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

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Abstract

The present work, aiming to exploit oleaginous fungi for biodiesel production. Ten fungal strains were isolated from two petroleum polluted soil samples and screened for their abilities to accumulate lipid. Lipid rich three species viz, *Aspergillus terreus*, *Aspergillus niger* and *Aspergillus flavus* were found to be the highest lipid producers. Potential isolates were identified at the species level by morphological (macroscopic and microscopic) examination and molecularly confirmed by using 18S rRNA gene sequencing. Improvement of lipid accumulation by optimization of various parameters of culture conditions. The results reported clearly that the most suitable medium conditions for highest lipid production (38.33%) of *Aspergillus terreus* as the most potent lipid producer composed of 5% sucrose, 0.5 g/L ammonium nitrate with initial pH 6.0, after seven days of incubation in a static condition. The three promising fungal isolates have been taken for fatty acids analysis by gas chromatograph (GC) after transesterification. Fatty acid methyl esters (FAME) profile indicated the presence of higher saturated fatty acid fractions compared to polyunsaturated fatty acids. 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 chromatograph analysis of *A. terreus* lipid indicated 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%), respectively. Therefore, fatty acid profile of *A. terreus* has confirmed its potentiality as feedstock for producing lipid for biodiesel manufacturing.

Introduction

Massive consumption of fossil fuels has already caused serious concern over global warming caused by greenhouse gases emission (Campbell-Lendrum and Prüss-Ustün 2019; Matsakas et al. 2017). Biodiesel is the most efficient renewable and sustainable substitute for fossil diesel fuel that is focused on biomass (Mahlia et al. 2020). Biofuel offers an eco-friendly attractive alternative to fossil fuels (Khan and Hussain 2017; Nisar et al. 2017). Recently, research attention has been shifted to the biofuel production on a global scale from single cell oils (SCO) produced by oleaginous microorganisms (Meng et al. 2009; Papanikolaou 2019; Ramírez-castrillón et al. 2017; Tao et al. 2006). These oleaginous species have the ability to accumulate and store 20–25% of lipids of its total dry biomass (Athenaki et al. 2018; Hu et al. 2009), mostly consisting of triacylglycerols (TAG) that form the storage fraction of the cell. Microbial lipids (SCO) that are produced by oleaginous microorganisms and considered as a promising feedstock for biodiesel production including microalgae, bacteria, fungi, and yeasts (Bharti 2019; Cho and Park 2018; El-Kassas et al. 2016; El-Sheekh and Hamouda 2016; Hamouda et al. 2016; Hoekman et al. 2012; li et al. 2008; Meng et al. 2009; Subramaniam et al. 2010). Microbial oils have several advantages such as productivity is usually higher than the plants or vegetable oils, they can be effectively developed in bioreactors, have short life cycles, show rapid growth rates, easy genetic modifications for certain products, and light independency or other climatic varieties (Dourou et al. 2018; Sitepu et al. 2014).

Oleaginous filamentous fungi are an attractive source for biodiesel synthesis and suggested as a favorable feedstock for a sustainable biodiesel industry (Hoffmeister and Keller 2007; Papanikolaou and Aggelis 2011; Peng and Chen 2008; Reis et al. 2019; Zhao et al. 2008). Many fungal species are considered as SCO and able to accumulate lipids, including *Aspergillus oryzae, Aspergillus awamori, Mortierella isabellina, Mortierella alliacea, Humicola lanuginose, Trichoderm a reesei, Penicillium commune*, and *Mucor circinelloides* (Antonio et al. 2013; Bhanja et al. 2014; Hussein et al. 2017; Li et al. 2008; Magdum et al. 2015; Rossi et al. 2011; Shafiq and Ali 2017).

Lipid accumulation by oleaginous fungi using different renewable substrates such as glycerol (Papanikolaou and Aggelis 2002; Polburee 2015; Ramírez-castrillón et al. 2017; Rivaldi et al. 2017), molasses and whey (Bellou 2012; Economou et al. 2011a; Vieira 2014), wastes from food industry, agro industrial residues, lignocellulosic biomass, seed oils and wastewater (Babakur 2019; Chuppa-Tostain et al. 2018; Cuevas et al. 2020; Campos et al. 2020; Dourou et al. 2018; Economou et al. 2011b; Ekas et al. 2019; Shafiq and Chechan 2019; Tsegaye 2018a, 2018b; Yousuf 2010). Glucose is the carbon source most normally utilized to enhance the growth of oleaginous fungi and lipid accumulation (Ochsenreither et al. 2016; Saxena et al. 2008; Zhao et al. 2008).

The fatty acid profile of microbial lipids is quite similar to that of conventional vegetable oils (Papanikolaou 2012; Rude and Schirmer 2009). Lipids of filamentous fungi have a unique fatty acid profiles are rich in some valuable polyunsaturated fatty acids, such as □-linoleic acid that cannot be synthesized by many other oleaginous microorganisms (Ratledge 2013; Subramaniam et al. 2010; Subhash and Mohan 2011). Gamma - linoleic acid (GLA; 18:3) is as an essential fatty acid in humans and has been reported to be effective for the prevention of a variety of diseases including cardiovascular diseases, rheumatoid arthritis, hyper-cholestromia, atopic eczema and asthma (Murad et al. 2010). Also, it was often utilized in dietary supplements and for infant nutrition (Huang et al. 2009).

Two main types of biofuels are biodiesel and ethanol which have become the most environment-friendly alternative fuels like petroleum, diesel and jet fuel for transportation (Wormslev 2016). Biodiesel is increasingly attracting worldwide attention due to the cost-effective

and eco-friendly, as biodegradability; a decrease of sulfur and aromatic hydrocarbons content, which reduced their toxic emission of CO, CO_2 during fuel combustion (Demirbas 2008; Knothe 2008; Kotasthan 2017). Biodiesel involves the mixture of fatty acyl methyl/ethyl esters (FAMEs), obtained typically from transesterification of vegetable oil that can be used for existing conventional diesel engines regardless of its origin and feedstocks from which it is derived (Alptekin 2017; Patel et al. 2017).

The focus of the present work was to isolate, identify and characterize oleaginous fungal species from oily polluted soil samples, estimate the lipid content of these isolates, study the effects of physical and nutritional parameters to detect and maximize the accumulation of intracellular lipids of the promising fungal strains to enhance the quality of biodiesel. Finally, investigate the fatty acid methyl ester (FAME) composition after transesterification of fungal oil. According to the literature, fatty acids from the promising oleaginous isolated fungus are significant for biodiesel production.

