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Mycodegradation of Crude Oil by Fungal Species Isolated from Petroleum Contaminated Soil

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ABSTRACT: In the present study, investigated the abilities of four fungi species isolated indigenously contaminated soil for crude oil biodegradation. The species fungi belongs to *Aspergillus niger, Candida glabrata, Candida krusei and Saccharomyces cerevisiae*. All the fungal species obtained in this study were found to be more predominant in the polluted soil and appeared significant differences in the percent of crude oil biodegradation. *Aspergillus niger* recorded the highest biodegradation of 94%, then *Candida krusei* 61% and *Candida glabrata* 60% whereas the lowest biodegradation rate was demonstrated by *Saccharomyces cerevisiae* 58 after 7 days of incubation. There was no significant in dry weights of fungi which utilized Petroleum hydrocarbon as a carbon and energy source.

KEYWORDS: Biodegradation, contaminated soil, fungi

I. INTRODUCTION

Petroleum is one of the most important energy resources for industry, daily life and a raw material of the chemical industry. Petroleum is a complex mixtures consist of thousand compounds called hydrocarbons [1,2]. Oil pollution from many operation such as oil refineries, petrochemical plants, petroleum production, transportation and use contribute highly to produced a large amount of hazardous waste which reach to the environment causing huge disturbances in components of the ecosystems [3]. The problems of soil contamination with petroleum hydrocarbons often result in significant decline in its quality and such soils become not useful for use [4]. In Iraq, especially Basrah Governorate there are many oil fileds distributed in different areas such as Allihas, Rumaila, Majnoon and others, adding to the South Refineries Company receiving crude oil from production sites by a network of pipelines, and often accompanied the transport and refining of crude oil occurrence pollution problems, especially soil contamination, leading to serious health and environmental risks. Also contamination of soil with crude oil has been known to affect on the soil properties as it hinders plant growth. Microorganisms are nature's original recyclers have the ability to utilize hydrocarbons as sole sources of carbon and energy for metabolic activities. Therefore used in cleanup of the environment as one of the safe and inexpensive biological methods such as biodegradation which can be described as effective method for the treatment of oil contamination because the majority of molecules are biodegradable [5]. The essence of bioremediation is remove the pollutants and restore the environment to its habitable form. Therefore physical methods of remediation are not employed [6].

The presence of oil contamination significantly impacted microbial community composition and diversity, regardless of the soil matrix type [7]. In addition to divers in microbial populations due to the presence and concentration of contaminants, community composition and diversity are influence by properties of the subsurface and other environmental factors including geographic region and soil pH [8]. Furthermore, it has been shown that the type of TPH-contaminants influences the makeup of biodegrading populations [9,10]. Hydrocarbons in the environment are biodegraded primarily by bacteria, yeast, and fungi. Recently, many researchers studied the important of fungi in biodegradation of pollutants and the most common fungi which have been recorded as a biodegrades belongs to following genera:, *Aspergillus, Penicillium, Amorphoteca, Candida, Fusarium, Neosartorya, Mycotypha, Rhizopus, ,Botryris* [11,12,13,15,].

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The aim of current study to investigate the capability of fungal and yeast species to utilize crude oil as carbon source for growth in mineral salt media.

II. MATERIALS AND METHODOLOGY

2.1 Source of Crude oil

Crude oil, which is used to test capability of fungi isolates on biodegradation was obtained from the Rumaila oil fields that produce light crude oil and equipped to refinery for the refining.

2.2 Source of soil samples

The soil samples that were used in this study were obtained from the contaminated soil surrounding the Basrah refinery filled with crude oil and sludge. soil samples were collected from different location just 5 cm below the soil surface by hand trowel with care was taken in sampling to avoid contamination of the samples. Samples were transported to the laboratory in plastic bags for fungi isolation immediately. Necessary the samples were stored under refrigeration at 4ºC until isolation, which was no later than 48 hours after sampling.
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2.3 Oil degrading fungi

Four fungal species were used in this study for degrading of crude oil. The fungi species were obtained from isolation of indigenous fungi by use serial dilution method on the contaminated soil sample plated in the potato dextrose agar (PDA).

