



Evaluating Oleaginous Fungi for Sustainable Biodiesel Production: Screening, Identification and Optimization of Lipid Production

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HEREIN, we assessed oleaginous fungi for biodiesel production. We isolated 10 fungal strains from two petroleum-polluted soil samples and screened their ability to accumulate lipids. *Aspergillus terreus*, *A. niger*, and *A. flavus* were found to be the most potent lipid producers. All isolates were identified at the species level by morphological (macroscopic and microscopic) examination and molecularly confirmed by performing 18S rRNA sequencing. Furthermore, we optimized culture conditions to improve lipid accumulation. *Aspergillus terreus*, the most potent lipid producer, showed the highest lipid production (38.33%) in the presence of 50g/L sucrose and 0.5 g/L ammonium nitrate (initial pH, 6) after seven days of incubation under static conditions. Gas chromatography was performed for fatty acid analysis after lipid transesterification. Fatty acid methyl/ethyl ester profile indicated that saturated fatty acids were more common than polyunsaturated fatty acids. The total concentration of fatty acids was 107.98, 38.29, and 37.48mg/100g of lipids accumulated by *A. terreus*, *A. niger*, and *A. flavus*, respectively. Gas chromatographic analysis of *A. terreus* lipids indicated that oleic acid (C18:1, 18.51%) was the most abundant, followed by stearic acid (C18:0, 15.91%) and myristic acid (C14:0, 14.64%). The fatty acid profile of *A. terreus* confirmed it to be a promising new commercial biodiesel feedstock.

Keywords: Biodiesel, Fatty acid profile, Maximize lipid accumulation, Oleaginous fungi, Promising fungal isolates.

Introduction

There is a growing concern on global warming due to the increased worldwide consumption of fossil fuels, which is associated with the emission of greenhouse gases (Matsakas et al., 2017; Campbell-Lendrum & Prüss-Ustün, 2019). Biodiesel is the most efficient renewable and sustainable substitute for fossil diesel fuel (Khan & Hussain, 2017; Nisar et al., 2017; Mahlia et al., 2020). The use of alternate vegetable oils has been widely reported to be suitable for biofuel production (Kibazohi & Sangwan, 2011; Abdelrazk & Ahmed, 2020). Biofuel production from single cell oils produced

by oleaginous microorganisms, including microalgae, bacteria, molds, and yeasts, has recently garnered considerable attention (Tao et al., 2006; Meng et al., 2009; Ramírez-Castrillón et al., 2017; Papanikolaou, 2019). Oleaginous microorganisms show the ability to accumulate and store 20%–25% of lipids of their total dry biomass (Hu et al., 2009; Athenaki et al., 2018), mostly consisting of triacylglycerols, which form the storage fraction of cells. Microbial lipids (i.e., single cell oils) are a promising feedstock for biodiesel production (Li et al., 2008; Meng et al., 2009; Subramaniam et al., 2010; Hoekman et al., 2012; Abdel-Hamied et al., 2018; Cho & Park, 2018; Dorya et al., 2018; Bharti, 2019).

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Oil production by microorganisms has several advantages; for example, the oil production level is usually higher than that of plants. Furthermore, microorganisms can be easily cultivated in bioreactors, have short life cycles, and show rapid growth rates; besides, certain genetic modifications are easily possible, and their growth is not dependent on light or other climatic variables (Sitepu et al., 2014; Dourou et al., 2018).

Oleaginous filamentous fungi are suggested as a favorable feedstock for a sustainable biodiesel industry (Hoffmeister & Keller, 2007; Peng & Chen, 2008; Zhao et al., 2008; Papanikolaou & Aggelis, 2011; Reis et al., 2019). Many fungal species, including *Aspergillus oryzae*, *A. awamori*, *Mortierella isabellina*, *M. alliacea*, *Humicola lanuginosa*, *Trichoderma reesei*, *Penicillium commune*, and *Mucor circinelloides*, are used for the production of single cell oils and able to accumulate lipids (Li et al., 2008; Rossi et al., 2011; Antonio et al., 2013; Bhanja et al., 2014; Magdum et al., 2015; Hussein et al., 2017; Shafiq & Ali, 2017). Oleaginous fungi use various renewable substrates for lipid accumulation, such as glycerol (Papanikolaou & Aggelis, 2002; Polburee, 2015; Ramírez-Castrillón et al., 2017; Rivaldi et al., 2017), molasses, whey (Economou et al., 2011a; Bellou, 2012; Vieira, 2014; Abdel-Hamied et al., 2018), food industry waste, agro-industrial residues, lignocellulosic biomass, seed oils, and wastewater (Yousuf, 2010; Economou et al., 2011b; Moubasher et al., 2016; Chuppa-Tostain et al., 2018; Dourou et al., 2018; Tsegaye, 2018a, 2018b; Babakur, 2019; Ekas et al., 2019; Shafiq & Chechan, 2019; Campos et al., 2020; Cuevas et al., 2020; Marwa et al., 2020). These fungi most commonly use glucose as the carbon source to enhance growth and lipid accumulation (Saxena et al., 2008; Zhao et al., 2008; Ochsenreither et al., 2016).

