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Promising oleaginous filamentous fungi as biodiesel feed stocks: Screening and identification

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ABSTRACT

Some fungi exhibit the capacity to accumulate intracellular lipids in excess of 70 percent of their biomass during metabolic stress periods. Using Nile-red viable colony staining assay, several fungal isolates collected from different Egyptian ecosystems were screened for its lipid production. Four isolates F1, F2, F3 and F4 were identified as promising lipid producers. Lipid production was performed using basal based medium containing glucose and yeast extract as carbon and nitrogen sources. The maximum lipid contents were 50, 49, 46 and 71% of isolates F1, F2, F3 and F4, respectively. Both morphological and molecular examination identified the isolates F1, F2, F3 and F4 as Drechslera sp., Fusarium sp., Fusarium sp. and Aspergillus fumigates, respectively. Identification and determination of produced fatty acids by GC/MS revealed the presence of long chain fatty acids. Fatty acid profiles showed presence of hexadecanoic acid, octdecanoic acid, and 11-octadecenoic acid in all isolates, while 9, 12-octadecadienoic acid was present only in the lipids of strain F4 with percentage value 40%. In conclusion, this work revealed the possibility of using the promising fungal isolates in biodiesel production.

Key words: Biodiesel, Lipids, oleaginous, fungi

INTRODUCTION

Because of the environmental and health disadvantages resulting from the use of the unsustainable fossil fuels, finding new energy alternatives should be clean, sustainable, easily available and environment-friendly is the main goal of many researchers in recent years around the world [1]. Among the various natural alternatives to fossil fuels, biodiesel constitutes a renewable one that is compatible with current commercial diesel engines and has clear benefits relative to diesel fuel including enhanced biodegradation, reduced toxicity and a lower emission profile [2]. However, the high costs of the biodiesel raw materials (edible oils) greatly restrict the expansion of its production on a large scale. In fact, this problem opened a significant debate about the dilemma of the use of edible crops in the production of biofuels in general and biodiesel in particular. That's where there is general agreement that the use of such crops in production of biofuels is a threat to the global food security. Therefore, it is necessary to explore new raw materials that reduce the biodiesel price without competing with food production [3].

In this concern, microbial lipids can represent a valuable alternative feedstock for biodiesel production, and a potential solution for a bio-based economy. Recently, the development of processes to produce single cell oil (SCO) by using oleaginous microorganisms has triggered significant attention [4, 5]. These organisms accumulate lipids, mostly in the form of triacylglycerols (TAG). The occurrence of TAG as reserve compounds is widespread among all eukaryotic organisms such as fungi, plants and animals, where it was rarely described in bacteria [6].

In this context, fungi are an attractive source of lipids for use in biodiesel synthesis [7, 8]. Several species of fungi are able to accumulate significant amounts of intracellular lipid [9]; this lipid production can be optimized by adding supplementary nutrients to culture media and/or by altering culture conditions during growth [10]. Where, biomass-based biofuel production represents the most important approach to face high energy prices and the potential depletion of crude oils reservoirs, to reduce greenhouse gas emissions, and to enhance sustainable economy [11]. So, oleaginous filamentous fungi are suggested as a favorable feedstock for a sustainable biodiesel industry [12, 13].

Consequently, there is a need to identify new filamentous fungi with a high lipid-producing ability and improve its biodiesel production efficiency. So, the main objectives of the present study were: (i) isolation and identification of filamentous fungi capable of yielding high amount of storage lipids from different Egyptian ecosystems, (ii) analysis of fatty acid profiles of the different positive isolates and testing their potential utilities as biodiesel feedstock.

MATERIALS AND METHODS

2.1 Samples collection and fungi isolation

Three soil samples from Bani-Sweif, Qena, Asyut governorate, and one water sample from river Nile at El-Minya governorate, Egypt were collected. Samples were collected 5-15 cm below the surface and then were stored at 4°C until use. About 1 g of each soil sample was individually suspended in 1 mL of sterile distilled water, then soil and water samples were serially diluted to 10 fold and plated on yeast peptone dextrose (DOX) agar plates (in g/L: D-xylose 100, yeast extract 1, KH_2PO_4 2.0, $MgSO_4.7H_2O$ 0.75, Na_2HPO_4 1, $CaCl_2.2H_2O$ 0.2, $FeCl_3$ 0.01, $ZnCl_2$) with the initial pH 6 and supplemented with 50 mg/L Rose Bengal (4,5,6-Tetrachlorofluorescein) and 100 mg/L chloramphenicol. The plates were incubated at 30°C for 7 days in an incubator. Several fungal colonies were obtained from different plates and it was purified by single colony transferred repeatedly to a new agar plate until pure cultures were confirmed.

