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Isolation of Fungi From Oil-contaminated Soil from Maysan Province, Southern Iraq

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ABSTRACT

Maysan province is one of the most important oil-producing provinces in Iraq, therefore, soil contamination as a result of extraction and processing of the oil is one of the most important environmental problems for the soil and the living organisms present in it. Twenty-sevenof fungal species were isolated and identified from soil contaminated with crude oil. Among them, 24 species (88.8%.) belonged to Ascomycota (anamorphs stages) and three (11.2%) Zygomycota. A total of 235 isolates were recovered from sites of study. *Aspergillus* showed a high number of species (6) and 78 isolates, followed by *Alternaria* (4 species, 64 isolates). *Aspergillus niger* has the highest occurrence and frequency values (42.5% and 21.70% respectively) and 51 isolates, and *Alternaria alternate* (39.16%, 20% and 47 isolates), followed by *Rhizopus oryzae* with a frequency and occurrence of 17.5% and 8.93% respectively and 21 isolates, on other hand, 8 species revealed the lowest occurrence and frequency reaching 0.83%, 0.42 respectively. Eight species were isolated described as new records from Iraq, which are *Alternaria tenuissima, Bibolaris australiensis*, *Curvularia lunata, Nigrospora oryzae, Rhizopus oryzae*, *Stachybotrys chartrum*, *Syncephalastrum racemosum* and *Ulocladium botrytis*. The biodiversity of fungi isolated from oil-contaminated soil was compared with previous studies. Brief descriptions of the new recorded fungi were given.

Key words : *Fungi, Oil-contaminated soil, Crude oil, Ascomycota, Aspergillus, Iraq.*

Introduction

The broad use and consumption of crude oil lead to contamination of various environmental systems, including soil and water (Behnood *et al.*, 2013). Contamination of soil with crude oil and hydrocarbon compounds occurs through leaks and spills that occur as a result of cracks in transport pipelines and storage tanks for oil and its derivatives as a result of drilling, extraction, transportation, storage, refining and export operations, which are frequently induced, which leads to soil pollution (Parsad and Katiyar, 2010; Essabri *et al.*, 2019). Therefore, this led to direct global attention towards its seepage into the environment. The pollution changes in the soil biological and physicochemical characteristic due to the oil may be toxic to microorganisms and plants in soil (Borowik *et al.*, 2017; Raheem and Kasim, 2020). Microorganisms such as fungi and bacteria are able to survive these contaminated environments are those that have improved specific physiological and enzymatic responses that permit them to use hydrocarbon as a substrate (Alrumman *et al*., 2015; Lafta and Kasim, 2020).

Fungi are found in almost all environments, however the vast majority of its are found in soil. Furthermore, filamentous fungi are capable of growing on wide spectrum of materials by producing extracellular enzymes, even able to grow under ambient environment (Juhasz and Naidu, 2000; Abd Al-nabi and Kasim, 2020). The fungi found in the oil-contaminated soil possesses high capacityto tolerant of extreme conditions by using several mechanisms such as produce several enzymes, resistant units and pigments (such as melanin) (Ruibal *et al*., 2009; Sheifert *et al*., 2011). Furthermore, fungi are considered to be better degraders of crude oil and oil-derived products than bacteria because fungi can degrade high molecular weight polycyclic aromatic hydrocarbons (Thenmozhi *et al.*, 2013) and can tolerate high concentrations of pollutants without affecting their enzymatic activity (Liu *et al*., 2011). Das and Chandran (2011) showed that fungi isolated from oil spill soils can decrease oil pollution.

Enzymatic approach of fungi involved in biodegradation of toxic compounds of crude oil into environmentally friendly compoundsby eliminating some functional groups either *in-vivo* or *in-vitro* process (Balaji *et al.,* 2014). Meantime, several enzymes can be produced by fungi which found in oil-contaminated soil such as lipase, protease, phytase, laccase and peroxidase.