The fatty acid (FA) profile of microbial lipids is quite similar to that of conventional vegetable oils (Rude & Schirmer, 2009; Papanikolaou, 2012). Lipids of filamentous fungi show a unique FA profile; they are rich in some valuable polyunsaturated FAs, such as γ -linoleic acid, which cannot be synthesized by other oleaginous microorganisms (Subramaniam et al., 2010; Subhash & Mohan, 2011; Ratledge, 2013). γ -Linoleic acid (18:3) is as an essential FA in humans and has been reported to be effective

in preventing various diseases, including cardiovascular disorders, rheumatoid arthritis, hypercholesterolemia, atopic eczema, and asthma (Murad et al., 2010).

The two main types of biofuels are biodiesel and ethanol, and they are the most environment-friendly alternative fuels (Wormslev, 2016). Biodiesel is increasingly attracting attention across the globe as it is cost effective, eco-friendly, and biodegradable; however, it has a low content of sulfur and aromatic hydrocarbons. Thus, toxic emissions of CO and CO₂ are lower during fuel combustion (Demirbas, 2008; Knothe, 2008; Kotasthan, 2017). Biodiesel involves the mixture of fatty acyl methyl/ethyl esters (FAMES), typically produced from the transesterification of vegetable oils that can be used for existing conventional diesel engines regardless of its origin and feedstock from which it is derived (Alptekin, 2017; Patel et al., 2017).

Herein, we aimed to isolate, identify, and characterize oleaginous fungal species from petroleum-polluted soil samples, estimate lipid content, and study the effects of physical and nutritional parameters to detect and maximize intracellular lipid accumulation of promising fungal strains to enhance the quality of biodiesel. We also investigated FAME composition after fungal oil transesterification. According to the literature (Li et al., 2008; Papanikolaou & Aggelis, 2011; Reis et al., 2019) FAs from potent oleaginous fungi are of significant importance for biodiesel production.

Materials and Methods

Sample collection and fungi isolation

We isolated 10 fungal isolates from two petroleum-polluted soil samples collected from the front of Moharam-Bek and Amrya gas stations in Alexandria, Egypt. The samples were collected in sterile containers and transferred to the laboratory for analyses. To isolate oleaginous fungi, they were serially diluted to 10⁻⁵-fold; 1 mL of this diluted sample was cultured on potato dextrose agar (PDA) supplemented with 2mL gentamicin, followed by incubation at 28°C for 3–6 days (Kumar et al., 2011). The obtained colonies were then morphologically evaluated, and distinct pure colonies were subcultured on PDA slants and stored at 4°C for further analysis.

Screening oleaginous fungal isolates for lipid production and dried biomass

Pure fungal isolates were cultured on basal media: 0.5g/L yeast extract, 0.4g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2g/L KH_2PO_4 , 0.5g/L CaCl_2 , 0.05g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 50g/L glucose. The initial pH was 6 to select the highest lipid producers. A sample of mycelium from the PDA slant was transferred to a tube containing 10mL sterilized distilled water and thoroughly mixed; 0.5mL of this suspension was then collected using a micropipette and added to a 250mL conical flask containing 50mL basal media, followed by incubation at 30°C for 7 days under static conditions. Subsequently, fungal growth was harvested by filtration (Whatman no. 1). The fungal mat on the filter paper was collected and washed thrice with distilled water to remove all media residues. The biomass was dried in a hot air oven at 60°C to constant weight.

Extraction of fungal lipid and determination of total lipid content

Lipids were extracted from the screened fungal isolates using the Bligh and Dyer method (1959), with slight modifications, using a 2:1 ratio of chloroform/methanol (v/v). Then, 100mg of dried biomass was ground using a mortar and pestle. Subsequently, 10mL chloroform and 5 mL methanol were added; 8mL of this solution was added to a known amount of ground dried mycelium and thoroughly mixed by vortexing for 5min. Saline (7.3g NaCl in 10mL distilled water) was prepared, and 2mL saline was added to each sample tube (containing dried mycelium), followed by mixing via vortexing for 5min. The samples were centrifuged at 3000rpm for 15min, and the lower layer of methanol, water, and NaCl was removed using a Pasteur pipette. The residual solvent was dried and the ratio of extracted lipids in compare to the cell dry weight was determined as follows (Magdum et al., 2015).

Percentage of lipid content (%) = $\frac{\text{Weight of lipid (g)}}{\text{weight of dried biomass (g)}} \times 100$

Morphological and molecular identification of the most potent fungal isolates

The most promising lipid producers were morphologically identified based on culture performance and microscopic examination at the Assiut University Mycological Center, Assiut, Egypt (Larone, 2002). The most promising oleaginous fungi were identified on

the basis of the 18S rRNA sequences. Fungal strains were cultivated on PDA at 28°C for 5 days. A small amount of fresh culture was scraped and suspended in 100µL autoclaved distilled water in a sterile 2mL Eppendorf vial, which was then boiled in a water bath at 100°C for 15min. The internal transcribed spacer region of the rRNA gene was amplified using PCR at SolGent Co., Daejeon, South Korea, using two universal primers: ITS1 (forward, 5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (reverse, 5'-TCCTCCGCTTATTGATATGC-3'). The purified PCR products (amplicons) were reconfirmed using a size nucleotide marker (100bp) by electrophoreses (White et al., 1990). The sequences were further analyzed using the Basic Local Alignment Search Tool on the National Center of Biotechnology Information website. A phylogenetic tree was constructed using the neighbor-joining program in MEGA 5.05.