Materials And Methods

Samples collection and fungi isolation

Ten fungal isolates were screened from two different oil-rich soil locations in the front of Moharam-Bek and Amrya gas stations which were polluted by petroleum products effluent in Alexandria, Egypt. Samples were collected in sterile containers and taken to the laboratory for analysis. Serial dilutions of the collected samples were carried out until 10^{-5} folds to isolate oleaginous fungi. One ml of the dilute was pour plated on Potato Dextrose Agar medium (PDA) supplemented with 2 mL of gentamicin and then incubated at 28 ºC for 3–6 days (Kumar et al. 2011). Morphological appearances of the inoculated plates were observed and distinct pure colonies were subcultured on PDA slants and stored at 4 ºC for further study.

Screening of oleaginous fungal isolates for lipid production

Pure fungal isolates were cultured on basal medium (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 6.0 to select the highest lipid producers. A portion of mycelium from PDA slant were transferred to a tube containing 10 mL sterilized distillated water and agitated then 0.5 mL of this spore suspension taken by micropipette and added to 250 mL conical flasks containing 50 mL of the basal medium and then incubated in 30 °C for seven days under static condition. After incubation the culture fungal growth was harvested by filtration (Whatman no.1), and the mats biomass were collected and washed three times with distilled water to remove the medium residues. The biomass was dried in a hot air oven at 60 °C until constant weight.

Extraction of fugal lipid and determination of total lipid content

The lipids were extracted from the screened fungal isolates dried biomass by the method of Bligh and Dyer method (1959) with slight modification using chloroform: methanol 2:1 (v/v). One hundred milligrams of the dried biomass was crushed with a mortar and pestle. Mixture of 10 mL of chloroform, and 5 mL of methanol were mixed, 8 mL from this mixture withdrew and added to a known amount of crushed dried mycelium then vortexed for 5 minutes. Saline solution (7.3 g of NaCl, 10 mL of distillated water) was prepared, withdrew 2 ml of the prepared saline solution then added to each sample tube (containing dried mycelium) then vertexing for 5 minutes. Samples 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 determine the ratio of extracted lipids in compare to the cell dry weight (Magdum et al. 2015).

Percentage of lipid content (%) = weight of lipid (g) / weight of dried biomass (g) x 100

Morphological and molecular identification of the most potent fungal isolates

The promising lipid producers were kindly identified morphologically at the Mycological Center, Assiut University, Assiut, Egypt. Identification was performed considering some specific morphologic characteristics, such as colony diameter, color, texture appearance, and microscopic examination was done by lactophenol cotton blue staining including conidiophore, vesicle, metulae, phialides and conidia (Larone 2002).

The promising selected oleaginous fungi were molecularly identified based on the 18S rRNA sequences. Fungal strains were cultivated on PDA medium at 28 °C for 5 days. A small amount of the fresh culture was scraped and suspended in 100 µL autoclaved distilled water in a sterile Eppendorf vial (2 ml capacity) and boiled in a water bath at 100 °C for 15 minutes. The non-living fungal strain was sent to the Molecular Biology Research Unit, Assiut University, Egypt for DNA extraction using patho gene-spin DNA/RNA extraction kit provided by Intron Biotechnology Company, Korea. The (ITS) region of the rRNA gene was amplified by polymerase chain reaction (PCR)

at SolGent Company, Daejeon South Korea using two universal fungal primers ITS1 (forward) and ITS4 (reverse). Primers have the following composition: ITS1 (5' - TCC GTA GGT GAA CCT GCG G – 3'), and ITS4 (5'- TCC TCC GCT TAT TGA TAT GC -3'). The purified PCR product (amplicon) was reconfirmed using a size nucleotide marker (100 base pairs) by electrophoreses on 1% agarose gel. Then these bands were eluted and sequenced with the incorporation of dideoxynucleotides (dd NTPs) in the reaction mixture. Each sample was sequenced in the sense and antisense directions using ITS1 and ITS4 primers (White et al. 1990). Sequences were further analyzed using Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information (NCBI) website. The phylogenetic tree was constructed by the neighbour-joining program in MEGA 5.05 software.

Optimization of culture conditions to maximize lipid production

To enhance biodiesel production from the tested fungal strains, the influence of the studied nutritional and environmental parameters on the fungal growth and lipid production are discussed under static condition for the promising fungal isolates (*A. terreus, A. niger* and *A. flavus*).

Effect of carbon and nitrogen sources on lipid production

The effect of different carbon sources on the lipid production was studied using the basal culture medium supplemented with (5%) glucose as a control. An equimolar amount of eight different carbon sources were tested as fructose, lactose, sucrose, maltose, dextrose, starch, wheat bran, and corn. Also for nitrogen source, yeast extract was replaced on equal nitrogen bases (0.5 g/L) by six different nitrogen sources as ammonium nitrate, sodium nitrate, peptone, ammonium acetate, casein, and soybean one at a time.

Effect of initial pH and incubation period on lipid production

The initial pH of the culture medium was adjusted with 1N HCl or 1N NaOH before autoclaving at different values ranging from 4 to 9 under static condition for 7 days. The effect of incubation period was detected at different time intervals 3, 5, 7 and 9 days.

Effect of incubation temperature on lipid production

The effect of different incubation temperatures was evaluated as following 20 °C, 25 °C, 30 °C, 35 °C, and 40 °C for 7 days.

Methylation of lipid and transesterification

The transesterification reaction of the obtained tested lipids was performed according to Radwan (1978) with slight modification. The lipid sample (5 mg) is dissolved in benzene (2 ml) in a test tube fitted with a condenser, and 1% sulfuric acid in methanol (2.0 ml) is added, close the tube well and place in water bath at 90 °C for an hour and half. Cool, add 8 mL water and 5 mL petroleum ether shake and separate out the ethereal layer in a dry tube.

Gas chromatography (GC)

Gas chromatographic analysis of FAMEs for promising fungal isolates were performed using Hewlett Packard (HP) 6890 GC system at Central Laboratories of General Health Institute, Alexandria, Egypt.

