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Myco-diesel Production by Oleaginous Fungi

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ABSTRACT

The results of isolation and identification of Oleaginous fungi from different kinds of cereals (fenugreek, mustard , Corn Black grain and sesame) from different locations at Baghdad city , the number of fungal genera and species isolated, was varied in the percentage of occurrence and frequency , the highest occurrence percentage of *Penicillium* sp. 50% , 42.8% and 44.4% in samples of cereals corn ,black grain and sesame respectively. Regarding the screening of fungal Isolates for extracellular lipase production on solid agar ranged within producer, weak producer and non-producer . *Aspergillus fumigatus, Trichoderma harizanum ,Penicillium sp. , Aspergillus terreus , Fusarium graminaerum , Aspergillus niger* and *Aspergillus flavus* have the highest activity for producing lipase ,all the seven fungal isolates grew well in liquid medium Yeast Extract Sucrose YES and showed good biomass of the dry weight the highest value 20.15 g / L to *Penicillium* sp. the total lipid contents were 20% or more of their dry weight ,except *A.niger* . Furthermore, qualitative analysis of the lipids contents by Gas Chromatography GC- were identified the presence of Palmitic ,Oleic acids , stearic acid and linoleic acid in all isolates that indicates the fungal oil obtained has properties similar to those of biodiesel .

Keywords: Oleaginous fungi , Myco-diesel , Yeast Extract Sucrose Medium , Transesterfication

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INTRODUCTION

Biodiesel is the most common biofuel in Europe. It is produced from oils or fats using transesterification and is a liquid similar in composition to fossil/mineral diesel. Chemically, it consists mostly of Fatty Acid Methyl (or ethyl) Esters (FAMEs). Feedstocks for biodiesel include animal fats, vegetable oils, and oleaginous microorganisms such as microalgae, bacteria and fungi (Li *et al.,* 2008; Hoekman *et al*., 2012).

Which contain more than 20 % fat per dry biomass as carbon storage. They change a carbon source obtainable in excess into intracellular tri Acyl Glycerol (TAGs) as soon as nitrogen restriction happens (Ratledge, 2004; Ageitos *et al*., 2011).

And have the ability to manufactures and accumulate high quantities of Tri Acyl Glycerols (TAG) inside their cells. This TAG can be simply converted to biodiesel through a process named transesterification through the conversion of methanol and TAG with potassium hydroxide as a catalyst (Socha and Sello,2010).

Oleaginous fungi for biodiesel production were used as feedstock in many researches such as yeast strains *Rhodosporidium* sp., *Rhodotorula* sp. , and *Lipomyces* sp. (Kavadia *et al*., 2001). and from molds some species of genus *Aspergillus* like *Aspergillus terrus* has a wide spectrum in production of biodiesel (Antonio *et al.,* 2013) , also *Trichoderm* spp., *Mucor circinelloides* and *Gliocladium roseum* (Magdum *et al*., 2015) .

Therefore this current study was undertaken to isolatation and identification of oleaginous fungi from different sources then test the ability of some oleaginous fungi for biodiesel or mycodiesel production.

MATERIAL AND METHODS

Isolation and Identification of Oleaginous Fungi from Different kinds of cereals

Samples of different cereals collected from different locations at Baghdad city such as sesame (*Sesamum indicum*), black grain(*Nigella sative*), fenugreek(*Trigonella foenum –graecum*), mustard (*Sinapis alba*), corn(*Zea mays*). The samples were initially subjected for surface sterilization with 0.6 % sodium hypochlorite solution for 2 min and rinsed twice with sterilized distilled water. Samples were dried with sterile filter paper , cultured on Potato Dextrose Agar (PDA) plates supplemented with antibiotic chloramphenicol at 10 pieces of each type of cereals per plate in triplicates and incubated at 30°C for 3-6days. Each fungal colony obtained was then sub cultured on PDA for subsequent characterization and taxonomic identification (Samson *et al*.,2010).

Fungi were identified and classified as depended on taxonomic keys (Raper and Fennell,1965; Simmons , 1967), the percentage of occurrence and frequency of isolation to each isolated fungal species were calculated according to the following formula:-

$$\textbf{\% occurrence of species} = \frac{\text{colonies number of species}}{\text{total number of species colonies}} \times \mathbf{100}$$

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

Screening Fungi Isolated for Lipid Production

A plate detection method containing a chromogenic substrate (Congo red) was used to screen the isolates for lipase production. The sterile medium was prepared and poured plates and allows solidifying. An agar plug (5 mm) was removed from the periphery of 7 days old cultures grown on PDA plates of each fungus , then placed in the center culture medium plates on to triplicate plates containing the screening medium , plates were incubated at 30°C until the fungal growth for 14 days in an incubator . Lipolysis was

indicated by the appearance of clear zone of inhibition around the disc of inoculation. The diameters of the colonies and clearance zones were measured after 14 days (Rees,1997; Haliru *et al* ., 2012).