Optimization of culture conditions to maximize lipid production

To enhance biodiesel production by *A. terreus*, *A. niger*, and *A. flavus*, the influence of nutritional and environmental parameters on fungal growth and lipid production was investigated under static conditions.

Effect of carbon and nitrogen sources on lipid production

The effect of different carbon sources on lipid production was assessed by supplementing the basal medium with 50g/L glucose (control). An equimolar amount of the following eight carbon sources was subjected to analyses: fructose, lactose, sucrose, maltose, dextrose, starch, wheat bran, and corn. Regarding nitrogen source, yeast extract was replaced with an equal amount of nitrogen bases (0.5g/L) from six different nitrogen sources (one at a time): ammonium nitrate, sodium nitrate, peptone, ammonium acetate, casein, and soybean.

Effect of pH and incubation period on lipid production

The initial pH of the culture medium was adjusted with 1 N HCl or 1 N NaOH before autoclaving to values ranging from 4 to 9 under static conditions for 7 days. The effect of incubation period on lipid production was studied at different timepoints (3, 5, 7, and 9 days).

Effect of incubation temperature on lipid production

This experiment was carried out to evaluate the effect of different incubation temperatures on lipid production at different incubation temperatures (20°C, 25°C, 30°C, 35°C, and 40°C) under static conditions.

Methylation and transesterification of lipid

Lipid transesterification was performed using the method reported by Radwan (1978), with slight modifications. Lipid samples (5mg) were dissolved in 2mL benzene in a test tube fitted with a condenser, and 1% sulfuric acid in 2mL methanol was then added. The tube was tightly sealed and placed in a water bath at 90°C for 90min. After cooling, 8mL water and 5mL petroleum ether were added, and the ethereal layer was separated in a dry tube.

Gas chromatography (GC)

Gas chromatographic analysis of FAMES for promising fungal isolates was performed on a HP 6890 GC system at the Central Laboratories of General Health Institute, Alexandria, Egypt.

Results and Discussion

Screening oleaginous fungal strains for lipid production

Overall, 10 fungal isolates were obtained: five from Moharam-Bek and five from Amrya gas stations in Alexandria, Egypt. They were tested

for growth and lipid accumulation under static conditions (Table 1). The highest lipid content (24.14%) was achieved by a brown fungal isolate (F1) from Moharam-Bek gas station, followed by a black isolate (F6) from Amrya gas station (12.32%) and an olive-green isolate (F2) from Moharam-Bek gas station (6.25%). Qiao et al. (2018) revealed that *M. circinelloides* showed the highest yield and maximum lipid content under static conditions. These results were congruent with those reported by Ali and El-Ghonemy (2014), Kirrolia et al. (2012), and Pandey et al. (2000), who found that lipid accumulation was higher under static conditions than under shaking conditions. Shafiq & Ali (2017) and Somasekhar et al. (2003) reported that many species of oleaginous fungi were able to accumulate a significant amount of intracellular lipid. The stored intracellular lipid content is used to maintain generations of cells, leading to the production of lipid-free biomass (Park et al., 1990; Subhash & Mohan, 2014). This phenomenon is known as lipid turnover (Fakas et al., 2007; Huang, 2009; Wu et al., 2010).

Morphological and molecular identification of promising fungal isolates

The promising fungal isolates were considered to be potent lipid producers. They were morphologically (macroscopic and microscopic examination) identified at the Assiut University Mycological Center, Assiut, Egypt. The sample coded ARS-1 was *A. niger*, ARS-2 was *A. terreus*, and ARS-3 was *A. flavus* (Fig. 1).

TABLE 1. Screening fungal isolates for oleaginity and fungal biomass determination

Isolate No./ soil source	Colony colour	Dry biomass (g/ 50mL)	Lipid concentration (g/L)	Lipid content (%)
F1/(MS)	Brown	0.29	1.40	24.14
F2/(MS)	Green	0.24	0.30	6.25
F3/(MS)	Black	0.20	0.20	5.00
F4/(MS)	Black	0.21	0.08	1.90
F5/(MS)	Green	0.18	0.10	2.78
F6/(AS)	Black	0.28	0.69	12.32
F7/(AS)	Brown	0.30	0.22	3.67
F8/(AS)	Green	0.20	0.15	3.75
F9/(AS)	Brown	0.30	0.32	5.33
F10/(AS)	Green	0.17	0.12	3.53

