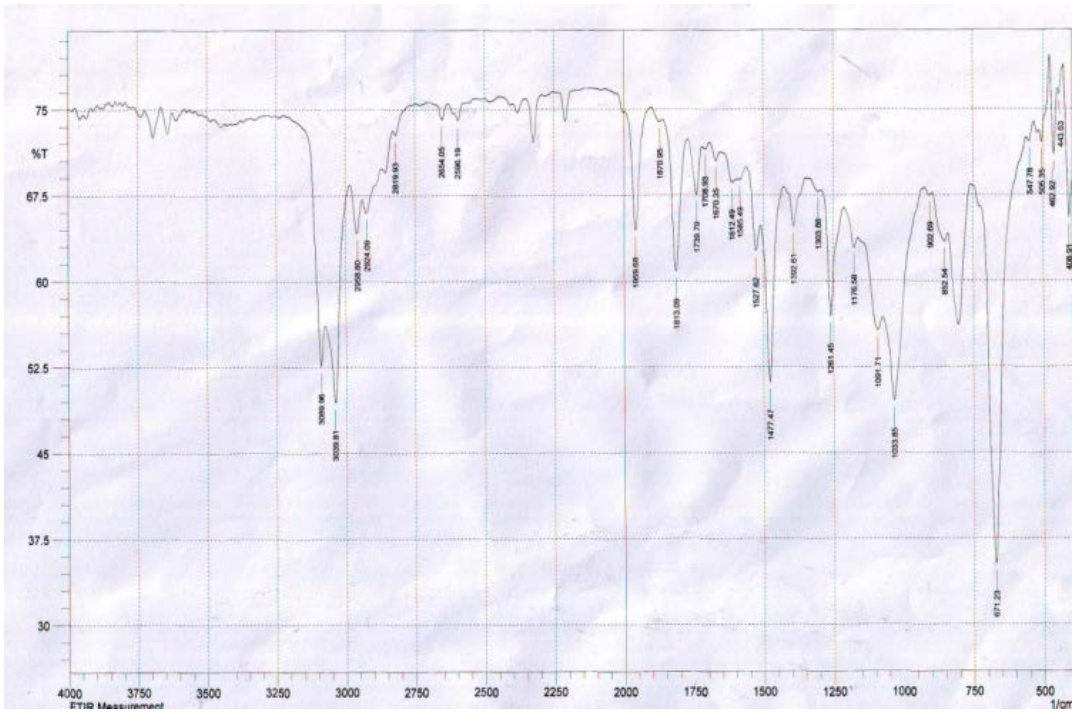

Figure 5. Biodegradation of kerosene by *A.niger* after 28 day incubation

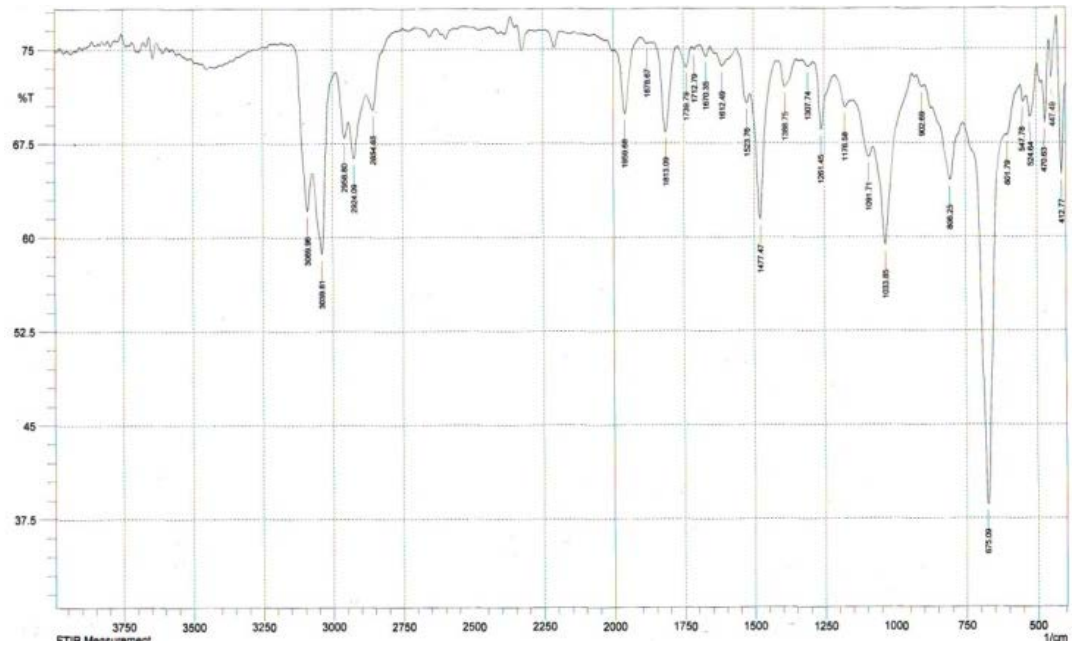

Figure 6. Biodegradation of kerosene by *R.stolinifer* after 28 day incubation

The results showed that the culture of fungi degraded the kerosene in mineral salts media. The highest percentage loss of kerosene concentration by the cultures of fungi was 93% by *A. niger* and 88% with *R.stolinifer* after 28 days of biodegradation (Table 3, Figure 2, Figure 3) and figure FTIR of non-degraded kerosene (uninoculated) revealed three prominent peaks represented hydrocarbons due to the > CH_2 symmetric (2854, 2924, 2958 cm^{-1}). A-CH_3 symmetric and asymmetric bend for an aliphatic hydrocarbon chain and for either a linear aliphatic hydrocarbon chain or a methyl benzene derivative was observed at 1462, 1377 cm^{-1}. A ring vibration at 1022 and 1546 cm^{-1} represented alkyl cycloalkanes and aromatic hydrocarbons, and peaks 636, 721, 798, 883 cm^{-1} represented mono-, tri- and tetra-substituted benzene derivatives (Figure 4). TPH extracted after incubation for 28 days showed bands at 3039 and 3089 cm^{-1} indicated aryl and vinyl and seven sharp bands between 671-1477 cm^{-1}, indicated the formation of aliphatic and aromatic aldehydes. Two bands formation between 1739-1959 indicated ester and carboxylic acid (Figure 5, Figure 6). This result was similar to the findings of [10,17] which showed that *Aspergillus versicolor* and *Aspergillus niger* exhibited biodegradation of hydrocarbons higher than 98%. No significant difference was observed in the changes in pH values obtained on kerosene during utilization by the fungal isolates from 0h of the 28th days of incubation. *A.niger* had the lowest pH of 4.6 after 28 days of incubation, but the *R.stolinifer* had the highest pH value of 6.3 after 28 days incubation (Table 4).

Table 4. Change in PH produced by fungal strains during utilization of Kerosene

	Time (days)				
	0.0	7	14	21	28
Fungi					
A.niger	7	6.7	5.8	5.0	4.6
R.stolinifer	7	6.9	6.8	6.7	6.3

4. Discussion

Study on the fungal species showed that *Aspergillus niger* and *R.stolinifer* were capable of consuming kerosene as a sole carbon. The similar results were reported by [18]. [13] reported that *A. flavus* and *P. notatum* are capable of growth and utilize the crude oil more than the other tested fungi.