2.4 Isolation of fungi

Oil contaminated soil samples were homogeneously mixed with removal of stones and other unwanted soil debris using 2 mm sieve. Serial dilution was performed on the soil samples, 1g soil was weighted and transferred to the flask containing 99 ml saline solution to attain a dilution of 10^{-2}. The mixture was shaken vigorously and was allowed for the soil to settle at the bottom of the flask to prepared other dilutions consecutively. 0.2 ml of the solution from each dilution was plated on sterile sabouraud dextrose agar (SDA) containing chloaramphenicol (250 mg/l), the plates were incubated at 30°C for 3 days or more depending on the rate of growth. The grown cultures were carefully and sub-cultured onto fresh PDA plates and incubated until the fungus begins to sporulation followed by subsequent sub culturing and incubation a number of times until pure cultures consisting of only one type of fungus respectively were obtained for biodegradation screening. A part of the pure culture was then transferred into PDA slant incubated at 30 °C for three days and stored as stock cultures at 4 °C in the refrigerator [16,17,14].

2.5 Screening for degradation of crude oil

Degradation studies course were carried out using Bushnell Hass Mineral Salts medium (MSM) containing $MgSO_4$ (0.2 g/l),$CaCl_2$ (0.02 g/l), KH_2PO_4 (1 g/l), K_2HPO_4 (1 g/l), $FeCl_2$ (0.05 g/l) and NH_4NO_3 (1 g/l). The pH was adjusted to 7. The BHMS medium was supplemented with 1% (v/v) crude oil as the sole carbon source [18]. Two agar plugs $1cm^2$ of the 24 h pure cultures of each the fungal isolates were inoculated into MSM (50 ml/250 ml Erlenmeyer flask) containing sterile crude oil 1% (v/v) as a sole source of carbon and energy (without redox indicator) [19,20]. All flask were Incubated with constant shaking about 120 rpm for 7 days at 30 ± 2°C. The oil in the flasks were monitored daily

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for dispersion and emulsification of oil in synchronous with fungal growth. The control flasks had no organism were incubated at same condition [21].

2.6 Identification of fungal isolates

Four fungi isolates were used for these study one of them filamentous fungi which examined under light microscope after preparations and identified depending morphological characters. Filamentous species was identified according to Hoogde and Guarro [22] and Watanabe [23]. For identification of *Candida* species using HiCrome Candida Differential Agar (HiMedia Laboratories Pvt. Ltd., India) according to manufacturer instructions. Isolates returned to other genera identify by using biochemical test [24,25].

2.7 Biodegradation assay of the selected fungi
1. Preparation of fungal culture

Two agar plugs $1Cm^2$ of the pure cultures of each of the four best potential species were inoculated in 50ml of the Potato dextrose broth in a 100ml Elementary flask and incubated at 30°C for three days in a rotary shaker at 120 rpm. This was used as inoculum at the 4% (v/v) for oil degradation by specific species.

2. Biodegradation of crude oil by fungi

There are one filamentous fungus and three yeast species showed more efficiency on crude oil degradation. To obtain more potential species for oil degradation, secondary screening was performed. About 100ml of MSM media was prepared in a 250ml Elementary flask. Two percents of mother culture of the fungus and yeast species were used to inoculate different sets of MSM supplemented with 1% of crude oil was added in the media. The flasks were incubated in a temperature regulated chamber at 30°C for 7 days and shaken at 120rpm. The experiment was done in replicates. Control flask without the fungi inoculum was prepared under the same cultural conditions. After incubation The degradation efficiency of the isolates were studied using gravimetric method after oil extraction.