MS = Moharam-Bek Station, AS = Amrya Station

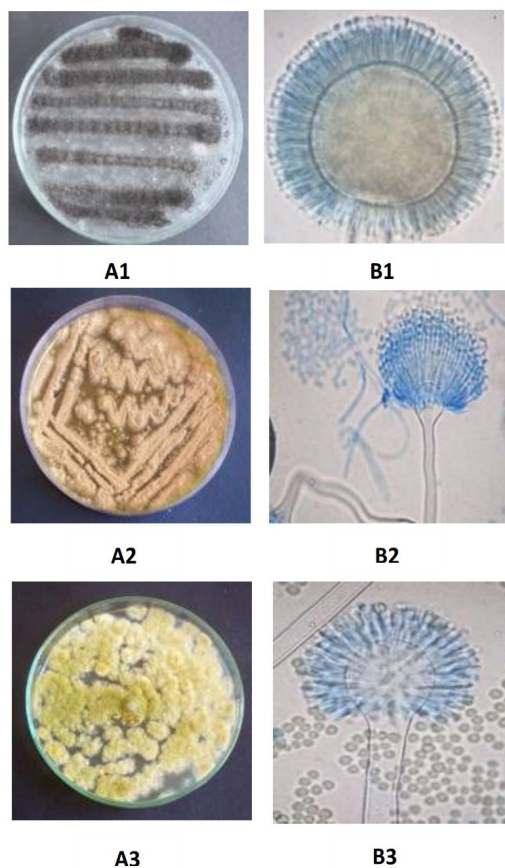


Fig. 1. Oleaginous fungi (A) in PDA medium and (B) on microscopy with magnification of 1000x: (ARS- A1) *A. niger*, (ARS-A2) *A. terreus*, and (ARSA-3) *A. flavus*.

The molecular identification of fungi and assessment of similarities with closely related fungal strains were achieved using the Basic Local Alignment Search Tool. The 18S rRNA sequence and its homologous sequences were analyzed using MegAlign (DNASar) 5.05. A phylogenetic tree was constructed after alignment with related strains with percentage of similarity (Fig. 2).

Optimization of culture conditions to maximize lipid production

Effect of different carbon sources on lipid production

As evident from Table 2, *A. terreus* produced the maximum amount of lipid (31.5%) in the presence of sucrose as the sole source of carbon, while wheat bran led to the highest dry biomass (0.48g/50mL). *Aspergillus niger* and *A. flavus* effectively used glucose, followed by lactose, as the sole source of carbon to produce the maximum amount of lipid (12.32% and

8.33%, respectively). Baqir et al. (1997), Abu-Elreesh & Abd-El-Haleem (2014), El-Haj et al. (2015), Al-Hawash et al. (2018), Carvalho et al. (2018), and Assawah et al. (2020) reported that glucose serves as the best source of carbon to yield maximum lipid production by *Aspergillus* spp., and this finding is in agreement with our data pertaining to *A. niger*. On the other hand, Abdelhamid et al. (2019) found that *P. commune* produced the maximum amount of lipid (34.92%) in the presence of xylose.

Effect of different nitrogen sources on lipid production

As evident from Table 3, the highest values for biomass and lipid content (0.2g/50mL and 38.33%, respectively) were achieved by *A. terreus* in the presence of ammonium nitrate. In contrast, *A. niger* produced the maximum amount of lipid (12.32%) in the presence of yeast extract. These data were congruent with those reported by Abu-Elreesh & Abd-El-Haleem (2014). On the other hand, Xing et al. (2012) and Ramírez-Castrillón et al. (2017) found that ammonium sulfate was the most suitable nitrogen source. Besides, Abdelhamid et al. (2019) observed that *P. commune* produced the maximum amount of lipids (43.06%) in the presence of peptone.

Effect of initial medium pH on lipid production

The external pH of the medium is an important environmental factor affecting plasma membrane permeability, metabolic activity, and cell growth (Amanullah et al., 2001; Minhas et al., 2016). In the present study, the highest values of biomass and lipid production were obtained at pH 6 for *A. terreus* (0.24g/50mL and 38.33%, respectively) and *A. flavus* (0.19g/50mL and 16.84%, respectively) (Table 4). Consistent with our data, Sukrutha et al. (2014) found that *Cunninghamella blakesleeana* showed maximum lipid production at pH 6. Furthermore, *A. niger* showed the highest lipid production (14.17%) at pH 7, i.e., the initial pH of the medium. This finding was in line with that reported by Abdelhamid et al. (2019), who found that *P. commune* showed maximum lipid production (33.16%) at pH 7. Similar observations were reported by Ali & El-Ghonemy (2014), Ruan et al. (2014), Ali et al. (2017), and Jiru et al. (2017), who found pH values ranging between 5 and 6 to be optimal for the growth of and lipid production by most oleaginous fungi.