Iraq is one of the most important producing and exporting countries of crude oil in the world, so it is natural for soil and water pollution to occur. Therefore, several studies have indicated isolation of fungi from oil-contaminated soil. Many fungi isolated from oil-contaminated soil in Basrah province (southern Iraq). (Hawash *et al*., 2018; AL-Dossary *et al*., 2019). Maysan (southern Iraq) is the second Iraqi province in the production of crude oil. However, there is no study regarding the isolation of fungi from soil contaminated with crude oil in this province. Our current study is aimed at isolating and characterizing fungi from oil-contaminated soil.

Materials and Methods

A total of 120 samples from oil-contaminated soil were collected from many sites of in Al-Mashrah district east Maysan province (southern Iraq) including oil refinery of Maysan and Buzrgan oil fields during November 2020 to April 2021. Soil samples were taken at a depth of 5-20 cm, placed in snap lock plastic bags and subsequently brought to the laboratory (Fungal Laboratory, Biological Department, College of Sciences, University of Misan).

Direct culture method was used for isolating fungi. 1 g of each sample sprinkled on tosterile potato dextrose agar plates (PDA) supplemented with 20 µg/ml of chloramphenicol to prevent bacterial growth and incubated at 25 °C. The cultures examined after 7-5 days for any fungal growth. When growth of mycelia was observed on the samples, a part of the mycelium was transferred to the Petri dishes containing PDA using a sterile loop and incubated at 25 °C for 7 days to get pure cultures. For examining the fungal mycelium that appeared in culture, slides were prepared by taking a portion of the mycelium using a sterile loop and put on a slide and stain with a drop of lactophenol or cotton blue lactophenol depending on the color of the mycelia and conidia. The specimen was examined under a light microscope. The isolated fungi were identified and described according to the available taxonomic keys. Permanent pure cultures and slides were preserved at the Department of Biology, College of Sciences, University of Misan.

The percentage of the occurrence and frequently of fungal species were measured using following equations (Krebs, 1972).

Percentage of occurrence =

$$\frac{\text{The number of samples that appeared to show one type}}{\text{The total number of samples}}$$

Percentage of frequency =

$$\frac{\text{The number of isolates of the same species}}{\text{the total number of isolates of all kinds}}$$

Results and Discussion

Taxonomic Study

Twenty-seven species of saprophytes fungi were isolated from oil-contaminated from soil oil refinery of Maysan and Buzrgan oil fields in the Province of Maysan during this study, 24 species belonged to Ascomycota (anamorphsstages) and three Zygomycota. Among them, 8 new first reports fungi in Iraq. These are *Alternaria tenuissima, Bibolaris australiensis*, *Curvularia lunata, Nigrospora oryzae, Rhizopus oryzae*, *Stachybotrys chartrum*, *Syncephalastrum racemosum* and *Ulocladium botrytis*, however these fungi were described and classified under light microscope by using international taxonomic keys. The taxonomic notes on isolated fungi are described below.

Alternaria tenuissima (Kunzee) Wiltshire,Trans. Br. Mycol. Soc., 18(2):157(1933).

Isolation No.: ZA02009. Fig 1.

Colonies aregreen, flat with regular edges, grow densely within 5-7 days. Hyphae septate, with thickness wall, golden, 4.5-2.5 µm wide. Conidiophore

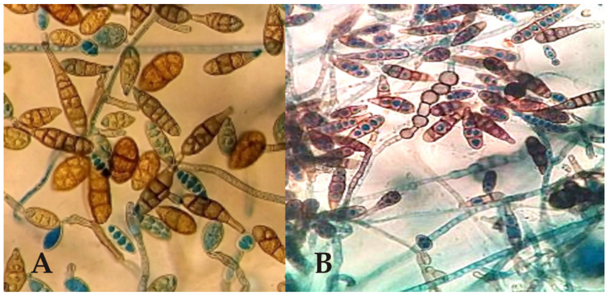

Fig. 1. *A. tenuissima* A: Conidia, B: Chlamydospores (arrow)

erected, branched, 15- 40 × 3-3.5 µm. Conidia arranged in chain, mostly unbranched, 2-5 conidia in chain, terminal conidium is smaller, oval, without beak. Other conidia brown to golden, elongated-oval, 100-40 x 20-10 µm, 2-7 transverse septa, septae 1-2 longitudinal septa, with long beak. Chlamydospores present on aged cultures, frequently globose, 8-12 µm.

