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ORIGINAL ARTICLE

Study of Lipase Production and Lipids Accumulation of Oleaginous Fungi Isolated from Oil-rich Soil in Basrah

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ABSTRACT

Objectives: The present study was aimed for rapid identification of lipids accumulation of oleaginous fungi isolated from oil-rich soil in Basrah /Iraq.

Methods: Soil samples were collected from oil-rich soil of Basrah-Iraq; dilution plate method was used to identification of fungi, as well as the percentage of frequency and occurrence was determined after identification of fungal isolates according of morphological features. Congo red agar was employed for evaluation of lipase production of fungal isolates, while lipid accumulation of fungal isolates was estimated by using Sudan black B technique. As well as, four fungal isolates that revealed highest lipid accumulation were underwent of DNA extraction and the result of genomic DNA was amplified with two universal primers ITS1 and ITS4, PCR products were purified and sequenced, furthermore, sequencing results of fungal species were identified in " BLAST " provided by the NCBI.

Results: The study was showed that isolation of 8 filamentous species due to Deuteromycetes. *Aspergillus flavus* was revealed high percentage of frequency with 18.18%, while the high percentage of occurrence for *Scytalidium japonicum* by 27.5%. Lipolytic activity test was showed that the majority of fungal isolates have the ability of lipase production 90%, however, the highest precipitation zone was due to *A.flavus* 55mm, followed with *A.niger* 47mm. As well as all selected isolates were given positive results when staining with Sudan Black B stain that used for intracellular lipids detection. Furthermore, the result of molecular identification showed that recorded *Aspergillus flavus* KAIN2 as new strain in Gene Bank that gets accession number MN508371.

Conclusion: *Aspergillus flavus* and *Scytalidium japonicum* were showed high percentage of frequency and occurrence. The majority of fungal isolates were appeared lipase activity, while all of them were showed ability of lipid accumulation when staining by Sudan black B.

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INTRODUCTION

Microbial lipids such as bacteria, algae, yeast and filamentous fungi can be valuable alternative raw materials for the production of biodiesel, and the potential solution for oil decrease in the world [1].Among those organisms that are able to accumulate lipids in their cells in large proportions are fungi. These organisms store important compounds in the form of triacylglycerol (TAG). TAG is found as large-scale backup compounds among all eukaryotic organisms

such as fungi, plants and animals, while it is rarely described in bacteria [2,3].

Fungi are important organisms that possess a group of characteristics that distinguish them from the bugs of the organisms. The most important of these characteristics is the ability to collect or accumulate high amounts of lipids. Fungi that have the ability to collect lipids in more than 20% of its biomass called oleaginous fungi [4].

The use of fungal lipids for raw materials to production of biodiesel has several characteristics, including containing saturated and unsaturated fatty acids, which are very similar to fatty acids found in vegetable oils such as (palmitic acid, palmitolic acid, stearic acid, oleic acid, linoleic acid, mystric acid, and arachidic acid etc.), easy to culturing and growth, does not require large land , no competition for food as well as short life cycle [1, 5, 6, 7].

Therefore, rapid detection of accumulation of intracellular lipids is essential to differentiate oleaginous species from non-oleaginous species, since, lipase production test used for many applications including detection of lipids accumulation [8, 9, 10]. Furthermore ,The commercial uses of lipase including production of biopolymer , pulp paper, food, and detergent [11, 12]. On the other hand, the use of Sudan Black B stain is one of rapid tests to determine lipids accumulation, thus, this dye saves time and not require a fluorescent microscope in comparison with other dyes [13, 14].

MATERIALS AND METHODS

Samples collection: Soil samples were collected from oil-rich soil of Basrah-Iraq; soil samples were underwent isolation by employing soil dilution plate method according to [15]. One ml of each concentration (10^1-10^3) was added to petri dish, then melted PDA was dispensed into plates, the plates were incubated at 25ºC for 3-7 days.