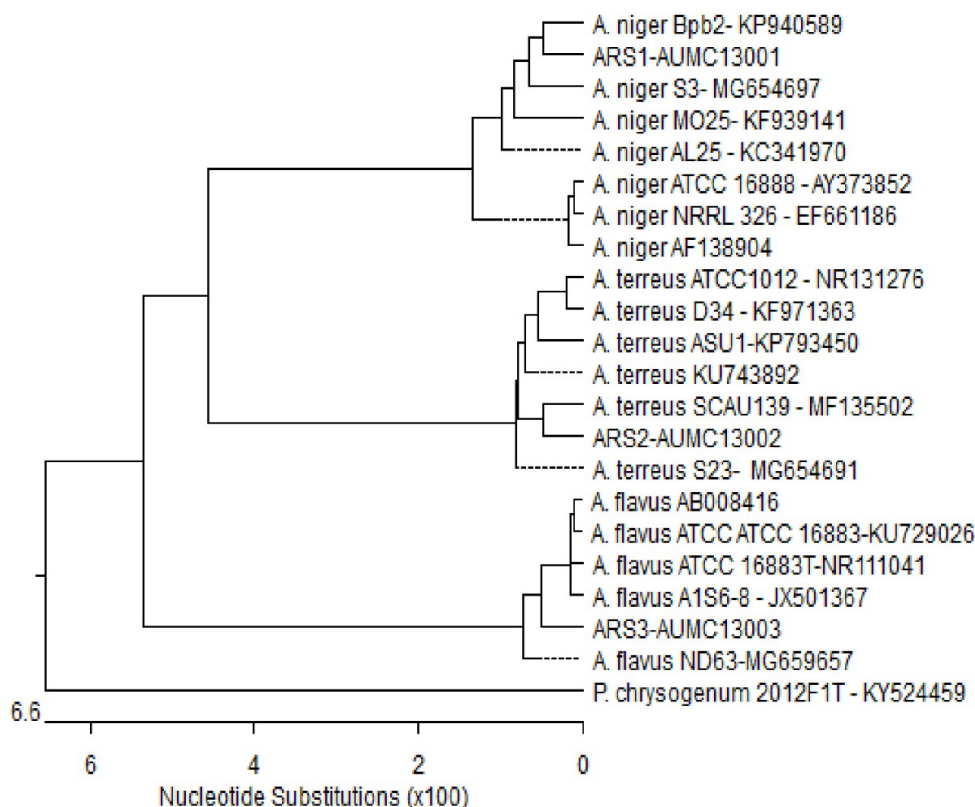


Fig. 2. Phylogenetic tree of 18S rRNA sequences of fungal strains (ARS-1, ARS-2 and ARS-3) aligned with closely related sequences accessed from the GenBank [A.= *Aspergillus*, P.= *Penicillium*) *P. chrysogenum* was included as out-group strain]

TABLE 2. Effect of different carbon sources on lipid production

Carbon source	<i>A. niger</i>			<i>A. flavus</i>			<i>A. terreus</i>		
	Biomass (g/ 50L)	Lipid concentration (g/L)	Lipid content (%)	Biomass (g/50mL)	Lipid concentration (g /L)	Lipid content (%)	Biomass (g/50mL)	Lipid concentration (g/L)	Lipid content (%)
Glucose	0.28	0.69	12.32	0.24	0.30	6.25	0.29	1.40	24.14
Fructose	0.22	0.36	8.18	0.22	0.34	7.73	0.24	1.26	26.25
Lactose	0.11	0.15	6.82	0.24	0.40	8.33	0.41	1.69	20.61
Sucrose	0.14	0.22	7.86	0.30	0.29	4.83	0.20	1.26	31.50
Maltose	0.27	0.18	3.33	0.26	0.10	1.92	0.22	0.32	7.27
Dextrose	0.24	0.49	10.21	0.29	0.38	6.55	0.38	1.66	21.84
Starch	0.20	0.30	7.5	0.11	0.15	6.82	0.12	0.29	12.08
Wheat bran	0.21	0.15	3.57	0.21	0.15	3.57	0.48	0.72	7.50
Corn	0.08	0.10	6.25	0.17	0.07	2.06	0.29	1.04	17.93

Influence of incubation period on lipid production

The lipid content of each strain differs depending on its growth rate. In this study, lipid accumulation and biomass gradually increased during the first 7 days of incubation, reaching maximum values at the seventh

day of incubation for *A. terreus* (38.33% and 0.24g/50mL, respectively) and *A. flavus* (16.84% and 0.19g/50mL, respectively) (Table 5). These results were in accordance with those reported by Sukrutha et al. (2014), who investigated maximum lipid production by *C. blakesleeana*, which was

found to be 28% after 6 days of static incubation. Our results pertaining to *A. niger* were similar to those reported by Kumar & Banerjee (2013) and Ali et al. (2017); they found that *Aspergillus* spp. showed maximum lipid production after 5 days of incubation. In addition, Abdelhamid et al. (2019) found that *P. commune* showed the highest lipid production (46.36%) after the fifth day of incubation.

Influence of incubation temperature on lipid production

The highest fungal biomass and lipid production were observed at 30°C for all promising fungal strains. The maximum lipid production (38.33%) was achieved by *A. terreus* at 30°C, and this finding was in accordance with that reported by Carlile et al. (2001), Li et al. (2008), Ali and El-Ghonemy (2014), and Subhash & Mohan (2014). Patel & Desai (2019) found 32°C to be the optimal incubation temperature for extracellular enzyme production for polysaccharide degradation and lipid accumulation by fungi, and Abdelhamid et al. (2019) reported that *P. commune* showed the highest lipid accumulation (41.18%) at 26°C.