Teleomorph: no known

Isolate examined: from soil of Buzrgan oil fields, 11Nov.2020.

Our isolates are in conformity with Jasniæ *et al.* (2011) except little differences, however, this may be attributed to many reasons such as the type of medium,we used PDA medium, while Jasniæ *et al.* (2011) used potato carrot agar (PCA), and environmental variations. This species can be easily recognized from the related species *A. alernata* by the shape, size and number of septa of conidia, however the conidia of *A. alernata* are smaller with shortbeak (Pastor and Guarro, 2008).

Bipolaris australiensis Bipolaris australiensis (M.B. Ellis) Tsuda and Ueyama, Mycologia 73: 90 (1981).

Isolation No.: FAO1986. Fig. 2.

Colony is wooly, brown to black, superficial, regular edges, and grow within 5-6 days, Hyphaepale brown, septated, 5-4 µm diam. Conidiophores macronematous, simple or branched,

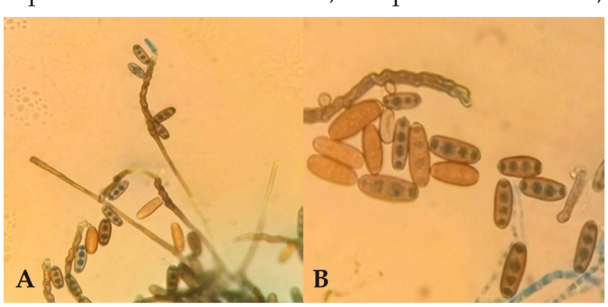

Fig. 2. *B. austsliensis* A: Conidia, B: Conidiophores

brown,50-150 x 3.5-4.5 µm, with short denticles, bearing conidia apically and laterally. Conidia cylindrical, pale brown, 15.5-30 x 4.5-7.5 µm, 2–3-septa.

Teleomorph: *Cochliobolus australiensis* (Tsuda and Ueyama) Alcorn.

Isolate examined: from soil of Buzrgan oil fields, 29 Sep. 2020.

The feature of the present isolate is in conformity with the designated species *B.australie* (Tsuda and Ueyama 1981). *Cochliobolus australiensis* the telomorph of *B.australie*. Conidia of *Bibolaris* can germinate from both end so-called bipolaris (Sciortino, 2017).

Curvularia lunata(Wakker) Boedijn, Bulletin du Jardin Botanique de Buitenzorg 13 (1): 127 (1933).

Isolation No.: AH01982. Fig 3.

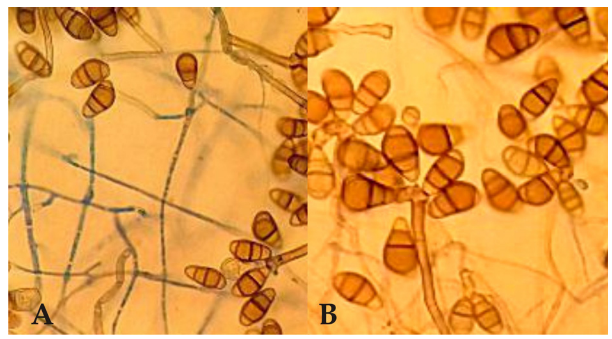

Fig. 3. *C. lunata* : A: Conidia, B: Conidiophore

Colonies smooth, brown to dark, superficial, regular edges, and grow within 5 days. Mycelium septated, brown, 2.5-3.5 µm wide. Conidiophore brown, straight or flexous,simple or branched, 150-300 x 3.5- 4.5 µm,bearing conidia apically and laterally, conspicuous pores left after secession of conidia. Conidia porosporous, oval, brown, 15-20 x 10-14 µm, mostly 4-celled, pale brown in terminal cells, dark brown and larger in 2 middle cells, especially curved, difficulty distinguishing the hilumbasally.