All plates were examined visually directly and with dissecting and light microscope .All fungal isolates were transferred to PDA plates for purification and identification. As well as the pure culture were transferred to PDA slants and maintained at 4ºC for further study , fungal isolates were identified on the bases of their morphological features and the percentage of frequency and occurrence of fungal isolates were determined as the following formula [10]:

$$\%\text{frequency of species} = \frac{\text{Number of species appearance in the sample}}{\text{Total number of species appearance}} \times 100$$

$$\%\text{ occurrence of species} = \frac{\text{Colonies number of species}}{\text{Total number of species colonies}} \times 100$$

Detection of Lipase activity: Screening media contain g/l (NaCl, 5; Ca Cl2, 0.1; Peptone,10 ; Agar, 20; congo red,0.5; and 1 ml of Castor oil) with initial pH,6.5 , was used to evaluate lipase production of fungal isolates [16]. 6 mm in diameter of each fungal isolates that previously grown in PDA was placed on the center of Congo red agar in triplicate for each isolates, in addition to control plat without fungal growth. After incubation period at 30°C for 8 days, the diameter of precipitated zone (lipolysis) around the colonies was considered as a measure of lipase production as the following formula:

Lipolysis (mm) = diameter of precipitated zone with colony - diameter of colony.

Lipids accumulation: The observation of lipids accumulation by fungi was determined with Sudan Black B staining according to [17]. Fungal isolates were stained with Sudan Black B stain then dried and removed stain by washing the slide with xylol .After that the slides were counterstained with safranin .Finally, the slides were washed with distilled water, dried and examined under light microscope. Subsequently, oleaginous fungal isolates that accumulated lipids and produced lipase were selected for further study.

Molecular identification: Four fungal isolates that appeared lipids accumulation with staining of Sudan black B were subjected to DNA extraction according to [18], as well as genomic DNA was amplified using two prefixes universal primers (ITS1 and ITS4) according to [19]. PCR products were purified and sequenced of (ITS1-5.8S – ITS4 rDNA) for PCR Products by Macrogen Company, South Korea " http://dna.macrogen.com . Sequencing results of fungal species were identified in " BLAST " provided by the NCBI " http://www.ncbi.nlm.nih.gov ".

Statistical analysis: Using SPSS statistical package for social sciences to data all analysis by using ANOVA at significant level $p \leq 0.05$.

RESULTS

Isolation and Identification of fungi: The current study was revealed that isolation of eight species of filamentous fungi, most of them due to Deuteromycetes . Isolated fungi were included *Alternaria alternata*, *Aspergillus niger, A.flavus*, *A.ochraceus*, *A. sydowii*, *Cladosporium herbarum*, *Penicillium chrysogenum*, *Scytalidium japonicum*, white sterile hyphae and white yeast. The result of Table 1 was showed that percentage of frequency and occurrence of fungal species, the most common species was *A. flavus* with 18.18% of frequency, while the highest occurrence was due to *S. japonicum* with 27.5 %.

Table 1: **Frequency and occurrence Percentage of fungal isolates isolated from oil-rich soils.**

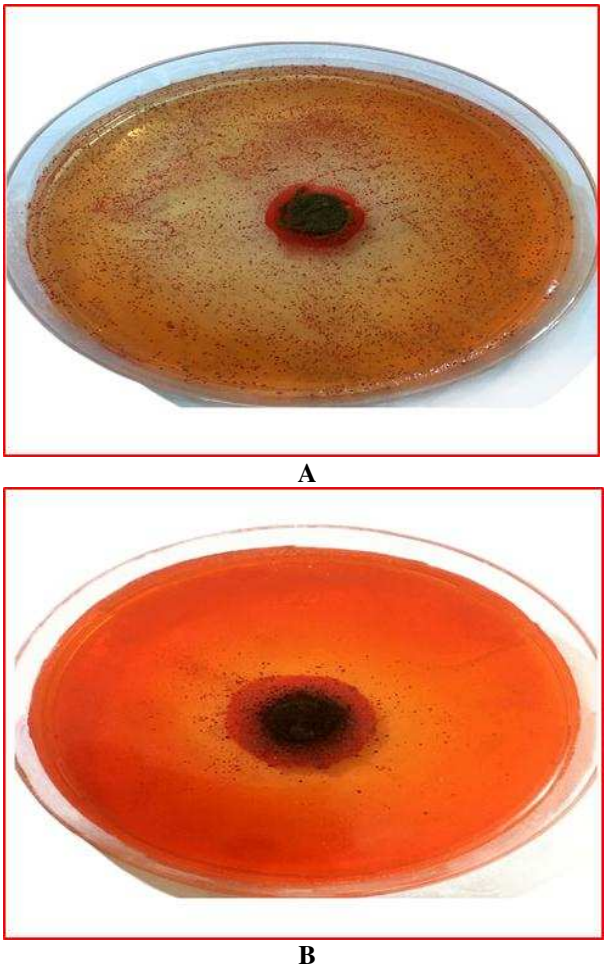
Fungal isolates	Frequency %	Occurrence %
Alternaria alternata	9.09	2.5
Aspergillus niger	9.09	2.5
A.flavus	18.18	15
A.ochraceus	9.09	2.5
A. sydowii	9.09	2.5
Cladosporium herbarum	9.09	2.5
Penicillium chrysogenum	9.09	17.5
Scytalidium japonicum	9.09	27.5
white sterile hyphae	9.09	25
White yeast	9.09	2.5

