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Evaluation of biodiesel produced from some oleaginous fungi oil using whole-cell lipase

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BIODIESEL, known as fatty acid methyl esters (FAMEs), is a sustainable and ecofriendly substitute for Petro-diesel. This study investigates biodiesel synthesis from oil derived from oleaginous fungi, specifically, *Fusarium verticillioides* AUMC 16026, using *Rhizopus arrhizus* AUMC 16025 and *Penicillium crustosum* Pmor whole-cell lipase as a biocatalyst. This research marks the first record of using *P. crustosum* Pmor whole-cell lipase as a biocatalyst for biodiesel synthesis. Twenty-two fungi were isolated and screened for their ability to accumulate lipids and produce lipase. *F. verticillioides* AUMC 16026, the most potent lipid producer, showed the highest lipid production when grown in the presence of sucrose and sodium nitrate (initial pH, 4) for 3 days at 30°C under static conditions. *R. arrhizus* AUMC 16025 had the highest lipase activity (4.67 U/g), followed by *P. crustosum* Pmor (3.67 U/g). Therefore, they were chosen to perform the fungal oil transesterification process. Gas chromatography was utilized for analyzing produced FAMEs. Analysis of biodiesel produced from *F. verticillioides* AUMC 16026 oil transesterified using *R. arrhizus* AUMC 16025 whole-cell lipase revealed that linoleic (27.28%) and acids (25.46%) were the most abundant methyl esters. Meanwhile, those produced using *P. crustosum* Pmor whole-cell lipase displayed that oleic acid (23.53%) and palmitoleic acid (21.28%) were the most abundant, followed by linoleic (18.88%) and palmitic acids (11.84%). The biodiesel generated by both lipases was of similar quality, and both were similar to that of biodiesel derived from vegetable and leftover frying oils. This indicated that *F. verticillioides* AUMC 16026 oil was a good feedstock for biodiesel production.

Keywords: Biodiesel, oleaginous fungi, whole-cell lipase, fatty acid methyl esters, and transesterification.

Introduction

Biodiesel, a greener substitute for Petro-diesel, is composed of mono-alkyl esters derived from long-chain fatty acids, primarily fatty acid methyl esters (FAMEs). It is an alternative fuel that reduces net greenhouse effects and is used in many countries. It can be utilized as a transportation fuel with multiple benefits over Petro-diesel as it is renewable, nontoxic, and biodegradable. Also, it lowers pollutants, requires no modifications to the engine or additional oil, and has higher flash points **(Khot *et al.*, 2018)**.

Lipids can be transformed into biodiesel through a process called transesterification using methanol and a catalyst, which can be either an acid, base, or enzyme. However, using chemical catalysts, such as acid and base, poses several challenges, including difficulty in recovering glycerol, removing the catalyst from the product, treating the wastewater, and producing soap from water and free fatty acids. Furthermore, chemical catalysts are energy intensive. To overcome these issues, researchers are focusing on the use of an enzymatic catalyst as a more sustainable solution. Enzymatic methanolysis using immobilized lipase or whole-cell microorganisms that produce lipase is gaining popularity. Although enzymatic transesterification reactions are efficient and produce pollution-free products, they are not yet suitable for large-scale biodiesel production due to the high cost of the enzyme itself. Purifying enzymes involves several treatment processes, such as stabilization, immobilization, and purification. Since it does not need to undergo extra purification or stabilization procedures, employing fungal whole-cell intracellular lipase (whole-cell lipase) as a catalyst is preferable to using the refined enzyme, therefore lowering production costs **(Ghaderinezhad *et al.*, 2014; Elhussiny *et al.*, 2020).**

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Microbial lipids, also known as single-cell oils (SCOs), are oils produced by oleaginous microorganisms from carbohydrates under specific conditions and stored within the cell **(Ratledge, 2004)**. Oleaginous microorganisms can synthesize and accumulate lipids exceeding 20% of their dry cell weight. They do this by accumulating triglycerides (fuel precursors), which are then converted into fatty acid mono-alkyl esters **(Cho and Park, 2018)**. Triacylglycerols (TAGs) are the major component of the SCOs followed by free fatty acids, monoacylglycerols (MAG), diacylglycerols (DAG), sterols, and polar lipids (glyco-, sphingo-, and phospholipids) **(Khot *et al.*, 2018).**

Oleaginous microorganisms are numerous and widely distributed, including bacteria, yeasts, molds, and algae. Fungi have manifold advantageous features that make them a prominent group of microorganisms for the production of single-cell oils (SCOs). These include their short life cycle, ease of growth, capability to accumulate lipids up to 80% of dry weight, independence from light energy, and their ability to utilize a diverse range of affordable and renewable carbon sources. These carbon sources include agroindustrial residues, lignocellulosic material, wastewater, mono-sugar, cereal, corncob, sweet sorghum, and crude glycerol **(Vivek, 2017; Yehia *et al.*, 2017; Abdelhamid *et al.*, 2019; Pajares *et al.*, 2024)**.