2.2 Screening for oleaginous fungi

After reaching to the pure culture, fungi were allowed to grow for 3 days. So, the amount of oleaginous microorganisms and the content of lipid accumulated could reach a certain level. Subsequently, the isolates that appeared earlier and grew the fastest were picked to visualize the intracellular lipids inside fungal cells. Fungal biomass was stored in a dark with 0.5 mL PBS solution and 0.05 mL Nile-red solution (Nile red 25 µg Nile-red/ acetone 1000 mL) for 30 min [14]. Then, stained lipid bodies were photographed using fluorescence microscope (IX-70, Olympus, Tokyo, Japan) equipped with a CCD camera (U-CMT, Olympus, Tokyo, Japan).

2.3 Classical and molecular identification of the fungal isolates

Observing the morphologic characteristics (color, texture appearance, and diameter of the colonies) and microscopic characteristics were performed to identify fungal isolates cultivated on potato dextrose agar medium as described previously [15]. To identify fungal isolates at the molecular level, the protocol used by Abd-El-Haleem [16] was used. The primers used for the amplification and sequencing of 18S-rRNA-encoding genes were those described by Suh and Nakase [17]. The PCR products were sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems).The sequences were analyzed using the BLAST program (National Centre for Biotechnology Information) to determine the closest available database sequences.

2.4 Biomass production and lipid extraction

To screen and select the highest biodiesel producer among purified fungal isolates, they were cultured in basal medium (in g/L: yeast extract 0.5, $MgSO_4.7H_2O$ 0.4, KH_2PO_4 2.0, $CaCl_2$ 0.5, $CuSO_4$ $5H_2O$ 0.05 and 5% glucose (w/v), with initial pH 6). Flasks were removed every 24 hours for seven days and microbial cells were harvested from the media by centrifugation and washed with distilled water three times, then freeze dried at –50°C. Exacted weight was taken, and then total lipids were extracted from the dried biomass with chloroform: methanol, volume ratio of 2:1. Ultrasonication to favor cell membrane disruption during extraction was done. The mixture containing extracted lipids was separated from residual biomass by centrifugation and the solvent fraction was transferred to a new tube. Then the residual of solvent was removed in a rotary evaporator followed by lyophilization to determine the ratio of extracted lipids in compare to the cell dry weight.

2.5 Biodiesel production and analysis by gas chromatography.

Microbial oil was extracted as mentioned above before transesterfication. The transesterfication reactions were carried out using sulfuric acid as catalyst in flasks at following conditions: 30:1 molar ratio of methanol to oil, 160 rpm, 5 h of reaction time, temperature at 55°C and 80% catalyst amount based on oil weight [18, 19]. The reaction mixture was cooled and undisturbed until two layers were formed in a separating funnel. The upper layer (biodiesel) was separated with petroleum ether and the final biodiesel product was obtained by evaporating the ether from the solution. The fatty acid methyl esters of biodiesel were analyzed by GC/MS. It was performed with Agilent 6890N

Gas Chromatograph connected to Agilent 5973 Mass Spectrometer at 70 eV (m/z 50–550; source at 230 °C and quadruple at 150 °C) in the EI mode with an HP-5ms capillary column (30 m ´ 0.25 mm i.d., 0.25 mm film thickness; J & W Scientific, USA). The carrier gas, helium, was maintained at a flow rate of 1.0 mL/min. The inlet temperature was maintained at 300 °C and the oven was programmed for 2 min at 150 °C, then increased to 300 °C at 4 °C/min, and maintained for 20 min at 300 °C. The injection volume was 1 mL, with a split ratio of 50:1. Structural assignments were based on interpretation of mass spectrometric fragmentation and confirmed by comparison of retention times as well as fragmentation pattern of authentic compounds and the spectral data obtained from the Wiley and NIST libraries.

RESULTS AND DISCUSSION

Some works have dealt with the use of oleaginous microorganisms for biodiesel production. However, utilization of oleaginous filamentous fungi as biodiesel producers has a recent history, which derives from studies focused to poly-unsaturated fatty acid production (PUFA) [3, 8, 20]. In this context, this work described isolation, screening and characterization of oleaginous fungi for biodiesel production.

