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To cite this article: Inaam M. N. Alrubayae and Kadhim F. Kadhim 2020 *IOP Conf. Ser.: Mater. Sci. Eng.* **928** 062011

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Determination of oleaginous from non-oleaginous fungi using enzymatic and microscopic techniques

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Abstract

The current study was showed that the most fungal isolates that isolated from agricultural soils belong to Deuteromycetes, where *Aspergillus terreus* was appeared with 21.73 % and 43.23 % frequency and occurrence ratio respectively followed by *A. niger* with 17.39%, 35.7% of frequency and occurrence respectively. Moreover, fungal isolates that included *A. fumigatus*, *A. terreus*, *Cladosporium ramotenellum* and *Lichtheimia corymbifera* were revealed the highest productivity in the catalyse medium for the production of lipase .on the other hand, *A. terreus* was recorded the highest lipase composition with 47 mm of precipitation zone around colony on Congo red agar, as well as it was gave positive results of Sudan black B stain. Furthermore, the selected oleaginous fungal isolates were showed accumulative capacity of lipids reached to 23 % when cultivated in wheat straw medium as natural fermentation medium.

Keywords : Oleaginous fungi, non-oleaginous fungi ,congo red, sudan black B

Introduction

The environmental policies to diminish CO₂ emissions have invigorated investigation and development of biotechnology to yield clean energy from renewable resources. Biodiesel is an elective technique, which is ecofriendly and created from inexhaustible sources like vegetable oils, plant oils, microorganisms or animal fats by transesterification with low sub-atomic weight alcohols. The employment of fungi is one of the most important renewable sources to achieve this goal, whereas in recent years there are many studies focused on different fungal groups and their ability to accumulate lipids within their biomass (1,2,3,4). Oleaginous fungi can be considered as a promising alternative to fuel, as it has the ability to accumulate lipids in its biomass as well as it has advantages over plants by not competing for food resources, growth on multiple sources and does not require arable land. Numerous of filamentous fungi and yeasts have the potentiality to synthesis and accumulate high amounts of lipids such as triglycerides



(TAG) in their biomass especially long chain fatty acids such as in (C16:1 , C18:1 , C16:0 ,C18:3 , C18:2 , C18 , C16) that similarity to vegetable oils, therefore, it is important to identification oleaginous fungi and know their species using different and cheap methods (3,5,6, 7) .

Accumulation of lipids in oleaginous fungi always occurs with limitations sources of nitrogen, phosphorus, zinc, iron and magnesium. Moreover, the scanty of nitrogen source (N) is the most effective and efficient source of lipids accumulation (lipogenesis) (8,9,10,11).

Fungi and yeasts are good sources of lipids assemblage in their biomass. These lipids are similar to vegetable oils, therefore can be invested to produce biodiesel in power generation and internal combustion engines rather than fossil fuels. Where the fossil fuels have become severely damaging to the environment (7,12,13,14,15).

Material and methods

Samples collection

Five samples of agriculture soils were selected for present study, all samples were collected with a small sterile shovel. Then the dilution process was carried out on the soil with three concentrations according to (16). Every sample in three concentrations with duplicate were cultured in PDA and incubated for 4-7 days at 25 ° C. The fungal isolates were purified and identified depending on the references of fungal identification (17,18). Finally the frequency and occurrence of the fungal isolates were calculated.

Detection of oleaginous fungi

The demarcation test of oleaginous from non- oleaginous fungi was performed according to detect the ability of isolates to produced lipase (the enzyme analyzing the lipids) and the accumulation of lipids bodies using Sudan black B dye as microscopic methods.

Congo red agar with castor oil as catalyst was used for lipase production trail; this medium was contained peptone as source of carbon (19). The accomplishment of lipase composition was measured by diameter of precipitated zone (mm) around the colonies of fungal isolates, as well as the lipids aggregation (lipids bodies) were determined using Sudan black B pigment as stated by (20).

Molecular identification

After morphological identification of all fungal isolates that depending on macroscopic and microscopic features, the molecular identification of oleaginous isolates were identified. Where the uppermost four isolates that give larger precipitation zone for further study of lipids accumulation were chosen to molecular identification.