Teleomorph: *Cochliobolus pallescens* (Tsuda and Ueyama) Sivan.

Isolate examined: from soil of Buzrgan oil fields, 3 Mar. 2021.

The description of this species conforms with Bodijn (1933). Species of this genus can be distinguished by the number of cells, septa, presence or absence of curvature, shape, size and color of conidia, Conidia *C. Protuberata* contains 4-5 cylindrical cells.

Nigrospora oryzae (Berk. and Broome) Petch, in: J. Indian bot., (1924).

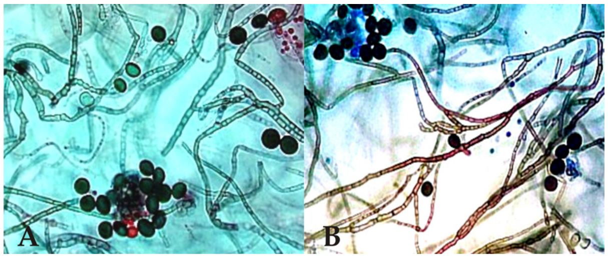

Fig. 4. *N. oryzae* A: Conidia, young (hyaline) old (dark), B: Hyphae

Isolation No.: ZHO2014. Fig. 4.
Colony wooly, dark brown, superficial, grow within 4-5 days. Hyphae septated, pale brown, branched, 2-6 µm diam. Conidiophoreun branched hyaline, globose, very short, bearing single conidium apically on hyaline vesicle. Conidiaglobose to oval, brown to black, 11 -15 x 7.5-10 µm.

Teleomorph: *Khuskia oryzae* Hudson.

Isolate examined: from soil of Buzrgan oil fields, 28 Nov. 2020.

The present species is precisely similar to those described by Petch (1924). Five species belong togenus *Nigrospora,* however the dimensions and the shape of conidia distinguish *N. oryzae* from other species (Watanabe, 2010).

Rhizopus oryzae Went and Prnisen Geerligs (1895).

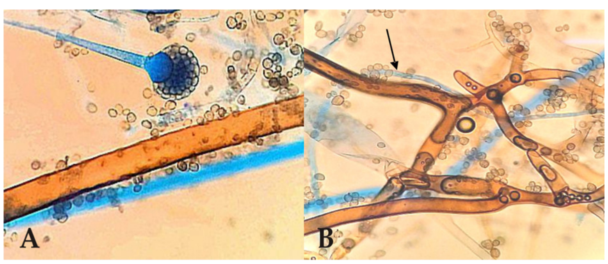

Fig. 5. *R. oryzae* A: Sporangia and Spores, B: Rhizoid (arrow)

Isolation No.: MA02006 Fig 5.
Colonies wooly, gray, grow within 3 days, Hyphacoenocytic, hyaline, 5-15 µm diam. Sporangi ophorema cronematous, erect, simple or branched, yellowish to dark brown, more than 1500 µm tall, 20-30 µm diam., at the base, gradually tapering upwards to a width of 14-16 µm at the apex, contain rhizoid attached to sporangiophore at the base. Sporangiaglobose, brown to black, 75-150 µm diam., columella spherical, brown Sporangiosporesoval to subglobose, palebrown, smooth wall, 5.5-10.5 µm diam.

Teleomorph: no known

Isolate examined: from soil of Buzrgan oil fields, 30 Oct. 2020.