The results showed in the present study that the culture of fungi degraded kerosene in mineral salts media. The highest percentage loss of kerosene concentration by the cultures of fungi were 93% by *A.niger* and 88% with culture of *R.stolinifer* after 28 days of biodegradation (Table 3). The same result was obtained by [19] in their study reported that *A. terreus* and *Fusarium sp.* were the percent degradation to aliphatic compounds reached to 100%, these greater capacity to remove crude oil due to the adaptation of these fungi to the pollutant composition, as well as to the enzymatic systems of the fungi [20]. The in vitro growth test of the isolated fungi showed aspecies-specific response. All of the both of the studied fungal strains were able to best growth in 10% v/v kerosene polluted media and therefore could be useful for the remediation of light soil pollution. Results of this research showed that the amounts of kerosene were decreased in the presence of the studied fungal strains considerably. It means that the fungal strains were able to degrade kerosene and consumption of its components. kerosene consists of paraffin, cycloparaffins, aromatic and olefinic hydrocarbons with carbon numbers predominantly in the C9 to C16 range and asphaltic compounds of varying molecular weight, complexity, and degree of susceptibility to microbial oxidation [21]. Mycelial organisms can penetrate insoluble substances such as crude oil and this increase the surface are available for microbial attack [22]. The reduction in pH of the culture fluids in flasks within 28 day incubation period confirmed chemical changes of the hydrocarbon substrates which must have been precipitated by microbial enzymes [23].

Hydrogen ion concentration is a major variable govering the activity and composition of fungi. Many species can metabolise over a wide pH range from the highly acidic to alkaline extremes. Thus the insensitivity of the fungi to high hydrogen ion concentration and narrow pH range of most bacteria account for the sharp drop in pH. Microbial degradation of hydrocarbons often leads to production of organic acids and other metabolic products [24]. Thus organic acids probably produced account for the reduction in pH levels [25].

5. Conclusion

Both strains *A.niger* and *R.stolinifer* were capable of consuming kerosene as a sole carbon The data obtained in the present investi-gation a dvance our knowledge of kerosene resistance in *Aspergillus niger* isolated from Iraqi marshes and may make romising candidates for further investigations regarding their ability to remove kerosene from contaminated enviro-nments.

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