3.1. Screening fungal isolates for total lipids production

Nile-red viable colony staining assay was used to screen for biodiesel producers. Previously, some works reported that Nile-red stain is emitting strongly positive red fluorescence signals only with hydrophobic compounds like lipids and intended to show any lipid particles inside the cells and could be detected by fluorescence spectroscopy [14, 21]. Figure 1 (A, B, C, D) showed that among all fungal isolates four of them F1 (Bani-Sweif governorate), F2 (Qena governorate), F3 (Asyut governorate) and F4 (El-Minya governorate) where the most powerful biodiesel producers. These results were confirmed through the optical microscopic observation. In addition, the presence of lipid granules inside the stained fungal cells was proofed by the appearance of strong fluorescence signals emitted from their stained lipidic organelles with different diameter under fluorescence microscopy.

3.2. Identification of the fungal isolates

Fungal taxonomy is traditionally based on comparative morphological features [22]. However, special caution should be taken when closely related or morphologically similar endophytes are identified, because the morphological characteristics of some fungi are medium dependent and cultural conditions can substantially affect vegetative and sexual compatibility [23]. In contrast, molecular techniques exhibit high sensitivity and specificity for identifying microorganisms and can be used for classifying microbial strains at diverse hierarchical taxonomic levels [24]. As shown in Figure 1, the observed macroscopic characters of the four selected fungal isolates F1, F2, F3 and F4 identified them as *Drechslera* sp., *Fusarium* sp., *Fusarium* sp. and *Aspergillus fumigates*, respectively. In the same line, 18S rDNA homology search values of the four isolates confirmed the morphological characteristics and showed similarity (>99%) with the following fungi *Drechslera nobleae*, *Fusarium solani*, *Fusarium neocosmosporiellum* and *Aspergillus fumigates*, respectively. Their GenBank accession numbers and the most relative published sequences are presented in Table 1.

Table 1: Similarities between isolates of the present study with its accession numbers and the most relative published sequences in the GenBank

No.	isolates	Accession number	Nucleotide length	Closest published species with its accession numbers	Similarity (%)
1	F1	KF703438	425	*Drechslera nobleae*, JN940959	99
2	F2	KF703439	479	*Fusarium solani*, JQ837837	99
3	F3	KF703440	507	*Fusarium neocosmosporiellum*, AB302198	99
4	F4	KF703441	486	*Aspergillus fumigates*, HM590663	99

3.3. Biomass production and lipid accumulation

It is known from the previous studies that the best carbon source for lipid accumulation in fungi is glucose under limitation of nitrogen [25, 26]. In the present study, fungal cells were grown in basal media containing glucose and yeast extract as carbon and nitrogen sources, respectively. All grown flasks were exposed to the same conditions, and three separate flasks were inoculated from the same culture in each case.