Determination of Biomass for Oleaginous Fungi

To select the highest lipid producer among them, isolates were cultured in basal medium YES , some tested fungal isolates (produced lipids) was assessed by determining the dry weight of the biomass .Test broth (YES) for each500ml medium were prepared in 1000 ml Erlenmeyer flask and added 5 discs in diameter 5mm for each selected fungal isolate and final pH was adjusted to 6.0,then incubated for 14days at 30ºC, and the biomass growth of fungi was observed on 14days. A clear biomass mat was obtained by filtered using Whatmman No. 2 filter paper inside a biological safety cabinet. The collected biomass was washed twice with distilled water and dried at 60ºC for 24 hours or until constant weight was achieved then the dry weight was estimated gravimetrically mg /L (Lai *et al*., 2004).

Extraction of Fugal Lipid

The lipids were extracted from dried biomass using chloroform, methanol 2:1(v/v) chloroform: methanol Both dried samples of selected fungal isolates were crushed with a mortar and pestle by simultaneously adding (10 ml of chloroform,5 ml of methanol) withdraw 8 ml from this mixture and added to 1gram of biomass was vortexed for 5 minutes and prepare solution saline (7.3g of NaCl ,10ml of water) withdraw 2 ml was added each tube following vortexing for 5 minutes .Then sample tubes were centrifuged at 3000 rpm for 15 minutes and the lower layer of methanol ,water and NaCl was removed by Pasteur pipette , residual of solvent was dried then estimated gravimetrically mg /L and to determine the ratio of extracted lipids in compare to the cell dry weight (Magdum *et al.,* 2015).

Biodiesel Production and Analysis by Gas Chromatography

The fatty acid compositions of the lipid produced by oleaginous fungal isolates that were extracted were determined by analysis of Fatty Acid Methyl Esters (FAMEs) depended upon the method of (Shin *et al.,* 2015) with some modification. The FAMEs were produced by transesterification reaction. 2 ml of methanol with sulfuric acid (2.5% V/V H2SO4/CH3OH) as a catalyst was added to the crude lipid 100 mg. The reaction was progressed for 45 min at 90 C (water bath). Then, 1 mL H2O and 2 mL n-hexane were added. The FAMEs were dissolved into the n-hexane. The solution was centrifuged at 2000 rpm for 15 min to compact the water from the hexane phase containing FAMEs, which was then transferred into glass vials by using Pasteur pipettes. The FAMEs in n-hexane were analyzed using a gas chromatograph after adding 0.1ml of solution (KOH, methanol 11% W/ V) and 1ml heptane to The FAMEs.

RESULTS AND DISCUSSIONS

Isolation and identification of different kinds of cereals , *Aspergillus* spp. and *Penicillum* sp. also were the predominant fungi , *Aspergillus niger* was the highest occurrence percentage about 77.7 % and *Penicillium* sp. 78.9% in samples of cereals fenugreek and mustard respectively while the lowest occurrence percentage were at *Aspergillus niger* and *Aspergillus flavus* about 5.2% both of them in sample of mustard , the highest occurrence percentage of *Penicillium* sp. 50% and 42.8% in samples of cereals corn and black grain respectively Table 1- this might be return to saprophytic nature (live upon dead or decaying organic matter) of these genera beside they possess the ability to grow at different temperatures and high enzymatic capacity. *Aspergillus* spp. were highly aerobic and were found in almost all oxygen-rich environments and usually are preferred saprophytic living on the surface of a substrate, as a result of the high oxygen tension. Commonly, fungi grow on carbon-rich substrates like monosaccharides such as glucose and polysaccharides such as amylose (Geiser, 2009).

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Table 1: The Percentage of Oleaginous Fungi that Isolated From Different Kinds of Cereals

Samples	Type of Fungi	Occurrence%	Frequency %
Fenugreek	*Aspergillus niger*	77.7	14.5
	Aspergillus flavus	11.1	2.08
	Aspergillus fumigatus	11.1	2.08
Corn	*Penicillium* sp.	50	14.5
	Aspergillus niger	14.2	4.16
	Aspergillus flavus	14.2	4.16
	Fusarium graminearum	21.4	6.25
Mustard	*Penicillium* sp.	78.9	10.4
	Aspergillus niger	5.2	2.08
	Aspergillus fumigatus	10.5	4.16
	Aspergillus flavus	5.2	2.08
Black grain	*Aspergillus niger*	28.5	4.16
	Penicillium sp.	42.8	6.25
	Cladosporium sp.	14.2	2.08
	Aspergillus terrus	14.2	2.08
Sesame	*Penicillium* sp.	44.4	8.33
	Pacelomyces sp.	11.1	2.08
	Cladosporium sp.	33.3	6.25
	Aspergillus niger	11.1	2.08