FAME profile and GC of promising lipid producers

All fungi showed qualitative and quantitative differences in their lipid profiles. GC led to the identification of transesterified FAs; however, oleic acid and linoleic acid were not found in the FA profile of *A. niger*. Our data (Fig. 3) indicated the presence of a higher fraction of saturated FAs than unsaturated FAs, which is a potential feature indicating the fuel quality of fungal based diesel

(Dai et al., 2007; Subhash & Mohan, 2011; Zheng et al., 2012; Subhash & Mohan, 2014; Shafiq, 2017; Shafiq & Chechan, 2019). The current FAME profile is in agreement to the commonly used vegetable oil feedstock for biodiesel such as soybean, rapeseed, palm and sunflower (Christophe et al., 2012). The total concentration of FAs was 107.98, 38.29, and 37.48mg/100 g of lipids generated by *A. terreus*, *A. niger*, and *A. flavus*, respectively.

Fatty acid composition of potent fungal lipid producers

The FA composition of *A. terreus*, the most potent fungal isolate, is presented in Fig. 4 and Table 6. GC indicated the presence of a high proportion of long-chain FAs, which is composed of diverse saturated FAs, such as 15.91% stearic acid (C18:0), 14.64% myristic acid (C14:0), 10.92% palmitic acid (C16:0), 10.61% tridecanoic acid (C13:0), and 8.78% pentadecenoic acid (C15:0). A limited percentage of mono-[e.g., 18.51% oleic acid (C18:1) and 4.59% pentadecenoic acid (C15:1)] and polyunsaturated [e.g., 13.2% linoleic acid (C18:2)] FAs was found. A higher percentage of saturated FAs (60.86%) than unsaturated FAs (36.3%) was estimated. These results were in agreement with those of Farias et al. (2018) who reported similar data (saturated FAs, 70.7%; unsaturated FAs, 29.3%). The total saturated FA content (60.86%) was higher than that found in plant oils, such as palm oil (44%), jatropha oil (21.52%), and soybean oil (15%) (Vyas & Chhabra, 2017), indicative of high-quality biodiesel.

TABLE 3. Effect of various nitrogen sources on lipid production

Nitrogen sources	Fungal strain	<i>A. niger</i>			<i>A. flavus</i>		<i>A. terreus</i>			
		Biomass (g/50mL)	Lipid concentration (g/L)	Lipid content (%)	Biomass (g/50 mL)	Lipid concentration (g/L)	Lipid content (%)	Biomass (g/50mL)	Lipid concentration (g/L)	Lipid content (%)
Ammonium nitrate		0.38	0.66	8.68	0.11	0.22	10.00	0.24	1.84	38.33
Sodium nitrate		0.26	0.48	9.23	0.19	0.64	16.84	0.23	1.69	36.74
Peptone		0.42	0.55	6.55	0.26	0.38	7.31	0.21	0.79	18.81
Ammonium acetate		0.11	0.21	9.55	0.20	0.35	8.75	0.10	0.40	20.00
Casein		0.20	0.33	8.25	0.10	0.28	14.00	0.19	0.61	16.05
Yeast extract		0.28	0.69	12.32	0.24	0.40	8.33	0.20	1.26	31.50
Soybean		0.19	0.33	8.68	0.10	0.23	11.50	0.18	1.11	30.83

TABLE 4. Effect of initial pH on lipid production

pH value	Fungal strain	<i>A. niger</i>			<i>A. flavus</i>			<i>A. terreus</i>		
		Biomass (g/50mL)	Lipid concentration (g/L)	Lipid content (%)	Biomass (g/50mL)	Lipid concentration (g/L)	Lipid content (%)	Biomass (g/50mL)	Lipid concentration (g/L)	Lipid content (%)
4		0.08	0.10	6.25	0.08	0.12	7.51	0.15	0.25	8.33
5		0.10	0.17	8.50	0.14	0.39	13.93	0.17	0.94	27.65
6		0.28	0.69	12.32	0.19	0.64	16.84	0.24	1.84	38.33
7		0.24	0.68	14.17	0.15	0.42	14.02	0.21	1.22	29.04
8		0.24	0.52	10.83	0.11	0.22	10.00	0.19	0.72	18.95
9		0.13	0.14	5.38	0.07	0.09	6.43	0.13	0.21	8.08

TABLE 5. Effect of incubation period on lipid production by promising fungal strains

Incubation period (day)	Fungal strain	<i>A. niger</i>			<i>A. flavus</i>			<i>A. terreus</i>		
		Biomass (g/50mL)	Lipid concentration (g/L)	Lipid content (%)	Biomass (g/50 mL)	Lipid concentration (g/L)	Lipid content (%)	Biomass (g/50mL)	Lipid concentration (g/L)	Lipid content (%)
3		0.06	0.1	8.33	0.09	0.12	6.67	0.06	0.11	9.17
5		0.15	0.56	18.67	0.18	0.48	13.33	0.16	0.75	23.44
7		0.24	0.68	14.17	0.19	0.64	16.84	0.24	1.84	38.33
9		0.28	0.31	5.54	0.19	0.37	9.74	0.23	1.33	28.91