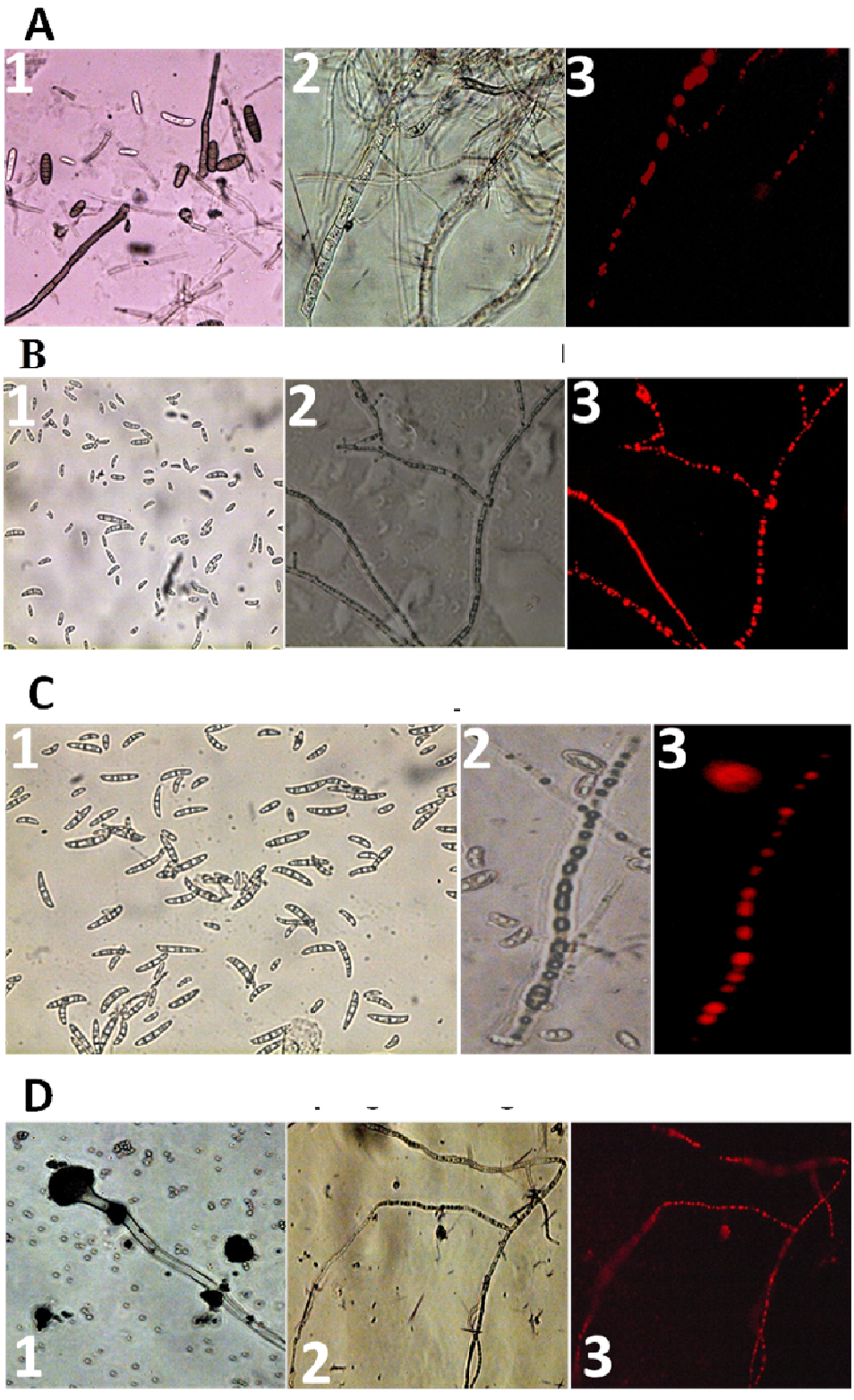

Figure1: Shows the morphological characteristics (A1, B1, C1, D1), phase contrast and Nile red stained cells by fluorescent microscopy to the same field (A2,3- B2,3-C2,3-D2,3) of the four positive isolates F1, F2, F3 and F4 respectively.

As illustrated in Figure 2, it was observed that the growth patterns behavior of strain F1 & F4 and F2 & F3 were getting closer in the way of growth. It was also noted that strains F 1, F2 and F3 reached their maximum amount of biomass (~ 8-10 g/l) after 7 days of incubation, while strain F4 reached its maximum growth rate (~ 9 g /l biomass) only after four days of incubation. The total lipid patterns of strain F4 was also differ from the patterns of strain F1,

F2 and F3 (Figure 3). The maximum yield of lipids was obtained after 6 days of incubation with strains F1, F2 and F4 and after 5 days with strain F3. This may be due to the fact that in case of high carbon and nitrogen ratio, glucose did not get exhausted, and biomass accumulation continued throughout the first four days [26].