The DNA of oleaginous isolates was extracted manually referring to (21). While ITS1 (F-5-TCC GTA GGT GAA CCT GCG G-3) and ITS4 (R-5-TCC TCC GCT TAT TGA TAT GC-3) primers was used to magnify of DNA indicating to (22), even though, PCR program that used to amplify the Internal Transcribed Spacer region (ITS 1-5.8S-ITS2) for oleaginous isolates, then PCR products of oleaginous isolates were sent to Macrogen Company(South Korea) to obtain the sequences and sequencing results were identified in BLAST provided by the NCBI “ <http://www.ncbi.nlm.nih.gov> ”.

Lipids production

In order to discrimination and extraction for lipids from fungi, wheat straw medium was used as alternative medium of fungal cultivation, this medium was prepared by adding 50 gm of wheat straw to 500 ml distilled water (D.W), then boiled, cooling, after that, the mixture was filtered through layers of medical gauze, finally the volume was completed to 1L of D.W, while, the pH was adjusted at 6.5. After activation on PDA medium, the oleaginous fungal isolates were transferred to Erlenmeyer flask (250 ml) containing 100 ml of autoclaved wheat straw fermentation medium and incubated at 30°C, 120 rpm for 8 days. Subsequently, the biomass was dried and calculated. As well as, by using methanol, chloroform system [1:2 methanol:chloroform] the lipids were extracted from dried fungal biomass according to (23) , finally the lipids were weighted.

Statistical analysis

ANOVA at significant level $p \leq 0.05$ was used for analysis all data by using SPSS statistical package.

Results

Identification of fungi

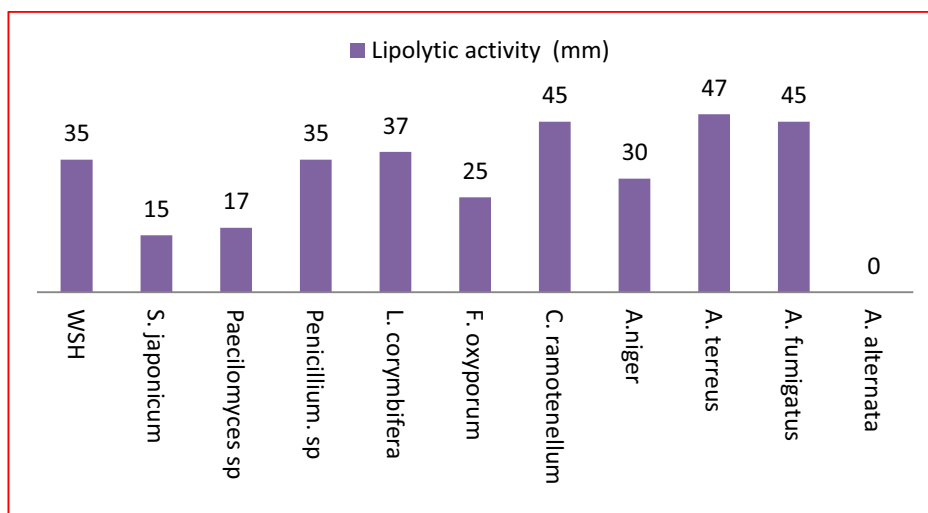
Eleven fungal species were isolated from agriculture soils which included (*Alternaria alternata*, *Aspergillus fumigatus*, *A. niger*, *A. terreus*, *Cladosporium ramotenellum*, *Fusarium oxyporum*, *Lichtheimia corymbifera*, *Penicillium* sp, *Paecilomyces* sp, *Scytalidium japonicum* and white sterile hyphae (WSH)). The current study was showed a clear variation in the frequency and occurrence of fungal isolates. *A. terreus* was appeared the highest frequency and occurrence with 21.73 % and 43.23% respectively followed by *A. niger* with 17.39% and 35.7% of frequency and occurrence respectively (table 1).