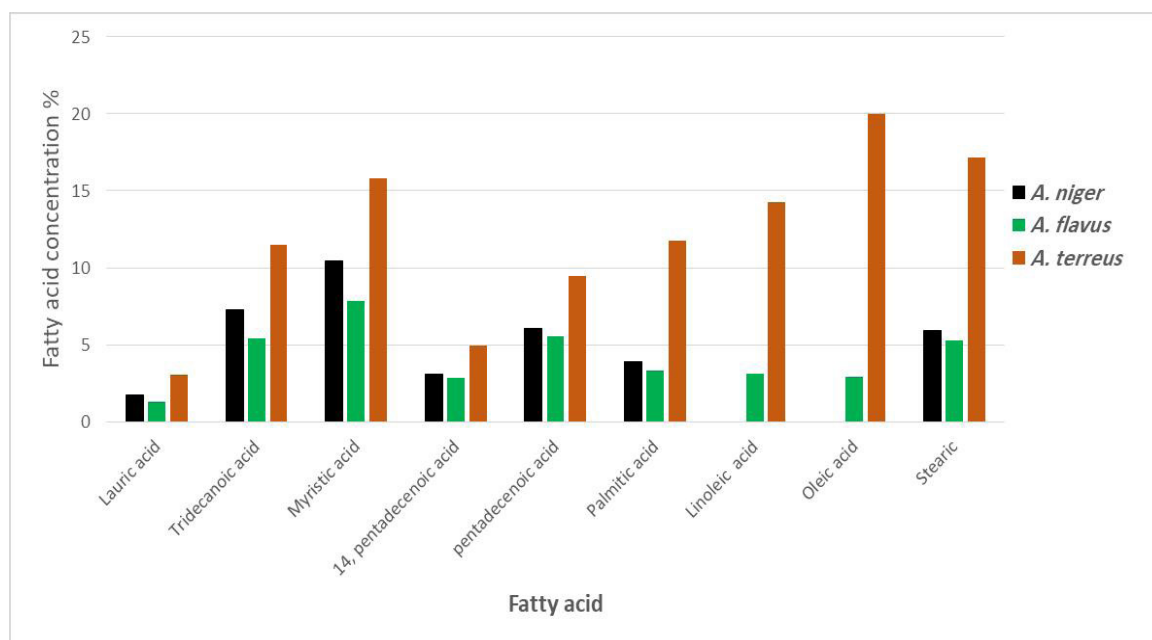


Fig. 3. Gas chromatography-profile of fungal FAME promising strains

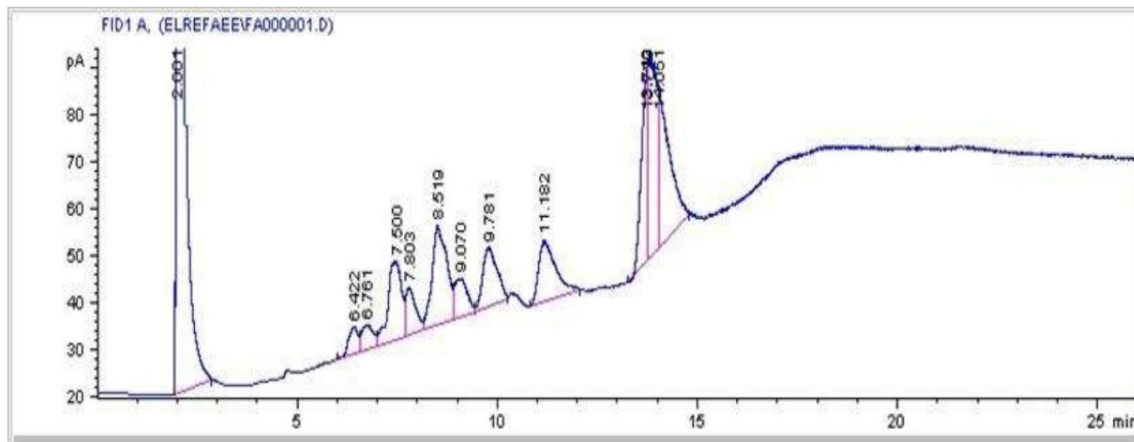


Fig. 4. Gas chromatography-profile analysis of *Aspergillus terreus* methyl esters

TABLE 6. Lipid profile and fatty acid concentration of *A. terreus*

Carbon number: Number of double bond	Fatty acid	Fatty acid (%)	Concentration of FA (mg/100g)
C12:0	Lauric acid	2.84	3.06
C13:0	Tridecanoic acid	10.61	11.46
C14:0	Myristic acid	14.64	15.81
C15:1	14, Pentadecenoic acid	4.59	4.96
C15:0	Pentadecenoic acid	8.78	9.48
C16:0	Palmitic acid	10.92	11.79
C18:2	Linoleic acid	13.20	14.25
C18:1	Oleic acid	18.51	19.99
C18:0	Stearic	15.91	17.18
Total concentration of FA in mg/100g of Lipid			107.98

Furthermore, our results indicated that the FAME profile of lipids produced by promising fungal isolates showed properties similar to those of biodiesel, validating its superior quality (Wu et al., 2010; Gadallah & Abd-El-Haleem, 2014; Babakura et al., 2019). The presence of long-chain FAs in the FAME profile has been reported to indicate enhanced biodiesel properties and also high fuel efficiency (Ziino et al., 1999; Dai et al., 2007; Vicente et al., 2009; Zheng et al., 2012; Çiçek & Yalçın, 2013).