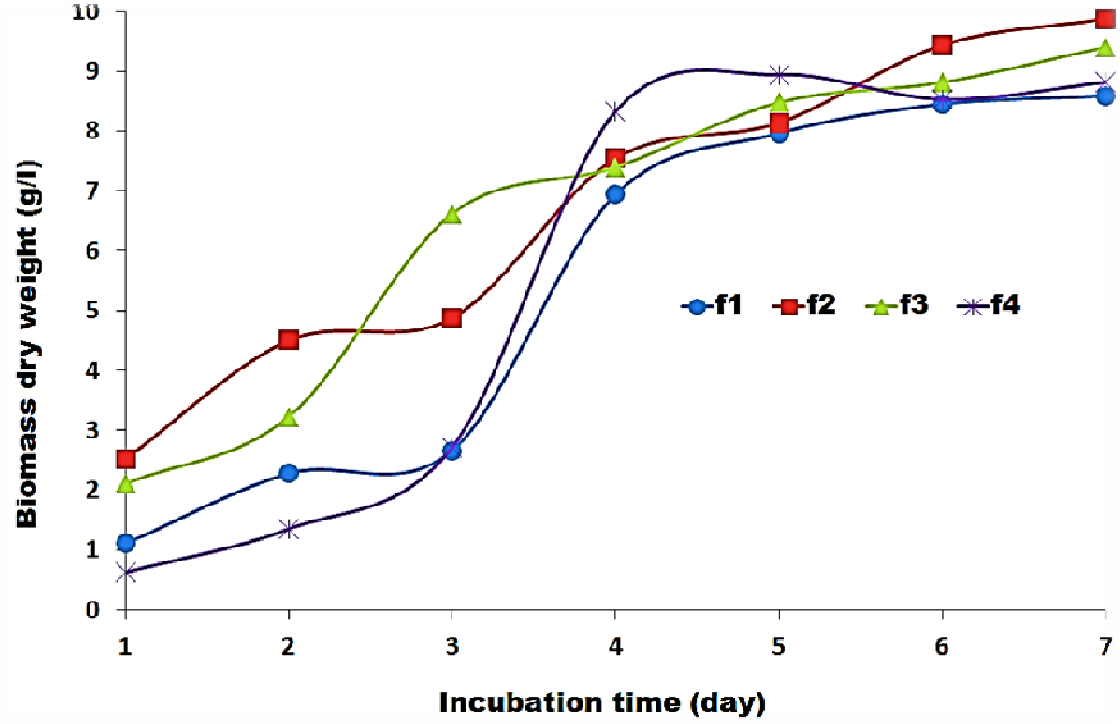

Figure2: Shows the growth curve of the four positive isolates on basal medium for 7th days of incubation

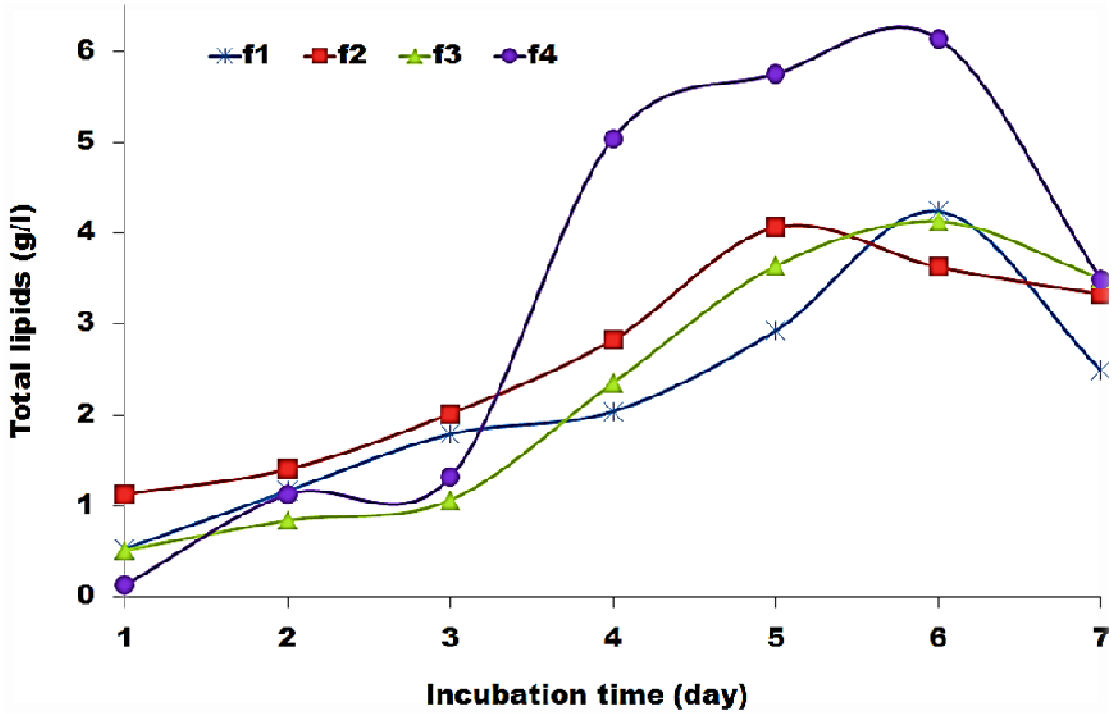

Figure3: Time course of lipid yield of the four different positive fungal isolates grown in basal medium containing glucose