Table (1): Frequency and Occurrence of fungal isolates from agriculture soils

| Fungal isolates | Frequency% | Occurrence% |
|----------------------------------|------------|-------------|
| <i>Alternaria alternata</i> | 4.34 | 0.47 |
| <i>Aspergillus fumigatus</i> | 8.69 | 0.94 |
| <i>A. niger</i> | 17.39 | 35.7 |
| <i>A. terreus</i> | 21.73 | 43.23 |
| <i>Cladosporium ramotenellum</i> | 4.34 | 0.47 |
| <i>Fusarium oxyporum</i> | 8.69 | 4.27 |
| <i>Lichtheimia corymbifera</i> | 4.34 | 0.47 |
| <i>Penicillium</i> sp | 13.04 | 6.65 |
| <i>Paecilomyces</i> sp | 4.34 | 0.47 |
| <i>Scytalidium japonicum</i> | 8.69 | 4.28 |
| White sterile hyphae | 4.34 | 2.85 |

Detection of oleaginous fungi

The results of lipase composition were revealed that a significant differences in the diameters of the precipitation zone around the colonies at $p \leq 0.05$ where *A. terreus*, *A. fumigatus*, *C. ramotenellum* and *L. corymbifera* were seen highest capability to yield lipase with diameters 47,45,45, and 37 mm around the colonies respectively, (fig. 1). In addition to, these four isolates revealed an obvious competence to lipids accumulation when detecting of fatty bodies inside fungal hypha by using Sudan black B stain, while, the lipids structures appear clear in a blue-black color under microscope (fig. 2).

**Fig (1):** Lipolytic activity of fungal isolates tested on Congo red agar.

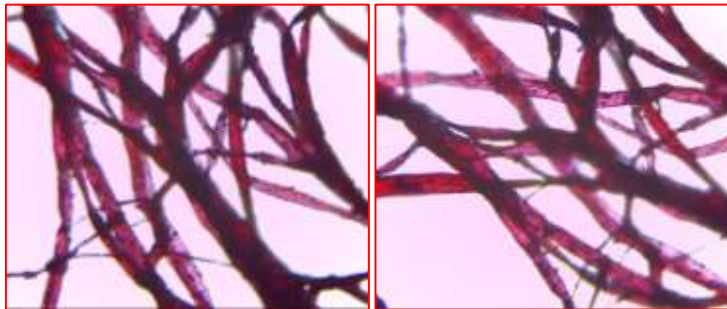


Fig (2): The intracellular lipids for *L. corymbifera*. Stained with Sudan black B stain

Molecular identification

Molecular identification results were revealed the similarity with morphological identification of four selected oleaginous fungal isolates. All PCR products of oleaginous isolates were showed at 600 -750 base pair .The result of sequencing of ITS1-5.8S-ITS2 rDNA with BLAST program was seen that *Aspergillus terreus* differ with their reference strain (*Aspergillus terreus* MH282506) in several positions of nucleotide sequences, so that, it was recorded in Gen Bank with accession number MN508370 as new strain for the first time of the world. *Aspergillus terreus* KAIN1 MN508370 was showed similarity with their reference by 98% and difference in some nucleotide at different position leading to mutations such as Transversion mutations as in following sequences :

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                260                               300
CTCTTGGTTCCTGGCATCGATGAA CAACGCAGCGAAATGCGATAACTAATGTGAATTGCAG
CTCTTGGTTCCTGGCATCGATGAA CAACGCAGCGAAATGCGATAACTAATGTGAATTGCAG

                440                               480
CGGCTCCCGGGGGACGGCA CCGAAAGGCA CCGCGGCACCGCGTCCGGTCTCGAGCGTA
CGGCTCCCGGGGGACGGCA CCGAAAGGCA CCGCGGCACCGCGTCCGGTCTCGAGCGTA

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Biomass production and lipids accumulation

The four selected oleaginous fungal isolates were showed insignificant difference at $p \leq 0.05$ for biomass production and accumulation of lipids when cultivated in WSM. Where the higher yield of biomass was appeared in *A. terreus* by (2g/l). However, *L. corymbifera* was showed higher value of lipids accumulation (23 %) followed by *C. ramotenellum* with (20 %), (fig.3).