This present isolate feature conforms with the designated species *R. oryzae* (Went and Prnisen, 1895). *Rhizopus* differs from *Mucor* in the presence of rhizoid and from *Lichtheimia spp.* in that rhizoid does not arise from a point opposite to the sporangiophore. *R.oryzae*is identical with *R. stolonifera* and *R. oligosporus* in most morphological characters. However, the sporangia and sporangiospores size is smaller in *R. stolonifera* and columella is biggerin *R. oligosporus* (Schipper, 1984).

Stachybotrys chartrum(Ehrenb.) S. Hughes, Canadian Journal of Botany 36 (6): 812 (1958).

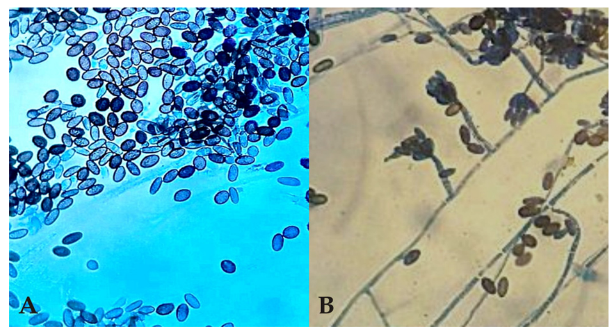

Fig. 6. *S. chartarum* A: young (hyaline), old (dark), B: mycelia and conidiophores

Isolation No.: SK01945 Fig. 6
Colonies are cottony, white to brown, grow within 4-6 days. Hyphaeseptate, hyaline, 3-5 µm diam. Conidiophore macronematous, branched or simple, brown, wall isthin becomethick with age, 50-100 x 3.5-4.5 µm, bear clusters of 3–7 phialides at the apex. Phialids are hyaline, cylindrical or ellipsoid, bearing clusters of conidia (3-10 conidia). Conidia hyalineor brown, oval, unicellular, rough-walled, 4-6 x 7-13µm.

Teleomorph: no known

Isolate examined: from soil of Buzrgan oil fields, 3 Dec. 2020.

*S. chartrum*was formerly known as *S. alternans* or *S. atra* (Masten, 2004). The characters of our isolate match well with Link and Hunhes (1958). This species is simply recognized by the size and color of conidia, however, two kinds of conidia, hyaline and brown (Andersen *et al.*, 2003).

Syncephalastrum racemosum Cohn, Kryptogamen-Flora von Schlesien 3-1(2): 217 (1886).

Isolation number: MD 02008 Fig. 7.

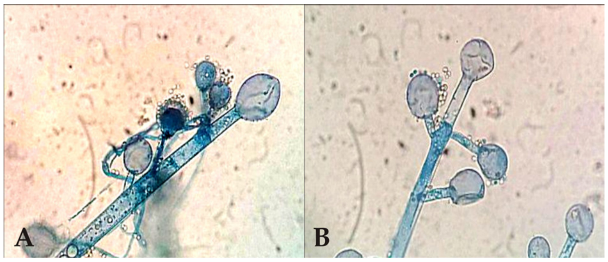

Fig. 7. *S. racemosum* A: sporangia, B: branched sporan giophore

Colonies are cottony, gray, slightly superficial, grow within 4 days. Mycelium hyaline,aseptate, 5-10 µm diam. Sporangiophoremacronematous, erect, simple or branched, more than 2000 µm long, 6.5-12.5 µm wide, and terminate in vesicle, rarely rhizoidal at the base. Vesicle hyaline to light brown, globose or subglobose, 20-50 diam. Merosporangia pale brown, cylindrical, 25-31.5 µm tall, with chains (1-8) of sporangiospores. Sporangiospores oval, dark brown, aseptate, thin-walled, 3.5-4.5 µm diam.
Teleomorph: no known
Isolate examined: from soil of Buzrgan oil fields, 17 Nov. 2020.

The characters of*S. racemosum*is agreed with Cohn ex. Schroter (1886). Syncephalastraceae has only *Syncephalastrum* which belong to Mucorales.
Ulocladium botrytis (Preuss) Woudenb. & Crous, Studies in Mycology 75: 206 (2013)
Isolation No.: FK 01955 Fig.8.