3. Crude oil extraction

After 7 days of incubation, fungi activities were stopped by adding 1% 1N HCl for extraction of crude oil, supernatants of fungi cultures were collected in separator funnel and organic solvent (Chloroform) was added then shaken carefully, this step repeated three time for fully extraction. Chloroform containing extracted oil was passed through anhydrous sodium sulphate to remove moisture. The solvent was vaporized overnight and the amount of oil measured using gravimetric method. This procedure carried out under same condition on the control flask and compared with zero time sample [26]. The percentage degradation of the crude oil was then calculated gravimetrically described by Ijah and Ukpe [27]. *Weight of crude oil (initial)- Weight of crude oil (after treatment) / Weight of crude oil(initial) x* 100.

2.8 Determination of dry weight

Dry weight of fungal species were determined by harvested of fungal cells from flasks containing liquid mineral salts broth amended with petroleum hydrocarbon after 7 days incubation. Mycelia collected from each flask and centrifuged at 5000 rpm (Hermle LaborTechnik GmbH, Germany) for five min. and dried in the oven (Memert-854 Gemmy, Taiwan) at 85°C, Dry weight was estimated to g/l.

III. RESULTS AND DISCUSSION

Four fungal species in this study isolated from contaminated soil that showed potentials for hydrocarbon biodegradation were identified as *Aspergillus niger, Candida glabrata, Candida krusei and Saccharomyces cerevisiae* after tested for insure their ability to biodegrade crude oil (Table 1,2). These fungi have been reported as hydrocarbon bio-degraders isolated from soil by Oudot *et al.* [28] ; April *et al.* [29] ; Obire and Anyanwu [30]. Isolated of fungi

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species from contaminated soil refer to the adaption of these fungi strains to petroleum compounds and degradation a wide range to these compounds [31]. Other reported from Atagana, [32] and Mohsenzadeh *et al*. [33] explained that organic compounds in soil were activated increase the growth of fungi and increases excreted extracellular enzymes and finally increases in biodegradation of crude oil.

Table. 1: Colour of *Candida* isolates on HiCrome Candida Differential Agar.

Species	**Colour**
Candida krusei	Red-rose
Candida glabrata	White
Other species	Florescent violet

Table. 2: Biochemical characterization of the yeast isolates

Biochemical test	**Results**
	Saccharomyces
Growth at 37 °C	+ ve
Germ tube production	- ve
Cycloheximide resistance	+ ve
Phenol oxidase production	- ve
Urea hydrolysis	- ve
Sugar fermentation	
Raffinose	+ ve
Glucose	+ ve
Galactose	+ ve
Sucrose	+ ve
Maltose	+ ve
Lactose	- ve
Trehalose	- ve
Fructose	+ ve

The ability to analyses compounds in crude oil by fungal species leads to utilized the carbon source in this components. The results showed the ability of fungal isolates to biodegradation of crude oil in the flask contained MSM, This flasks refer disappear large quantity and presence fragmentation of crude oil after. So there are several indicators to the ability of these fungi in biodegradation process, includes the changes in colour of culture media, dispersion or disappearance of crude oil and growing a biomass of fungal in the salt medium [16].

The screening method used in the present study depends on changing in the texture of crude oil that treated with fungal isolates, therefore can knowing isolates which have the ability to degrade crude oil in the MSM, confirmed the ability of the four fungi species to biodegrade crude oil. Although *Aspergillus* and *Candida* species were recorded in former studies as crude oil biodegrades, the present study confirmed that the fungus *Saccharomyces cerevisiae* demonstrated as a new record yeast in biodegradation of crude oil.

The results of the degradative ability of the isolated fungi showed that all the fungi utilized crude oil. This might be due to the fact that the fungal isolates were able to use the hydrocarbons as substrates for growth. Table 3 explain that the highest percent of biodegradation was reached to 94% after 7 days demonstrated by *A. niger*, while both *C. glabrata* and *C. krusei* showed convergent biodegradable value was amounted 60 and 61% respectively. whereas the lowest percent of biodegradation was recorded from *S. cerevisiae* 58% at same condition.