Screening Oleaginous Fungi Isolated for Lipase production on Solid Agar

The screening of fungal isolates for lipase production on Screening lipase medium is shown in Table 2. The lipolytic activity was indicated by the appearance of clear zone of inhibition around the disc of inoculation after 14 days of incubation. The results showed the ability to produce lipase in different types of cereals had varied activity between positive , negative and weak producer, all filamentous fungal isolates in sample of fenugreek gave positive result for three species of *Aspergillus.* While *Aspergillus niger* had a weak producer for lipase production in corn, mustard ,and black grain . The rest of isolates were varied according to the type of cereal and type of species in samples of corn , mustard , black grain and seaseam .

Table 2: Screening of Oleaginous Fungal Isolates in Different Cereals for Lipase Production on Solid Agar. The Symbol (+)Producer and (-) non Producer

Source	Type of fungi	Results for lipase production
Fenugreek	*Aspergillus niger*	+
	Aspergillus flavus	+
	Aspergillus fumigatus	+
Corn	*Penicillium* sp.	+
	Aspergillus niger	Weak producer
	Aspergillus flavus	+
	Fusarium graminearum	+
Mustard	*Penicillium* sp.	+
	Aspergillus niger	Weak producer
	Aspergillus fumigatus	+
	Aspergillus flavus	+
Black grain	*Aspergillus niger*	Weak producer
	Penicillium sp.	Weak producer
	Cladosporium sp.	–
	Aspergillus terrus	+

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Sesame	***Penicillium sp.***	**+**
	Pacelomyces sp.	**+**
	Cladosporium sp.	**Weak producer**
	Aspergillus niger	**_**

Microbial lipases are currently receiving much attention because of their biotechnological potential, such as broad substrate specificity, high yield and low cost production and so on. Therefore, they have been widely used in industrial applications, such as biodiesel production, organic synthesis, food, pharmaceutical, and detergents chemistry (Gupta *et al*. ,2004 ; Singh and Mukhopadhyay ,2012).

Biomass and total lipid yields

To determine the highest bio-mass and lipid yield of selected fungal isolates ,all the seven fungal isolates grew well in 14 days in liquid medium Yeast Extract Sucrose YES and showed good biomass under the given carbon rich, nitrogen limiting conditions. The dry weight of biomass was the lowest value 9.99 g / L to *A. niger* and the highest value 20.15 g / L to *Penicillium* sp. (Figure 1). The total lipid from the biomass was extracted and estimated (Figure 2). The highest yield of lipids was 9.75 g / L to *A.terreus* and the lowest yield of lipids was 1.29 g / L to *A.niger* .

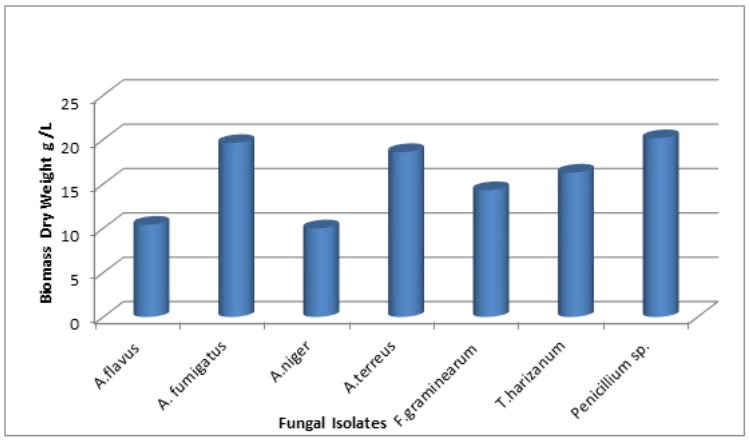

Figure 1:Fungal biomass Dry Weight g / L in Liquid Medium (YES) at pH 7 and Incubated for 14 days, 120 rpm at 30° C.

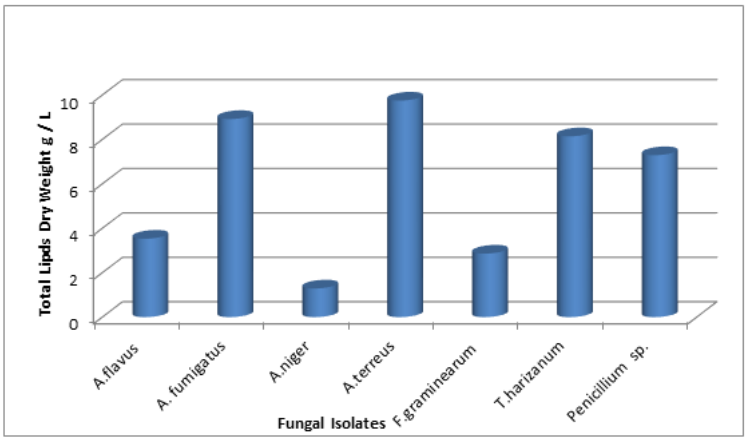

Figure 2: Total Lipid Dry Weight (g / L) in Liquid Medium (YES) at pH 7 and Incubated for 14 days, 120 rpm at 30° C