Results And Discussion

Screening of different oleaginous fungal strains for their lipid production

Ten locally fungal isolates were obtained, five from Moharam-Bek and five from Amrya oily polluted soil gas stations, Alexandria, Egypt. The selected fungal isolates were tested for their growth and lipid accumulation under static condition (Table 1). The highest lipid value (24.14%) was achieved by a brown fungus, isolate (F1) from Moharam-Bek station, followed by a black fungus, isolate (F6) from Amrya station (12.32%) and olive green fungus, isolate (F2) from Moharam-Bek station (6.25%). Qiao et al. (2018) revealed that the highest yield and the maximum lipid content produced by *Mucor circinelloides* in static condition. These results were congruent with Ali and El-Ghonemy (2014); Kirrolia et al. (2012); Pandey et al. (2000) who reported that lipid accumulation was higher in the static condition as compared to that generated in shaking condition. Shafiq and Ali (2017); Somasekhar et al. (2003) indicated that many species of oleaginous fungi are able to accumulate significant amounts of intracellular lipid. The stored intracellular lipid content is utilized to maintain generations of cells leading to the production of lipid-free biomass (Park et al. 1990; Subhash and Mohan 2014). This phenomenon is known as lipid turnover (Fakas et al. 2007; Huang 2009; Wu et al. 2010).

Table 1
Screening of different fungal isolates for their oleaginicity and determination of fungal biomass.

Isolate No./Soil source	Colony colour	Dry biomass (g/ 50 mL)	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				

Morphological and molecular identification of the promising fungal isolates

The promising fungal isolates were recorded as potent lipid producers. They are identified morphologically (macroscopic characteristics and microscopic examination) at Assuit University Mycological Center (AUMC), Egypt. Sample coded ARS-1 was *Aspergillus niger*, (ARS-2) *Aspergillus terreus*, and (ARS-3) *Aspergillus flavus* as shown in Fig. 1.

Molecular identification of fungi and similarities with closely related fungal strains were done using Basic Local Alignment Search Tool (BLAST). The 18S rRNA sequence and its homologous sequences were analyzed using MegAlign (DNA Star) software version 5.05. Phylogenetic tree was constructed after alignment with the related strains with a percentage of similarity (Fig. 2).

Optimization of culture conditions for the highest lipid production

Optimization of the culture conditions was a significant strategy to enhance the lipid accumulation with economical cost efficiency (Jiru et al. 2017).

Effect of different carbon sources on lipid production

The present results (Table 2) estimated that the maximum lipid production (31.50%) of *A. terreus* was achieved in presence of sucrose as a sole carbon source, while wheat bran led to the highest dry biomass (0.48 g/50 mL). Glucose was the suitable carbon source for *A. niger* with maximum lipid content and highest biomass (12.32%, 0.28 g/50 mL) followed by lactose as a preferred carbon source yielding a maximum lipid productivity (8.33%) for *A. flavus*. According to Abu-Elreesh and Abd- El-Haleem (2014); Al-Hawash et al. (2018); Baqir et al. (1997); Carvalho et al. (2018); El-Haj et al. (2015); Assawah et al. (2020) who estimated that glucose served as the best carbon source yielding a maximum lipid production for *A.* spp. which agree with our study on *A. niger*. On the other hand, Abdelhamid et al. (2019) suggested that maximum lipid accumulation reached 34.92% for *Penicillium commune* in presence of xylose.

Table 2
Effect of different carbon sources on promising fungal strains lipid production.

Fungal strain / Carbon source	*A. niger*			*A. flavus*			*A. terreus*		
	Biomass (g/ 50 mL)	Lipid concentration (g /L)	Lipid content (%)	Biomass (g/ 50 mL)	Lipid concentration (g /L)	Lipid content (%)	Biomass (g/ 50 mL)	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

Effect of different nitrogen sources on lipid production

The results as shown in Table 3 revealed that the highest values for growth biomass and lipid content (0.24 g/50 mL and 38.33%) by *A. terreus* were achieved in presence of ammonium nitrate. The favorable nitrogen source for *A. flavus* was sodium nitrate yielding maximum lipid production (16.84%), while peptone led to the highest dry biomass (0.42 and 0.26 g/50 mL) for *A. niger* and *A. flavus*, respectively. On the hand, yeast extract was the best nitrogen source for *A. niger* yielding maximum lipid productivity (12.32%). These data were congruent with Abu-Elreesh and Abd- El-Haleem (2014) reported that yeast extract was the best nitrogen source for *Aspergillus niger*. Ramírez-Castrillón et al. (2017); Xing et al. (2012) with disagreement with these results reported that ammonium sulfate was shown to be the most suitable nitrogen source. In contrast, Abdelhamid et al. (2019) proved the maximum lipids production (43.06%) for *Penicillium commune* in presence of peptone.

Table 3
Effect of various nitrogen sources on promising fungal strains lipid production.

Fungal strain / Nitrogen sources	*A. niger*			*A. flavus*			*A. terreus*		
	Biomass (g/ 50 mL)	Lipid concentration (g /L)	Lipid content (%)	Biomass (g/ 50 mL)	Lipid concentration (g /L)	Lipid content (%)	Biomass (g/ 50 mL)	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

Effect of initial pH on lipid production

The external pH of the medium was an important environmental factor affecting plasma membrane permeability, metabolic activity, and cell growth as reported by Amanullah et al. (2001); Minhas et al. (2016). As illustrated in Table 4 it was observed that the highest values for growth biomass and lipid production (0.24 g/50 ml, 38.33%) and (0.19 g/50 ml, 16.84%) at pH 6 for *A. terreus* and *A. flavus*, respectively. In this regard, Sukrutha et al. (2014) estimated that the maximum lipid productivity was achieved by *Cunninghamella blakesleeana* at pH 6. The initial medium pH 7 was the optimum value for maximum lipid production (14.17%) by *A. niger*. The present data for *A. niger* was in line with that investigated by Abdelhamid et al. (2019) as the maximum lipid yield (33.16%) by *Penicillium commune* at pH 7. Comparable results were estimated by Ali et al. (2017); Ali and El-Ghonemy (2014); Jiru et al. (2017); Ruan et al. (2014) who recorded that pH values ranging between 5 and 6 were found to be the suitable pH range for most oleaginous fungal growth and lipid production.

Table 4
Effect of initial pH on promising fungal strains lipid production.