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Table. 3: Biomass and biodegradation percent of the crude oil by fungal species

Fungal species	Biomass g/L	Biodegradation %	Degradation mg / day
Aspergillus niger	3.3	94	0.081
Candida krusei	3.5	61	0.049
Candida glabrata	3.8	60	0.045
Saccharomyces cerevisiae	3.52	58	0.047
Control	0	27	0

The results of current study *A. niger* as filamentous fungi was the perfect isolate demonstrated active ability to biodegrade crude oil (Figure 1), this result agree with results of Gesinde et al. [34] who indicated that *A. niger* have very active degradation capabilities of oil compounds. In other study, in comparison with different genera, *Aspergillus* species was the most efficient for utilizing of hydrocarbons in crude oil [30].
There are many global studies on the ability of fungi to degraded of crude oil. Ekundayo *et al.* [14] showed that the isolated fungi were capable of degrading the crude oil in varying degrees, but the active crude oil utilizing fungi was *A. niger.* In the same reports from Al Jawhari [31] who explain that the highest percentage loss of petroleum hydrocarbon concentration by the cultures of fungi was 95% with *A. niger* after 28 days of treatment. Al Nasrawi [13] also showed the same results. The results also showed the ability of *C. krusei* and *C. glabrata* for degradation of crude oil, the biodegradation percent amounted to 61% and 60% respectively. Reported from Chrzanowski et al. [35]showed that the a high degradation of hydrocarbons was 57% by *candida maltosa* after 7 days of incubation, also proved that the addition of surfactant led to a more efficient use of hydrocarbon biodegradation by the tested strains.

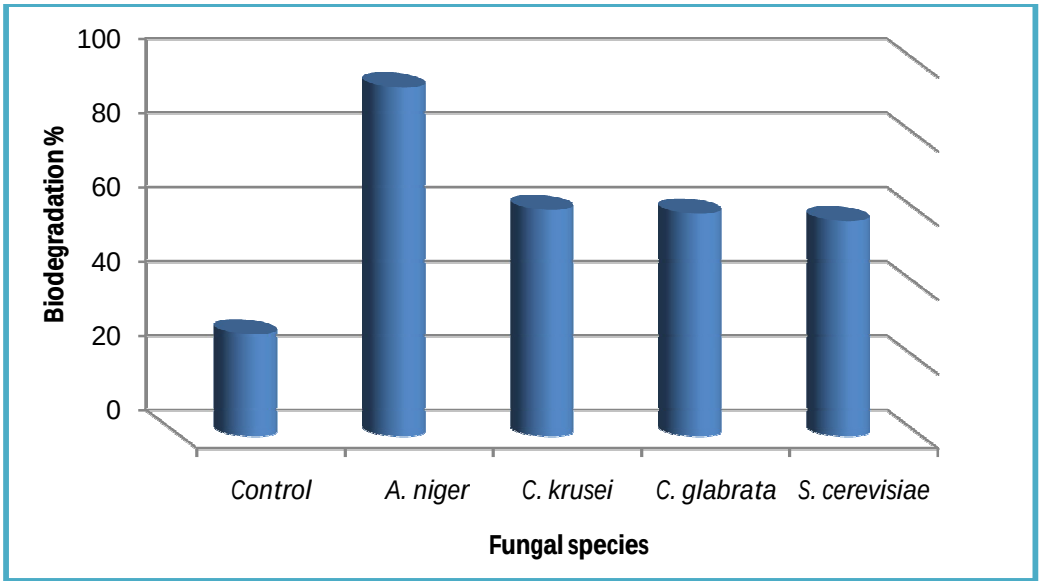

Fig. 1. Biodegradation percent of crude oil by fungal species after 7 days of incubation.