Detection of Lipase activity: The result of determination lipase production activity for fungal species was revealed variation of diameter of precipitation zone (lipolytic activity) around fungal colonies in Congo agar medium, with significant differences at p≤ 0.05 (Table 2). The majority of isolates were seen lipase activity (90%). However, the measure of diameter of precipitation zone of tested isolates was revealed that *A.flavus* was the highest species with 55 mm followed by *A. niger* 47mm, *A.ochraceus* 45 mm and *P. chrysogenum* with daimeter 42 mm (Figure 1).

Table 2: **Lipolytic activity of fungal isolates after 8 days incubation on Congo red agar.**

Fungal isolates	lipolytic activity(mm)
Alternaria alternata	0
A.flavus	55
A.niger	47
A.ochraceus	45
A. sydowii	5
Cladosporium herbarum	30
Penicillium chrysogenum	42
Scytalidium japonicum	10
White sterile hyphae	30
White yeast	5

Significant differences at p≤ 0.05



A

B

Figure 1: **Lipolytic activity that appeared as precipitated zone around the colonies after 8 days inoculation on Congo red agar (A : *A.flauvs*, B : *A.niger*) .**

Lipids accumulation: All four fungal isolates that given largest precipitated zone when grown on Congo red agar, were showed the capability of lipids accumulation that appeared as lipids bodies colored Black-blue within the cells when examined under microscope by using the colorimetric technique with Sudan Black B stain, (Figure 2).

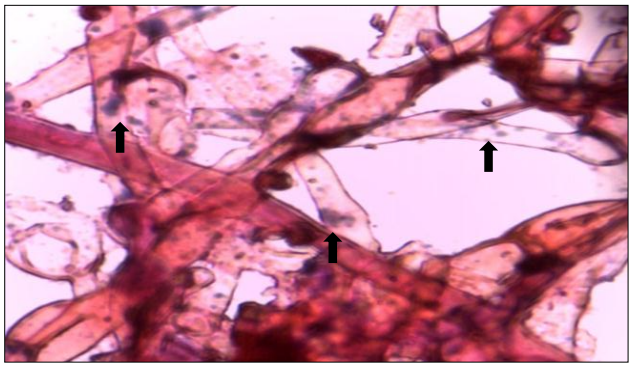


Figure 2**: Intracellular lipids after dyeing Sudan Black B for *A.flauvs* ; intracellular lipids.**

Molecular identification: Molecular identification was done to confirm the morphological identification of four selected isolates that used to previous experiments by using universal primers, ITS1 and ITS4. Figure 3 was showed the result of agarose gel electrophoresis analysis of DNA extracts from selected isolates, while the result of agarose gel electrophoresis analysis for (PCR) products was seen in Figure 4. All PCR products of oleaginous fungi were appeared between 600 -800 base pair. To evaluate the similarity of four selected fungal isolates, and the result of PCR products was compared with other reliable, sequences deposited in GenBank, which are part of the National Center Biotechnology Information, (NCBI). The result of sequencing of ITS1-5.8S-ITS4 rDNA with BLAST program was showed that *Aspergillus flavus* differ with their reference strains in several positions of nucleotide sequences, so that it was recorded as new strain which get accession number MN508371 in GenBank that have 98% similarity with the sequences of its reference strain GGV_BT03.

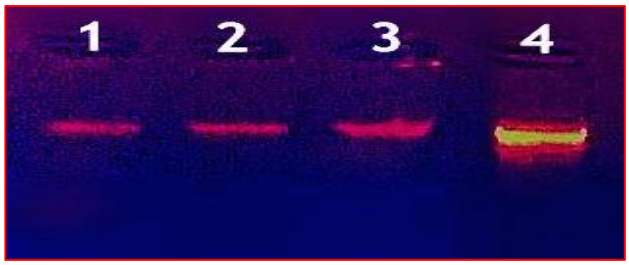


Figure 3**: 0.8% agarose gel electrophoresis of oleaginous fungi genomic DNA: (1-4) DNA products of fungal species.**