Fungal strain	A. *niger*			A. *flavus*			A. *terreus*		
pH value	Biomass (g/ 50 mL)	Lipid concentration (g /L)	Lipid content (%)	Biomass (g/ 50 mL)	Lipid concentration (g /L)	Lipid content (%)	Biomass (g/ 50 mL)	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

Influence of incubation period on lipid production

Incubation period has an observable effect on biomass lipid production. Lipid content of each strain differs depending upon its specific growth rate. In the current study, the lipid accumulation and dry biomass increased gradually during the first seven days of incubation and reached its maximum values (38.33%, 0.24 g/50 mL) and (16.84%, 0.19 g/50 mL) at the 7th day of incubation for *A. terreus* and *A. flavus*, respectively (Table 5). These results are in accordance with those reported by Sukrutha et al. (2014), who investigated the maximum lipid production was 28% after 6 days in static incubation of *Cunninghamella blakesleeana*. The fifth day of incubation was the optimum incubation period of *A. niger* yielding maximum lipid production (18.67%). Our results are in line with that detected by Ali et al. (2017); Kumar and Banerjee (2013), the maximum lipid production by *Aspergillus* spp. was achieved after 5 days of incubation. Also, study on *Penicillium commune* recorded the maximum lipid production (46.36%) after 5th day of incubation (Abdelhamid et al. 2019).

Table 5
Effect of different incubation periods on promising fungal strains lipid production.

Fungal strain	A. *niger*			A. *flavus*			A. *terreus*		
Incubation period (day)	Biomass (g/ 50 mL)	Lipid concentration (g /L)	Lipid content (%)	Biomass (g/ 50 mL)	Lipid concentration (g /L)	Lipid content (%)	Biomass (g/ 50 mL)	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

Influence of incubation temperature on lipid production

In the case of studying the effect of incubation temperature in the present survey, the highest fungal biomass and lipid productivity were reported at 30 °C for the all promising fungal strains, *A. terreus, A. niger* and *A. flavus*. The maximum lipid production for *A. terreus* as the most potent lipid producer reached 38.33% at incubation temperature of 30 °C. The results are in accordance with those recorded by Ali & El-Ghonemy (2014); Carlile et al. (2001); Li et al. (2008); Subhash and Mohan (2014). Patel and Desai (2019) estimated that the incubation temperature of 32 ºC was the optimum value for extracellular enzyme production for polysaccharide degradation and lipid accumulation of fungi. While, Abdelhamid et al. (2019) reported the maximum lipid accumulation percentage (41.18%) for *Penicillium commune* was estimated at 26 °C incubation temperature.

Fatty acid methyl ester (FAME) profile and gas chromatographic (GC) analysis of promising lipid producers

The fatty acid profile of the lipid samples was estimated by converting the fatty acids (FAs) to fatty acid methyl esters (FAMEs) after transesterification. The transesterified fatty acids were identified using GC analysis. The lipid profiles are differing qualitatively and quantitatively among all tested fungi. However, oleic acid and linoleic acid were absent in the lipids of *A. niger*. The presented data (Fig. 3) indicated the presence of a high fraction of saturated FAs than unsaturated FAs which is considered a potential feature to detect the fuel quality of fungal based diesel (Dai et al. 2007; Shafiq 2017; Shafiq and Chechan 2019; Subhash and Mohan 2011; Subhash and Mohan 2014; Zheng et al. 2012). This composition is quite 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 37.48 mg/100 g of lipid generated by *Aspergillus terreus*, *Aspergillus niger* and *Aspergillus flavus*, respectively.

Fatty acid composition of the potent fungal lipid producer

The fatty acid composition of *Aspergillus terreus* as the most potent fungal isolate was cited in (Fig. 4, Table 6). The GC- profile indicated the presence of high proportion of long-chain FAs, composed of a mixture of saturated FAs such as stearic acid (C18:0) 15.91%; myristic acid (C14:0) 14.64%; palmitic acid (C16:0) 10.92%; tridecanoic acid (C13:0) 10.61%; and pentadecenoic acid (C15:0) 8.78%. Limited percentage of mono-and polyunsaturated FAs was estimated such as oleic acid (C18:1) 18.51%; 14, pentadecenoic acid (C15:1) 4.59% as monounsaturated fatty acid (MUFA) and linoleic acid (C18:2) 13.20% as a polyunsaturated fatty acid (PUFA). A higher percent of saturated fatty acid (SFA) (60.86%) than unsaturated fatty acid (36.30%) was estimated. These results are in agreement with Farias et al. (2018) who reported that a higher percent of saturated fatty acid (70.7%) than unsaturated fatty acid (29.3%). The total SFA content (60.86%) was higher than other plant oils like palm oil (44%), Jatropha oil (21.52%) and soyabean oil (15%) (Vyas & Chhabra 2017) which indicates its high quality biodiesel.

Table 6
Lipid profile and fatty acid concentration of *Aspergillus terreus*.

Carbon number: Number of double bond	Fatty acid	Fatty acid (%)	Concentration of FA (mg/100 g)
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/100 g of Lipid			107.98

In the current study, a higher number of saturated fatty acids than polyunsaturated fatty acids was observed that indicates the fungal oil obtained has properties similar to those of biodiesel, which confirms its superior biodiesel quality (Babakura et al. 2019; Gadallah and

Abd-El-Haleem 2014; Wu et al. 2010). The presence of long chain fatty acids of FAME profile improves biodiesel properties, and hence, confirms high fuel efficiency (Çiçek and Yalçın 2013; Dai et al. 2007; Vicente et al. 2009; Zheng et al. 2012; Ziino et al. 1999).

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/100 g of lipid accumulated by *A. terreus*, *A. niger* and *A. flavus*, respectively. Gas chromatograph 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.

Declarations

Ethics approval and consent to participate:

Not applicable.

Consent for publication:

Not applicable

Availability of data and materials:

Not applicable.

Competing interests:

No potential conflicts of interest were reported by the authors.

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Authors' contributions:

All authors contributed to the study conception and design. Material preparation was performed by (Ahmed M. Elrefaey), data collection and analysis were performed by (Ghada A. Youssef & Ahmed M. Elrefaey).All authors contributed to the writing of this article. All authors read and approved the final manuscript.