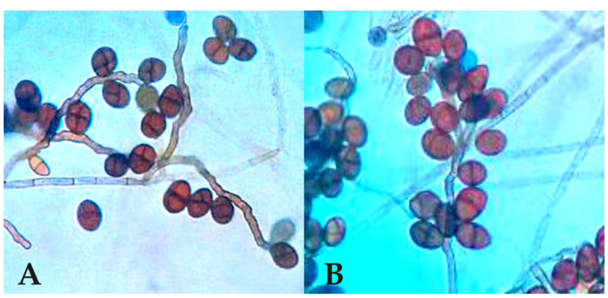

Fig. 8. *U. botrytis* A:Conidia, B: arrangement of conidia on conidiophore

Colony is cottony, brown to dark brown, with irregular edges, grows within 5-7 days. Hyphae septate, brown, 2-5 µm diam. Conidiophore simple or branched, golden color, 25-57 × 3-4 µm, bearing one to several conidia apically or subapically. Conidia oval, brown, 20-40 × 12-15 µm, 2 transverse septa, 1-2 longitudinal septa.
Teleomorph: no known.
Isolate examined: from soil of Buzrgan oil fields, 14 Oct. 2020.

The present isolates are exactly similar to those described by Preuss (1851). The main deference between *Ulocladium* spp.and *Stemphylium* spp. is the size and shape of conidia, and number of transverse and longitudinal septa. However, the septa of conidia of *Ulocladium* give a Y shape (Sciortino, 2017). Many species belong to *Ulocladium*, however, some differences in conidial measurements distinguish *U. botrytis* from other species, which are oval and golden color (Watanabe, 2010). This specie was erected as *Ulocladium botrytis* (Preuss, 1851) and transferred into *Alternariabotrytis* by Woudenberg *et al.* (2013).

Population Dynamic of Isolated Fungi

Twenty-seven taxa of fungi were isolated from oil-contaminated soil during this study, comprising 24 Ascomycota (88.8%.) and 3 species belonged to Zygomycota (11.2%). Moreover, all Ascomycota species were in anamorphic state. During this study, 16 genera were isolated, *Aspergillus* showed a high number of species (6) and 78 isolates, followed by *Alternaria* (4 species, 64 isolates) (Table 1).

This study revealed that 235 isolates had been isolated from the two study sites, *A.niger* -has the highest occurrence and frequency values (42.5% and 21.70% respectively), furthermore it appeared the highest number of isolates reached 51, followed by *A. alternate* (39.16%, 20% and 47 isolates), and then *R. oryza*e with a frequency and occurrence of 17.5% and 8.93% respectively and 21 isolates, followed by *P.lanusum*, showed a high occurrence (14.16%) and a frequency. In comparison, the lowest occurrence and frequency were found in 8 species reaching 0.83%, 0.42 respectively (Table 1).

Fungi have a great ability to adapt to various inappropriate conditions such as nutrient deficiency, severe environmental conditions, exposure to the maximum levels of radiation, toxic chemicals and pollutants such as crude oil (Moye-Rowley, 2003). This adaptation includes many mechanisms, highlighted that, are capable of producing a sexual structures abundantly (which can easily spread by water and air to reach the soil), resistance structures with thickness wall such as chlamydospores, sclerotia and resting spores, and can also be grew in unsuitable conditions as temperatures, pH and water content (Smits *et al.*, 1998). Furthermore, toxins and antibiotics may also be produced fungi,

Table 1: List of species isolated from oil-contaminated soilfrom Maysan province (southern Iraq)