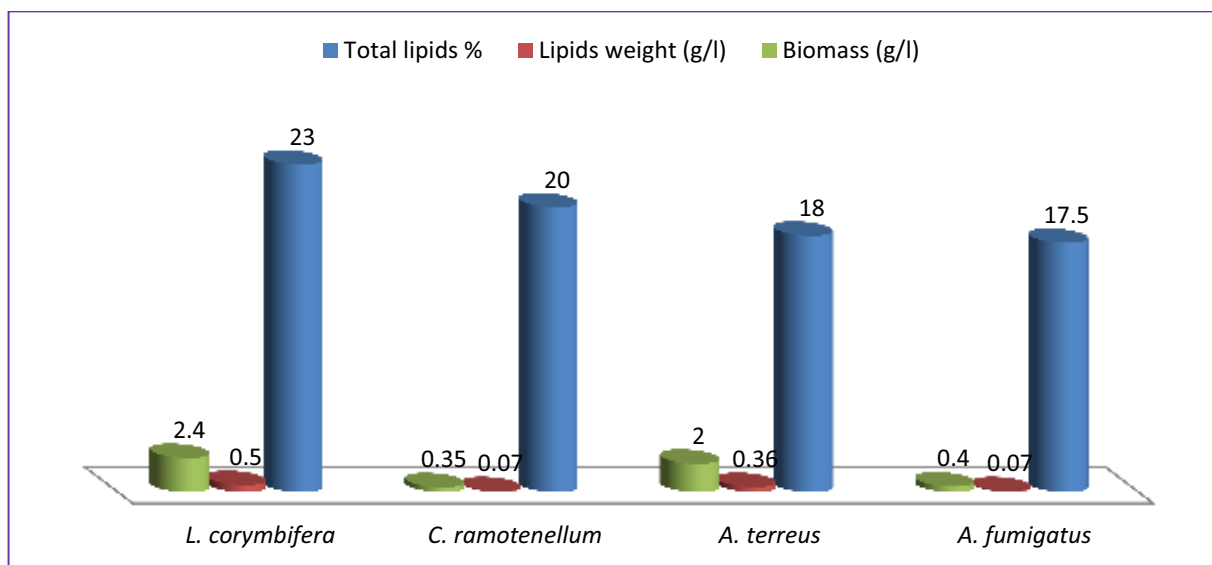


Fig (3): Biomass and lipids composition of oleaginous fungal isolates in WSM.

Discussion

In the current study, the majority of fungal isolates of agriculture soil due to Deuteromycotina, this group is widely distributed in many different environments, as well as *A. terreus* showed the high frequency and occurrence of fungal isolates, whereas the genus of *Aspergillus* was considered one of the most common genus in several natural environments, this may be due to distribution of *Aspergillus* genera and form hug number of conidia which able to live in harsh habitat, so that this finding was in agreement with many other studies (7,24,25).

Microbial lipids are the valuable alternative raw materials for the production of biofuel such as biodiesel, and the potential solution for petroleum decrease in the world. Fungi able to accumulation of lipids, hence fungi stored triacylglycerols (TAG) compounds in their cells which important of biodiesel production (2,26,27). Indeed the determination of oleaginous fungi from non-oleaginous fungi by efficient and quick methods is very necessary (6), this fact corresponding with the purpose of current study. Lipase (triacylglycerol acyl hydrolases, EC3.1.1.3) is a biological enzyme using for hydrolysis of triacylglycerols (the important part for biodiesel) to fatty acid (28, 29). Lipase is widely found in fungi (30,31,32) this fact coincides with the current study where 90.9% from fungal isolates can be yielded of lipase. Moreover, confirmation of lipids accretion by inexpensive and rapid technique is necessary to determine the oleaginous fungi from non-oleaginous fungi (6,23,33), achievement of this purpose by use of Sudan black B stain rather than of other high-cost dyes such as Nile red stain which need a fluorescent microscope. On the other hand, by these two techniques the tested oleaginous fungi have proven their ability of lipids accumulation (23%) from their biomass by using natural non-

stimulating medium for the production of lipids , Where these species can be stimulated to produce higher quantities of lipids using more rich enrichment media by increasing carbon sources and reducing nitrogen source (34,35)

Conclusion

The current study showed that the highest frequency and occurrence of *A. terreus*. In addition, the enzymatic technique of lipase production and microscopic pigmentation technique by used Sudan black B constituted a good techniques for screening between oleaginous and non-oleaginous isolates where the production of lipid accumulation for selected fungi reached 23% in a natural medium that was not considered good inducer for lipids accumulation of selected fungal isolates..

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