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As shown in Table -6 the Lipids content of fungal isolates *A.flavus* , *A.fumigatus* , *A. terreus* , *T.harizanum* , *Penicillium* sp. and *F.graminearum* were found to be oleaginous fungi , as the total lipid contents were 20% or more of their dry weight while *A.niger* was not oleaginous fungi the total lipid contents was 12.91% . The development of techniques to produce Single Cell Oil (SCO) by using oleaginous microorganisms such as fungi , bacteria and microalgae had triggered significant attention (Azocar *et al.,* 2010 ;Abu-Elreesh and Ad-El-Haleem , 2013) .

These microorganisms accumulate lipids, mostly in the form of Tri Acyl Glycerols (TAG) which is consider as reserve compounds in all Eukaryotic organisms like fungi, plants and animals, several species of fungi are able to accumulate significant amounts of intracellular lipid (somasekhar *et al.*, 2003) .

Table 3:The Best Biomass Productivity, Lipid Content and Lipid Percentage of the Different Fungal Isolates

Fungal Isolates	Biomass dry weight (g/L)	Total Lipids dry weight (g/L)	Total Lipids percentage to Biomass dry weight (%)
A.flavus	10.4	3.53	33.94
A.fumigatus	19.61	8.92	46.55
A.niger	9.99	1.29	12.91
A. terreus	18.59	9.75	52.44
F.graminearum	14.30	2.86	20.13
T.harizanum	16.28	8.14	50
Penicillium sp.	20.15	7.29	36.17

The selective oleaginous fungal isolates were grown in basal medium YES containing sucrose and yeast extract as carbon and nitrogen sources respectively ,and gave the best biomass of dry weight while the productivity of lipid was limited in six isolates among seven selected isolates ,this study is agreement with (Abu Elreesh and Abd- El-Haleem ,2014) who revealed the basal medium containing glucose and yeast extract was giving the best biomass of dry weight and lipid production after 7 days of incubation. Likewise (Ozsoy *et al.,*2015) showed that sugar beet pulp was a good substrate to cultivate *Mucor circinelloides* with high biomass of dry weight yield and oil yield.

Biodiesel production and analysis by Gas Chromatography –GC

In order to compare the potential utilities of the extracted total lipid as biodiesel feedstock , fatty acid composition FAME of the six selected oleaginous fungal isolates *A.fumigatus* , *A. terreus* , *A. flavus* , *T.harizanum*, *Penicillium sp*. and *F. graminearum* in liquid medium YES which were extracted by acid methanolysis during transesterfication process of fungal lipids extract to Fatty Acid Methyl Esters (FAMEs) which were determined by Gas Chromatography –GC .As shown in Table 4 ,fatty acid profiles by GC with Retention Time RT showed the presence of Palmitic acid and Oleic acid mostly in all selective oleaginous fungal isolates on YES medium which gave the highest concentrations among the others types of fatty acids Stearic acid , Linoleic and Myristic acid .

Table 4: Fatty Acid Composition of Extracted Total Lipids From Selected Oleaginous Fungal Isolates on YES Medium by GC

Fatty acids (FA)	% the extracted total lipids by GC of selected oleaginous fungal isolates						R.T
	A. fumigatus	*A. Terreus*	*T. harizanum*	*P.* sp.	*A. flavus*	*F. graminearum*	
Palmitic C16	65.37	15.26	36.41	20.41	16.31	17.39	4.80
Palmitolic C16.1	2.93	_	_	_	_		5.26
Stearic C18	27.19	_	_	_	_	2.59	8.49
Oleic C18.1	_	47.00	63.58	65.89	71.54	44.90	9.02
Linoleic C18.2	_	37.73	_	13.695	12.13	35.11	10.39

Higher number of Saturated Fatty Acid (SFA) than UnSaturated Fatty Acid (USFA) in the FAME analysis indicates that the fungal oil obtained has properties similar to those of biodiesel and the saturated forms give more favorable properties of biodiesel and for optimized biodiesel , it should contain both long chain saturated and poly unsaturated fatty acids, thus indicating the produced oil suitability for high quality biodiesel production (Papanikolaou *et al*., 2004 ; Wu *et al.,* 2010) the results are in agreement with many studies (Ziino *et al.,* 1999; Abu-Elreesh and Abd-El-Haleem , 2014 ; Subhash and Mohan , 2014).

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