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Figures

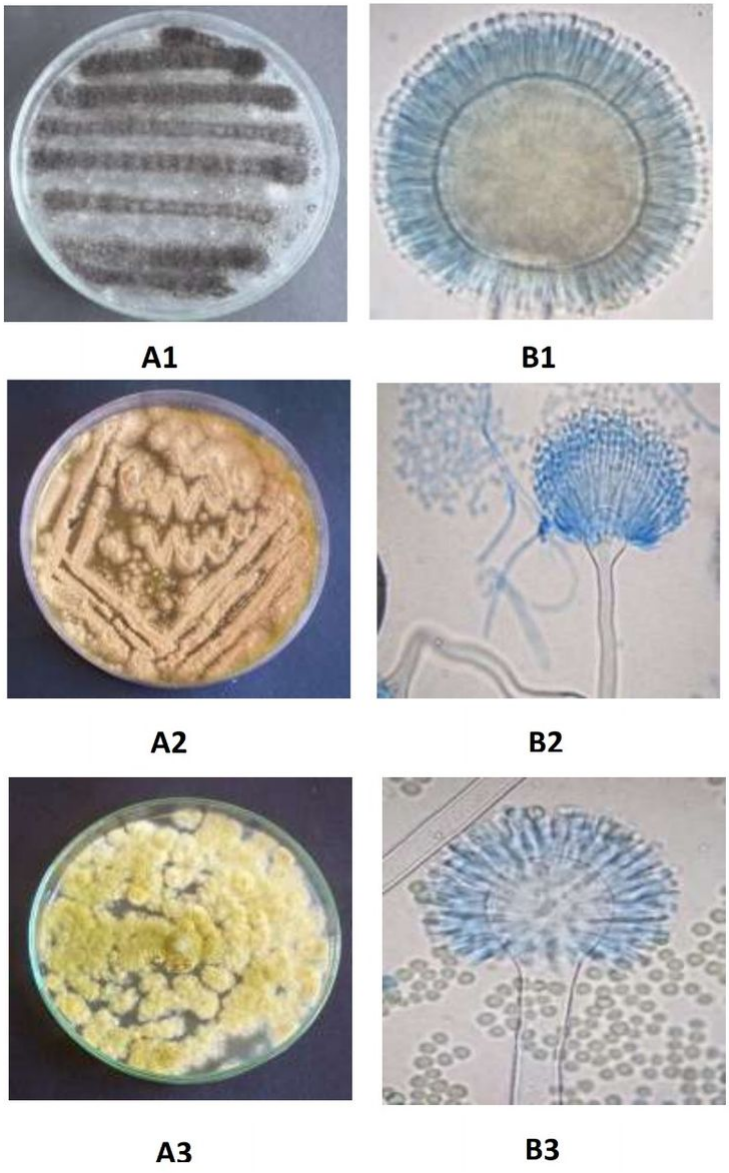
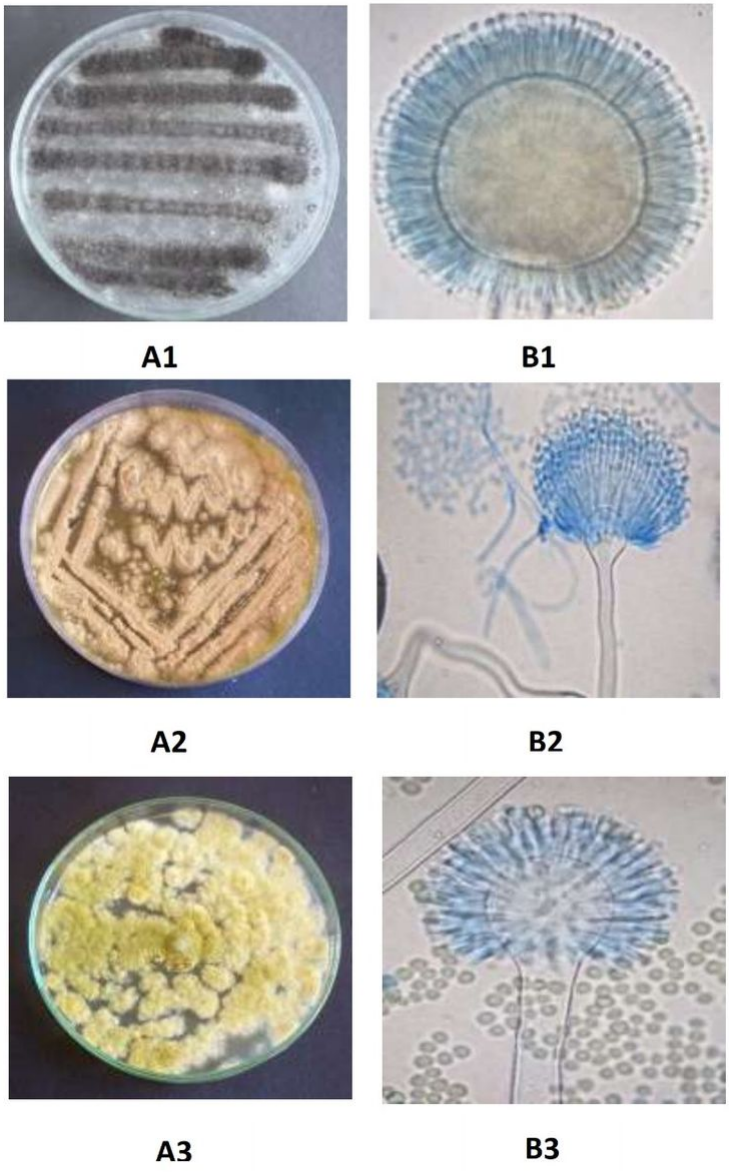


Figure 1

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.