According to weight losses of crude oil from MSM media by fungal species after 7 days of incubation (Table 4). *Aspergillus niger* was demonstrated the highest weight loss (9.42g/l) this result was similar to the findings of Al Nasrawi [13] which showed that *A. niger* exhibited weight losses 8.6% after three week of incubation, while the *C. glabrata* and *C. kruzei* were 6.16 and 6.06 g/l respectively, whereas the lowest weight losses was demonstrated by *S.*

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cerevisiae 5.82 g/l compared with control was 2.74 g/l. This indicates that these species have the potential to utilize crude oil as a carbon source.

Table. 4: Removing and remaining of crude oil after 7 days of incubation

Fungi species	Initial crude oil g/l	Removing oil g/l	Remaining oil g/l
Aspergillus niger	10	9.42	0.58
Candida krusei	10	6.16	3.84
Candida glabrata	10	6.02	3.98
Saccharomyces cerevisiae	10	5.82	4.18
Control	10	2.74	7.26

The difference in the removing and remaining of the crude oil before and after degradation by the fungi species compare with control samples showed in (Figure 2) which refer to different in ability of fungal species for degraded of crude oil, as a result of the variation in physiological and enzymes characteristics in this species.
Figure 4 also refer to disappear quantity of crude oil after incubation by fungal species and in the same time this figure refer amount of crude oil residual after incubation period and relationship with control. This result compare with quantity of crude oil after incubation at zero time (Bank). 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 [36].

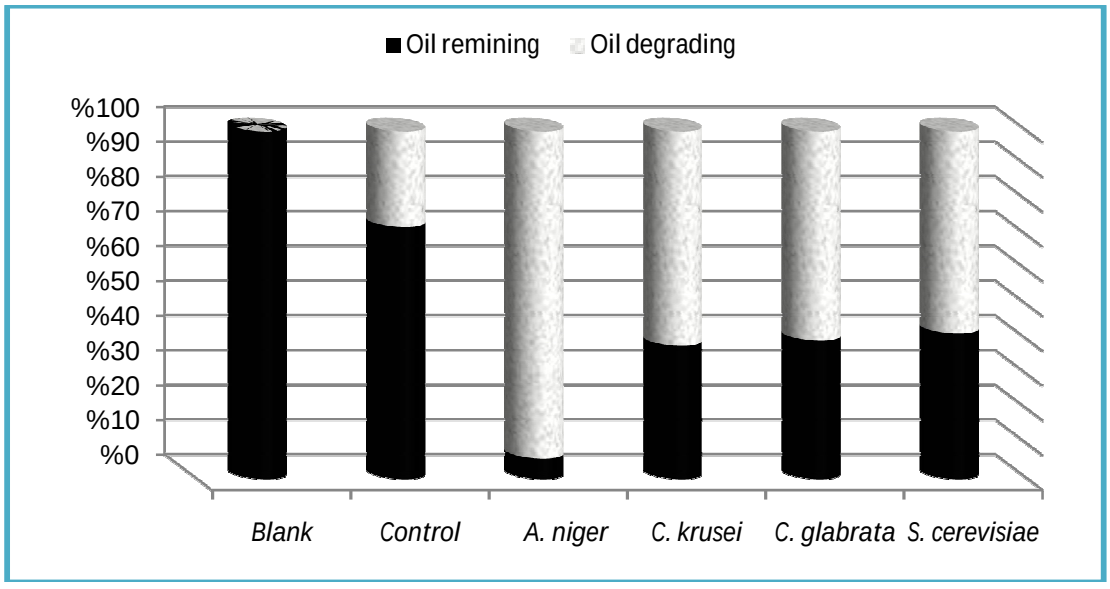

Fig. 2. Percent of removing and residual quantity of crude oil by fungal species after 7 days.

IV. CONCLUSION

Discharge pollutants from refinery of crude oil consider one of the critical problem to the environment due to impact of which on the 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 in particular with low cost, technique and wide public acceptance to cleaning up contaminated sites. Based on previous studies, some fungi have ability to

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degradation crude oil isolated from contaminated soil. 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 *Aspergillus niger* 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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