As shown in Table 2, the lipid content of strains F1, F2 and F3 was 50, 49 and 46% (lipid/cell dry weight), respectively. However, 71% of lipid content was achieved with strain F4. similar pattern of performance with respect to biomass production and lipid yield with *Alternaria* sp. and *Aspergillus* sp. was recorded [26, 27], where, the lipid yield was found to be as high as 7.8 g/L and 3.1 g/L corresponding to the highest biomass production of

14.6 g/L and 13.6 g/L, respectively. Also, 4.17 g/L in 96 h and 13.6 g/L in 48 h with a lipid productivity of 23% and 23.3% for *Mucor circinelloides* MU241, and *Aspergillus* sp. , respectively. While a SCO yield of 53% (w/w) of dry cell mass was obtained for *Mortierella isabellina*, when grown on glucose with concentrations ranging from 2% to 7% [28, 29].

Table 2: shows the best biomass productivity, lipid content and lipid percentage of the four different positive fungal isolates

No.	isolates	Biomass dry weight (g/l)	Total lipids dry weight (g/l)	Total lipids percentage to biomass dry weight (%)
1	F1	8.45	4.225	50
2	F2	8.14	4.062	49
3	F3	8.815	4.125	46
4	F4	8.53	6.137	71

3.4 Biodiesel production and analysis by gas chromatography

In order to compare the potential utilities of the extracted total lipids as biodiesel feedstock, fatty acid composition (FAME) of the four strains were extracted by acid methanolysis and its profile were determined by GC/MS [30]. As presented in Table 3, fatty acid profiles showed presence of hexadecanoic acid, octdecanoic acid, and 11-octadecenoic acid in all isolates. However, phthalic acid and tridecanoic acid was absent in the lipids of strains F1, F2 and F3, while 9,12-octadecadienoic acid was present only in the lipids of strain F4 with percentage value 40%. The major fatty acids in the lipids of strains F1, F2 and F3 was hexadecanoic acid followed by 11-octadecenoic acid exhibiting percentage values of 38, 51, 34 %, and 36, 35, 34%, respectively. This result was in agreement with [31] where hexadecanoic acid plus stearic acid was the most abundant fatty acid isolated from the fungus *Geotrichum*. In addition, [32] found that the lipid fraction of *C. japonica* VKMF was characteristic by octadecenoic acids reaching up to 50% of total fatty acids. Also, [33] showed that the lipid extract of *Fusarium* sp.ML-GEN.1 mainly contained oleic acid (41.66%), palmitic acid (23.26%), linoleic acid (19.18%).

Table 3: Show fatty acid composition and percentage of the extracted total lipids by GC-Mass of the four different positive fungal isolates

	Fatty Acids (FA)	Fungal Isolates				Retention Time	m/z (%)
		F1	F2	F3	F4		
1	Phthalic acid	-	-	-	5	149	-
2	Tridecanoic acid	-	-	-	3	74.0	-
3	Hexadecanoic acid	38	51	34	22	74.0	38
4	Octdecanoic acid	24	12	12	13	74.0	24
5	11-Octadecenoic acid	36	35	34	15	55.0	36
6	9,12-Octadecadienoic acid	-	-	18	40	67.0	-

Interestingly, the fatty acids profile of the four isolates indicates the presence of both saturated and unsaturated forms of fatty acids. Since the presence of unsaturated fatty acids only, show low oxidative stability [34]. Where, saturated forms tend to give more favorable properties of biodiesel, but at the same time the saturated forms has also some disadvantage like relatively poor low-temperature flow properties. However, for optimized biodiesel, it should contain both long-chain saturated and poly-unsaturated fatty acids [35, 36].

CONCLUSION

In the present study, several fungal isolates were screened for its lipid production. Four of them F1, F2, F3 and F4 were identified as promising lipid producers with a productivity reach to 50, 49, 46 and 71% respectively on a basal media containing glucose as a carbon source. In addition, the fungal isolate showed fatty acid profile that has potential utilities for biodiesel production as have been documented for vegetable oils, oleaginous yeasts and fungi.

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