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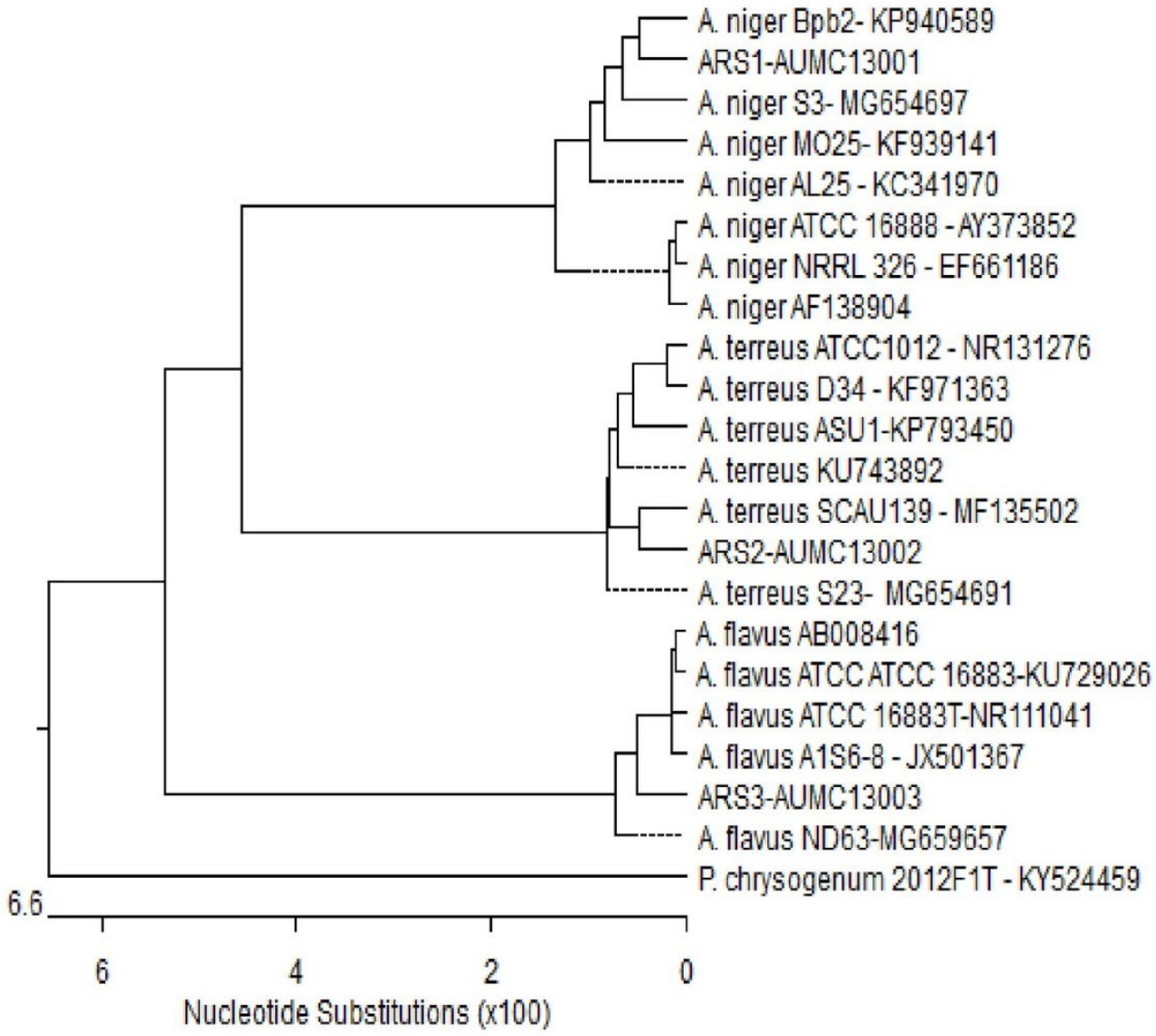
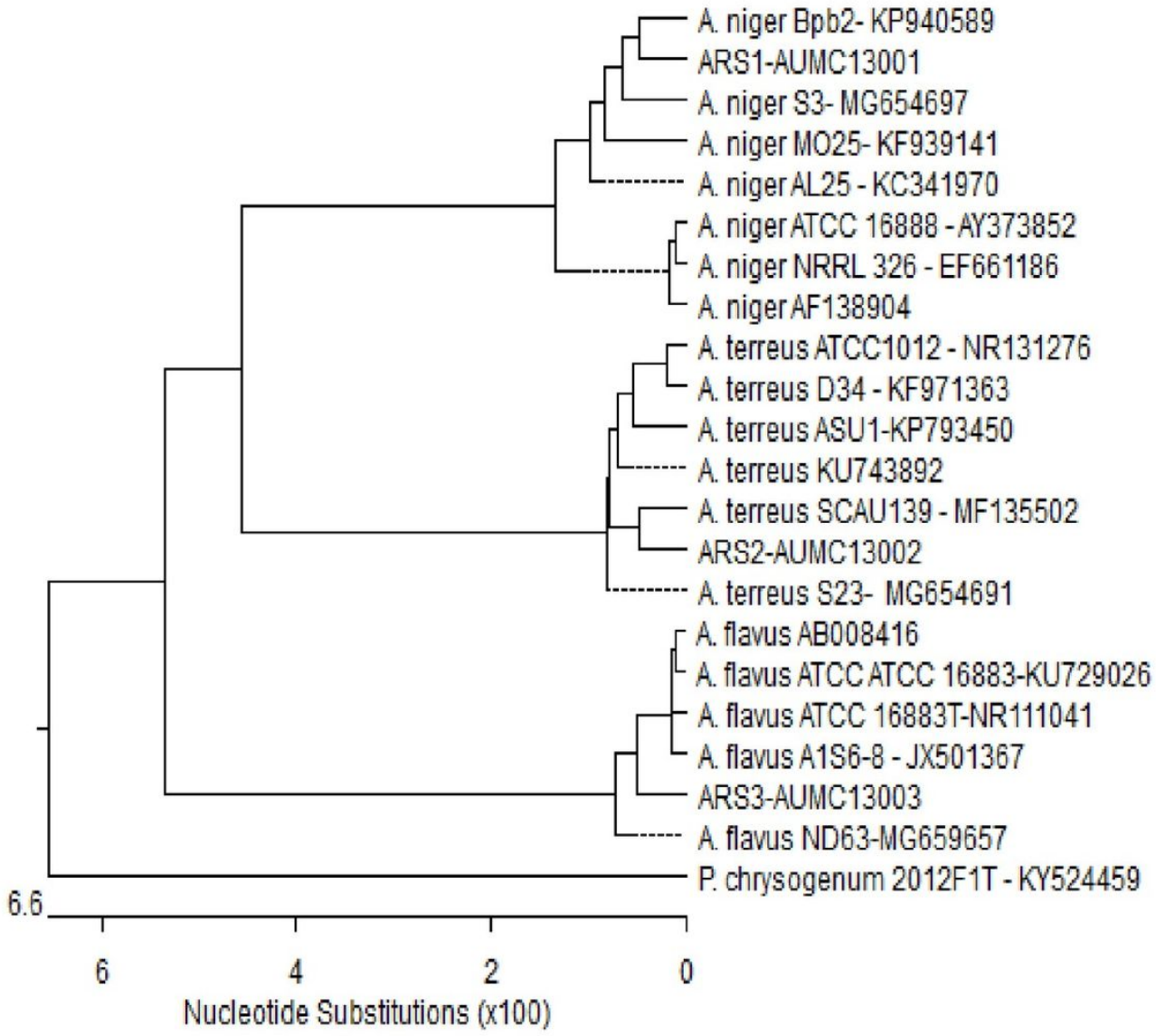


Figure 2

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.