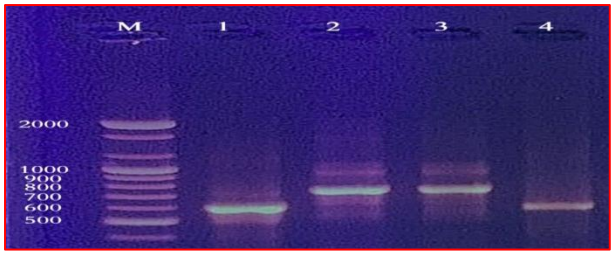
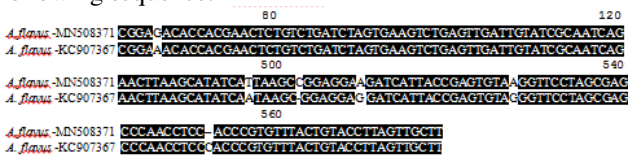


Figure 4: **2% Agarose gel electrophoresis analysis for PCR products of oleaginous fungi with using two primers ITS1,ITS4 ; M:DNA marker ; Lanes:1-4 amplification genes of fungal isolates.**

Description of *Aspergillus flavus* strain KAIN2: *Aspergillus flavus* KAIN2 was morphological similar to other isolates of *Aspergillus flavus*, while molecular identification by sequence of *Aspergillus flavus* KAIN2 MN508371 had shown similarity with *Aspergillus flavus*_KC907367 by 98% and variation in nucleotides at different positions, these differences were varied between deleting and adding nitrogenous bases some in addition to replacing some bases with others, leading to mutations such as Point Mutation (Frame shifts, Transition, and Transversion mutations) as in the following sequence:



Sequence result of *Aspergillus niger* isolates was showed high variation with reference isolate (MH855928.1) in 96.30%, so that, the isolate was not recorded in gen bank because need subsequent studies using other primers in spite of the morphological identification using special references for the classification of fungi proved that the isolate was due to the *Aspergillus niger*.

DISCUSSION

Oil-rich soil was considered poor soil that contain low organic materials with high amount hydrocarbons pollutants, this indicate to ability of fungi to resist such conditions. Furthermore, the present study was consistent with the study of [20], that showed fungi have the ability to live in harsh environmental such as temperature and salinity, where *Penicillium* spp and *Aspergillus* spp such as *A. flavus* is considered an important species that appeared resistant to salinity and drought , therefore this explanation was support current study [21, 22, 23, 24].

Many studies were pointed on the basic mechanism of lipid accumulation in oleaginous fungi [10, 25, 26]. Lipids accumulation was occurred when fungal isolates grown in nitrogen limiting media, this lead to suppression of isocitric dehydrogenase, so that, Krebs cycle was blocked. As well as extra carbon source was altered to triglyceride by sequences of enzymes such as citric acid lytic enzyme , malic acid enzyme and fatty acid enzyme [27]. Therefore, screening the ability of fungi to lipids accumulation by quick and efficient techniques is necessary and this agreed with the present study [13]. Lipase is a biological enzyme that works on the hydrolysis of TAG to fatty acid. The commercial uses of lipase comprise of a variety of different applications including production of biodiesel, biopolymer, pulp paper, food and detergent [11, 12]. Lipase is widely found in microorganisms, fungi is one of the most important sources for the production of this enzyme, so that, current study was confirmed with this fact [8, 28, 29]. In addition, lipase production tests are cheap tests that can be performed to determine the ability of fungi to collect lipids. Therefore, the precipitated zone (Lipolytic) is an indicator of enzyme analysis [9, 10, 16]. Moreover microscopic methods were described to detect lipids droplets that deposited as intracellular lipids include staining by fluorescent [30]. These techniques are requiring special stain like Nile red in addition to contrast microscope to recognize lipids accumulation, thus need expensive and specialized instrumentation. While Sudan Black B technique is simpler, rapid, and available requiring [13, 14, 31].

CONCLUSIONS

The current study was seen that *Aspergillus flavus* and *Scytalidium japonicum* were showed high percentage of frequency and occurrence respectively. On the other hand, Congo red agar and Sudan black B technique were subjected as easily, available and cheap methods to detect and evaluate lipase activity and the ability of fungal isolates for lipid accumulation, so that, the majority of fungal isolates were appeared lipase activity, while all of them were showed ability of lipid accumulation when staining by Sudan black B.

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