Fungal Species	No. of Isolates	Occurrence %	Frequency%
Alternaria alternate	47	39.17	20.00
A. chlamydospora	9	7.50	3.83
A.citri	4	3.33	1.70
A.tenuissima	4	3.33	1.70
Aspergillus flavus	8	6.67	3.40
A.fumigatus	1	0.83	0.43
A.niger	51	42.50	21.70
A.nidulans	3	2.50	1.28
A.terreus	12	10.00	5.11
A.versicolor	3	2.50	1.28
Bipolaris australinsis	4	3.33	1.70
B.saccharia	1	0.83	0.43
Cladosporium.cladosporioides	14	11.67	5.96
Curvularia lunata	2	1.67	0.85
Exserohilum holmii	3	2.50	1.28
Fusarium soloni	1	0.83	0.43
Nigrospora oryzae	1	0.83	0.43
Penicillium janthinellum	9	7.50	3.83
p.lanosum	17	14.17	7.23
Phoma glomerata	1	0.83	0.43
Stachybotrys chartarum	1	0.83	0.43
Stemphylium herbarum	1	0.83	0.43
Ulocladium atrum	2	1.67	0.85
U.botrytis	12	10.00	5.11
Rhizopus oryaae	21	17.50	8.94
Syncephalastrum racemosum	1	0.83	0.43
Mucor plumbeus	2	1.67	0.85
Total	235		100

and these may facilitate rapid colonization as well as can be considered as means of defense and competition in the same habitats (Serna-Chavez *et al.*, 2013).

The existence of populations of microorganisms in soil that respond to the presence of contaminating crude oil and hydrocarbons normally have more than one type of hydrocarbon utilizing microorganisms (Obire and Anyanwu, 2008). Many studies indicated that the most fungi able to biodegrading the crude oil, though at different averages (Al-Jawhari, 2014; Reyes-Ce´sar, *et al.*, 2013; Raheem and Kasim 2020).

Among 27 species isolated in this study, 24 belonged to Ascomycota, several studies indicated that the Ascomycota fungi is one of the most common and widespread fungal phyla in contaminated or uncontaminated soil with crude oil and play different environmental roles in these soils (Torn and Lynch, 2007; Hawksworth, 2012). This is due to their ability to produce many enzymes involved in decomposing organic materials of soil, especially oil-contaminated soil, which are capable of degrading high molecular weight compounds such as aromatic structures (Olukunle and Oyegoke, 2016).

Among the studied fungi, *A. niger*and *A. alternate* gave a higher number of isolates (51 and 47 respectively), many studies appeared that these species can colonize oil-contaminated soil and have the ability to secretes many enzymes to degrade crude oil, among them are Laccase, Peroxdase and Cytochrome p-450 monooxygenase (Chaudhry *et al.*, 2012; Durairaj *et al.*, 2016; Sabah *et al.*, 2016). In addition, we found that remarkable increase in fungi containing darkly pigmented (melanized) spores and hyphae, such as *Alternaria*, *Cladosporium*, *Curvularia* and *Stemphylium.* These fungi cannot always be growing at low water potentials and have the capability to withstand periodic wetting and drying (Deacon, 2013), meanwhile, melanin pigment makes them more resistant to inappropriate conditions (Moshera *et al.*, 2006).

Three species belonged to 3 genera (*Rhizopus*, *Syncephalostrum* and *Mucor*) of Zygomycota appeared in a field survey, this result agreed with Islam, (2017) who isolated 20 species, most of them Ascomycota. Bonugli-Santos (2015) indicate this likely due to the enzymatic activity was impaired of Zygomycota fungi compared with other fungi as Ascomycota especially when depletion of nutrients by competing microbes in soil. Furthermore, these fungi can be growing very quickly when nutrients are available, but its growth declined or stopped when it is depleted (Cajtham *et al*., 2008; Singh *et al*., 2012).

Conclusion

Oil spills are considered as one of the serious problems faced by human being due to impact of diffuse pollutants which cause decrease of environment health. The results indicate that many of the fungi isolated from oil-contaminated soil of Misan province were capable of using and degrading crude oil for nutrition. Consequently, oil-contaminated soils may be considered as habitats for many fungi especially Ascomycota which has enzymatic activity enable fungi to colonize such environments.

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