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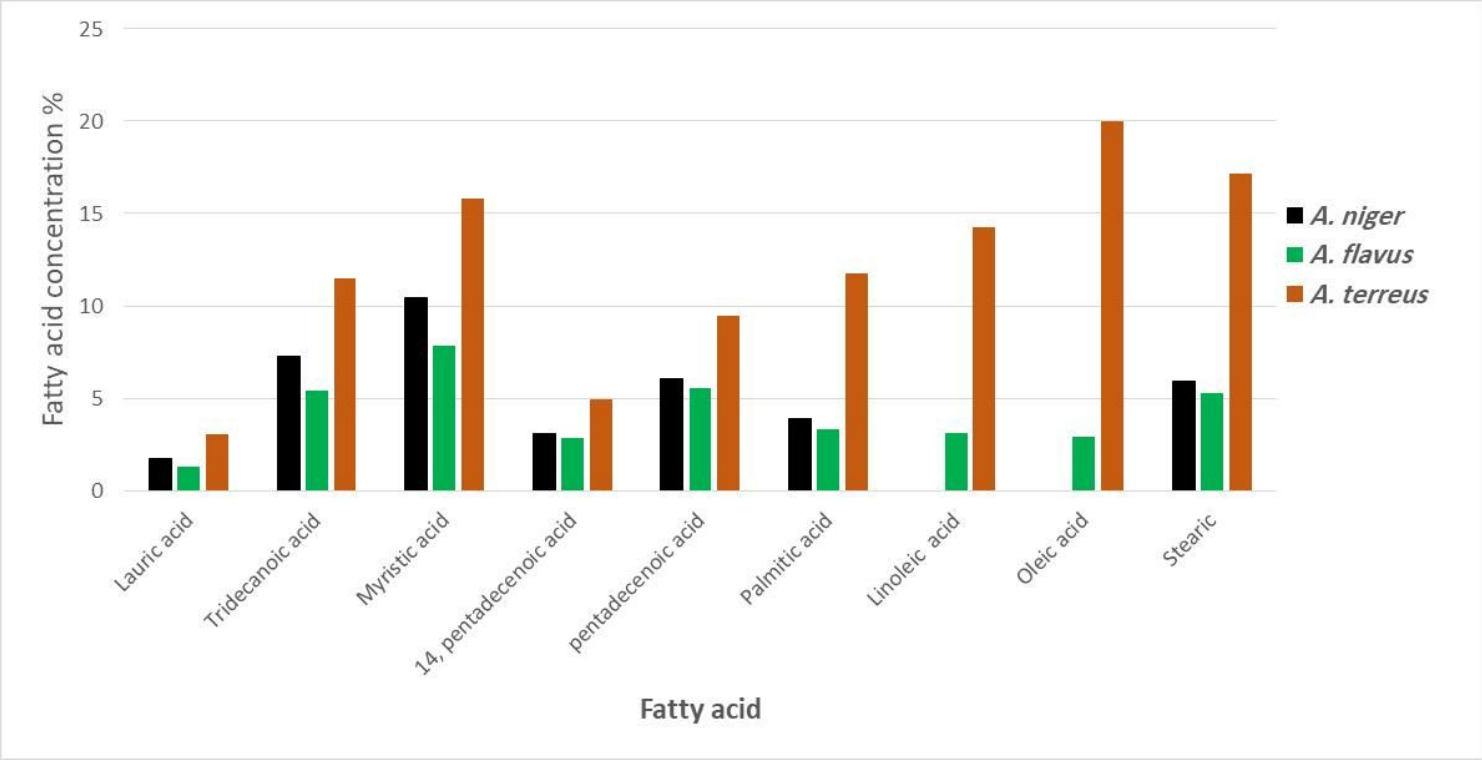

Figure 3

Gas chromatography - profile of fungal FAME promising strains.