Conclusion

Experimental data revealed the goals of the work, promising fungal isolates *Aspergillus terreus*, *Aspergillus niger* and *Aspergillus flavus* exhibited satisfactory lipid accumulation and constructed for biodiesel production. Optimization of cultural

conditions for maximum lipid production (38.33%) was achieved on the seventh day of growth at 30 °C incubation temperature in a static condition, using 5% sucrose, 0.5 g/L ammonium nitrate with initial pH 6.0 for *Aspergillus terreus* as a highest lipid producer. FAME profile indicated the presence of higher saturated fatty acid fraction compared to unsaturated fatty acids of the tested fungal species. The total concentration of fatty acids was 107.98, 38.29 and 37.48 mg/100g of lipid accumulated by *A. terreus*, *A. niger* and *A. flavus*, respectively. Gas chromatographic analysis of *A. terreus* lipid as the potent fungal strain revealed that oleic acid (C18:1, 18.51%) was the most abundant fatty acid, followed by stearic acid (C18:0, 15.91%) and Myristic acid (C14:0, 14.64%). Therefore, fatty acid profile of *A. terreus* has confirmed its potentiality as a new commercial biodiesel feedstock.

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Conflict of interest: Authors declare that there is no conflict of interest.

Author contributions: Samy El-Assar conceived of the presented idea. Samy El-Assar and Ghada Youssef developed the theory and performed the computations. Ahmed El-Refaey performed the material preparation and carried out the experiments. Samy El-Assar and Ghada Youssef were involved in planning and supervised the work. All authors discussed the results and approved the final manuscript.

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تقييم إنتاج البيوديزل الحيوى المستدام المستخلص من الفطريات الزيتية: فحص، تعريف، وتعظيم إنتاجية الدهون

غادة على أمين يوسف⁽¹⁾، أحمد محمد الرفاعي⁽²⁾، سامى عبد الحليم الأعصر⁽¹⁾
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يهدف هذا العمل إلى دراسة إنتاج وقود الديزل الحيوي بواسطة الفطريات الزيتية كبديل للوقود الأحفوري حيث تم عزل عشر عزلات فطرية من عينتين من التربة الملوثة بالبترول من الإسكندرية ، مصر. وتم فحص قدرة هذه العزلات على تراكم الدهون في كتلتها الحيوية. ولقد أوضحت النتائج أن عدد ثلاث من بين العشر عزلات لهم كفاءة عالية على إنتاج الدهون. وتم التعرف على هذه العزلات الثلاثة على المستوى المورفولوجي (العياني والمجهري) وتأكيدهما جزيئياً باستخدام التسلسل الجيني 18S rRNA وتم تعريفهم على إنهم أسبرجيليس تيريس وأسبرجيليس نيجر وأسبرجيليس فلافس. ومن ثم تم تحسين وتعظيم الظروف البيئية المختلفة عن طريق دراسة العوامل المؤثرة على إنتاج الدهون لتعطين أعلى إنتاجية للزيوت الفطرية المعزولة وتحسين نسبة تراكم الدهون. وأشارت النتائج بوضوح أن 5% سكروز، 0.5 جم / لتر نترات الأمونيوم مع درجة حموضة أولية 6.0 ، لمدة سبعة أيام من الحضانة (في حضان ثابت) عند درجة حرارة 30 درجة مئوية هي أفضل الظروف للحصول على أقصى إنتاجية للدهون (38.33%) في حالة فطر أسبرجيليس تيريس عن طريق تقنيات استرة الزيت الفطري. وأظهرت نتائج تحليل الأحماض الدهنية لهذه السلالات الفطرية إلى وجود أجزاء من الأحماض الدهنية المشبعة أعلى مقارنة بالأحماض الدهنية المتعددة غير المشبعة حيث بلغ التركيز الكلي للأحماض الدهنية 107.98، 38.29، 37.48 ملجم/ 100 جم من الدهن المتراكم بواسطة أسبرجيليس تيريس وأسبرجيليس نيجر وأسبرجيليس فلافس على التوالي. وأشار التحليل الكروماتوغرافي لدهن فطر أسبرجيليس تيريس أن حمض الأوليك (1: C18، 18.51%) كان أكثر الأحماض الدهنية وفرة، يليه حمض الإستيرك (0: C18، 15.91%) ثم حمض الميريستك (0: C14، 14.64%) على التوالي. وأكدت صورة الأحماض الدهنية لفطر أسبرجيليس تيريس وجود سلسلة طويلة من الأحماض الدهنية التي تعظم وتحسن خصائص وقود الديزل الحيوي. وتؤكد على قدرتها كمادة وسيطة تجارية جديدة لإنتاج دهون عالية الجودة مشابهة لخصائص الديزل الحيوي. وعلى ذلك فإن الزيت الفطري المستخلص من فطر أسبرجيليس تيريس يدخل في تصنيع وقود الديزل الحيوي.