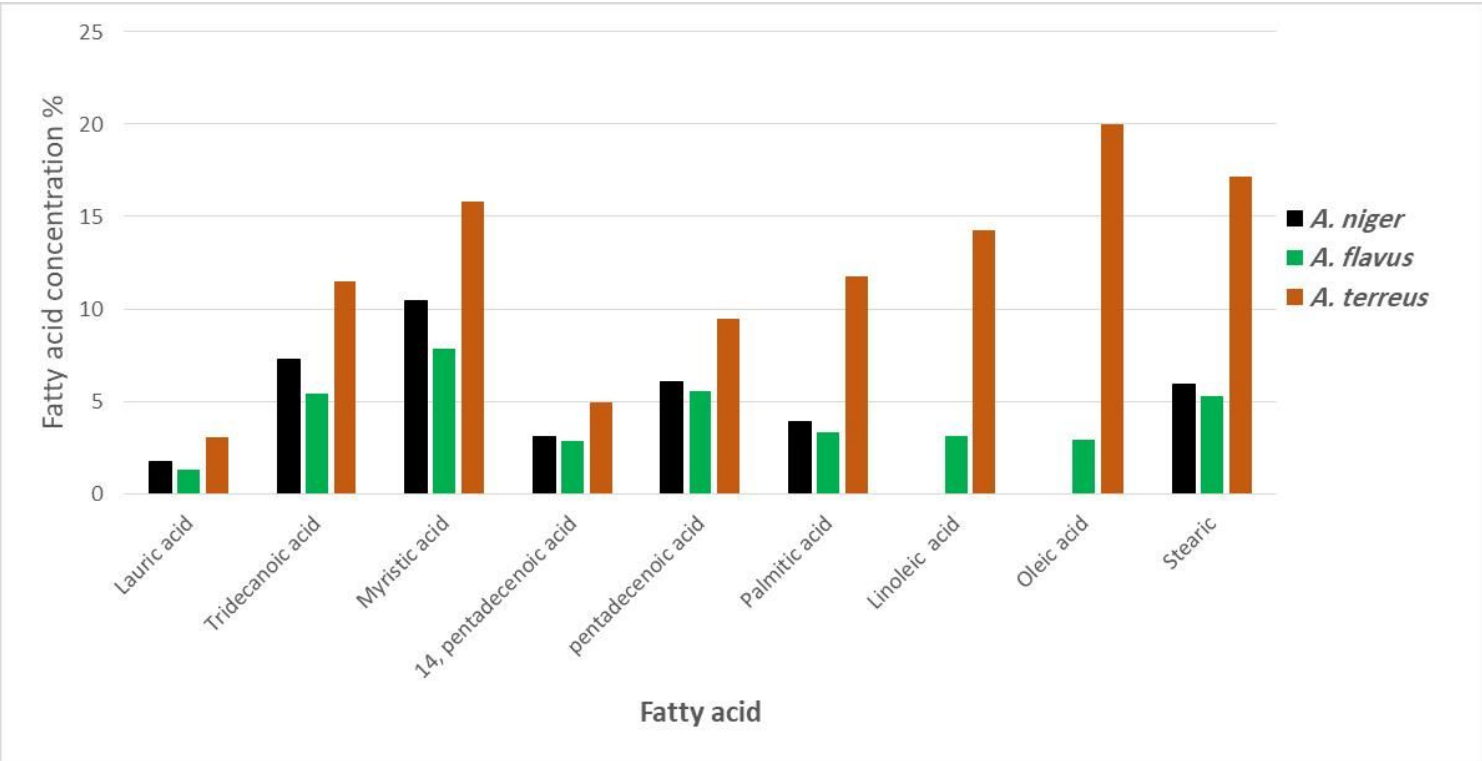

Figure 3

Gas chromatography - profile of fungal FAME promising strains.

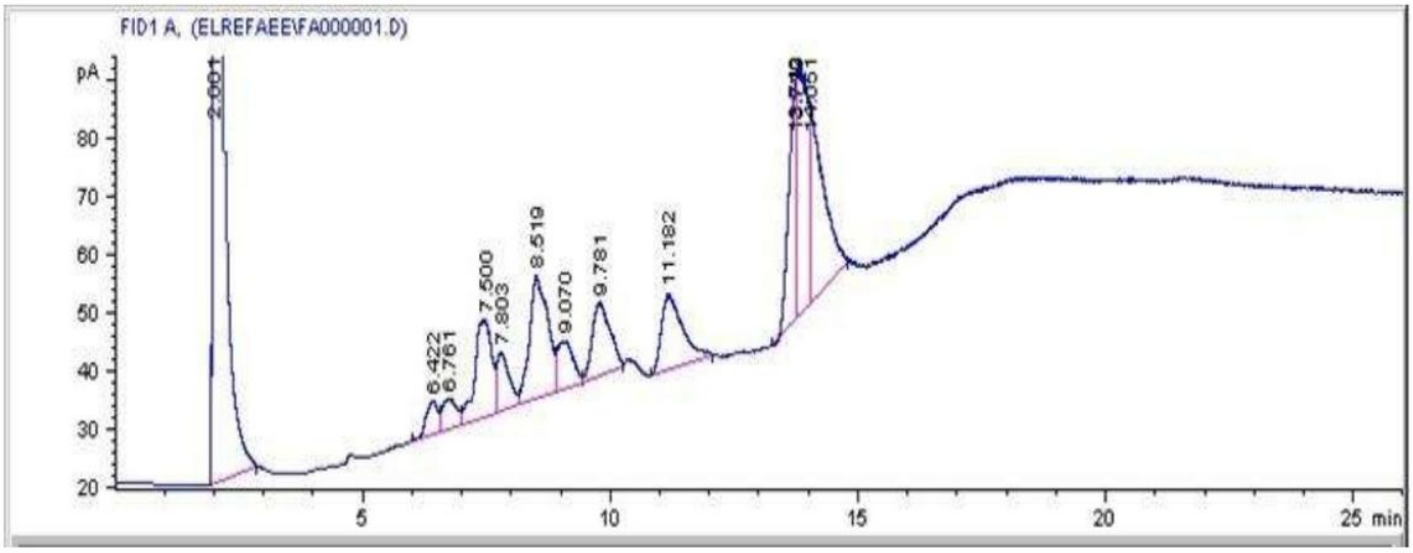

Figure 4

Gas chromatography-profile analysis of Aspergillus terreus methyl esters

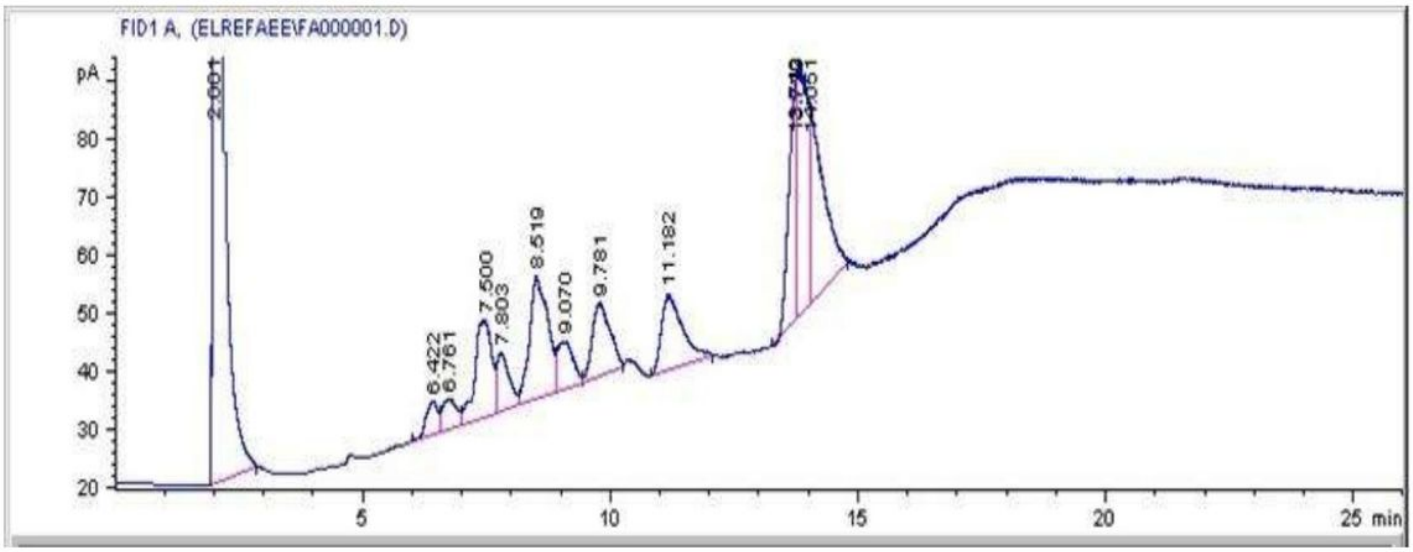

Figure 4

Gas chromatography-profile analysis of Aspergillus terreus methyl esters

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- graphicalabstractJPEG.jpg
- graphicalabstractJPEG.jpg