Several oleaginous filamentous fungi, mostly belonging to two major phyla (Mucoromycota and Ascomycota), have been identified: *Mortierella isabellina, M. alpina, M. vinacea, M. alliacea, Cunninghamella echinulata, Humicola lanuginosa, Rhizopus stolonifer, Mucor circinelloides, M. indicus, M. rouxianus, Aspergillus niger, A. oryzae, A. awamori, A. terreus, Colletotrichum corda, Fusarium solani, F. oxysporum, Trichoderma viride, Penicillium citrinum,* and *Neurospora intermedia* **(Dey *et al.*, 2011; Samadlouie *et al.*, 2012; Josu *et al.*, 2021; Mhlongo *et al.*, 2021; Miguel *et al.*, 2021).**

This study aimed to produce biodiesel using oleaginous fungal oil as a feedstock and fungal whole-cell lipase as a biocatalyst. This goal was achieved by the isolation and identification of fungal strains capable of yielding a high amount of storage lipids (oil) and those that had the ability for lipase production. Subsequently, the production of fungal oil by the chosen strains was optimized, and the potential transesterification of the produced oil into biodiesel using the most potent whole-cell lipase was also examined. Finally, the produced FAMEs were analyzed by GC.

Materials and Methods

1. Sample collection, isolation, and morphological identification of fungi

Fungi were isolated from soil polluted with waste oil from generators (sample 1 from El-Shorouk: N 30.1555, E 31.6460; sample 2 from Suez Road: N 30.088140, E 31.698060), oil-rich soil from gas station (First Settlement: N 30.059133, E 31.444072), and agriculture soil (El-Qalyubia: N 30.323858, E 31.125609). Fungi were also isolated from spoiled morta, spoiled jam, spoiled margarine, rotten sugarcane, and rotten grapes.

The fungal strain was isolated from the soil by serial dilution and the spread plate technique. Ten grams of each soil sample was individually suspended in 100 ml of sterile water, serially diluted from 10^{-1}- to 10^{-4}-fold, and spread over yeast extract peptone dextrose agar (YPD) plates. The composition of YPD medium was described by **Niehus *et al.* (2018)** as follows (g/l, w/v): 23 g glucose, 0.3 g peptone, 0.5 g yeast extract, 7 g KH_2PO_4, 2 g $Na_2HPO_4 \cdot 7H_2O$, 1.5 g $MgSO_4$, and 20 g agar (pH 5.5±0.2), corresponding to a C/N of 80. The plates were incubated at 28^{o}C for 4 days. Isolation of fungi from other sources was carried out by the streak plate method on the YPD agar medium. The plates were incubated at 28^{o}C for 4 days.

Fungal isolates were identified at the species level by observing culture characteristics (growth rate, pigmentation, and texture of colony) and microscopic characteristics (**Ellis, 1971; Ellis, 1976; Raper and Fenneli, 1977; Frisvad and Samson, 2004; Leslie and Summerell, 2006; Zheng *et al.*, 2007; Houbraken *et al.*, 2014; Visagie *et al.*, 2014; Pyrri *et al.*, 2021; Pitt and Hocking, 2022).**

2. Screening for lipid-producing and lipase-producing fungi

a- Sudan black B stain method

The three-day-old fungal isolates were smeared, heat-fixed, flooded with Sudan black B stain, and kept for 15 minutes. The excess stain was removed by washing the slide with 70% ethanol. The slides were counterstained with safranin for 30 seconds. The slides were washed with water, dried, and observed under an optical microscope at 100 x magnification (oil immersion lens) for the presence of blue-black or blue-gray fat globules within the cell **(Kamoun *et al.*, 2018)**.

b. Phenol-red agar plate method

Screening of lipase-producing fungi was conducted using the phenol-red agar plate method. Agar plates of phenol red supplemented with olive oil were prepared as follows (g/l, w/v): 0.1 g phenol red, 1 g $CaCl_2$, 10 g olive oil, 20 g agar, and pH adjusted to

7.3–7.4 with 0.1 N NaOH. Fungal isolates were inoculated onto the phenol red agar plates and incubated at 28 °C for 7 days. The plates were examined daily to check the change of color from red to yellow. A minor pH reduction during lipolysis will cause a color shift from red to yellow, signifying successful outcomes due to the release of fatty acids **(Ramnath *et al.*, 2017)**.

3. Molecular identification of the most potent fungal isolates

The most potent fungal isolates (*Fusarium verticillioides* AUMC 16026 and *Rhizopus arrhizus* AUMC 16025) were molecularly identified and submitted in the culture collection of Assiut University Mycological Center (AUMC), Assiut, Egypt, while *Penicillium crustosum* Pmor was molecularly identified in the Reference Lab of Animal Health Research Institute, Giza, Egypt. *Fusarium* was cultured on Czapek's yeast extract agar (CYA) medium and incubated at 28 °C for 5 days. In sterile Petri dishes with a diameter of 9 cm and 20 ml of autoclaved potato sucrose agar (PSA), *Rhizopus* was cultivated. The plates were then incubated for five days at 28 ºC. *Penicillium* was cultured on potato dextrose agar (PDA) medium and incubated at 28 °C for 3 days **(Pitt and Hocking, 2009)**. DNA was extracted using a Patho Gene-spin DNA/RNA extraction kit provided by Intron Biotechnology Company, Korea. The extracted DNA was kept in a 1.5 ml autoclaved Eppendorf tube before shipping to Sol Gent Company, Daejeon, South Korea, and PerkinElmer, California, USA, for polymerase chain reaction (PCR) and rRNA gene sequencing. PCR was performed using ITS1 (forward) and ITS4 (reverse) primers, which were incorporated into the reaction mixture. Primers had the following composition: ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC3'). The purified PCR product was sequenced with the same primers with the addition of dNTPs in the reaction mixture **(White *et al.*, 1990)**. All the sequences were submitted to GenBank. The obtained sequences were analyzed using the basic local alignment search tool (BLAST) from the National Center of Biotechnology Information (NCBI) website (http://www. ncbi.nlm.nih.gov).

4. Culture conditions for lipid production and optimization

To prepare the fungal biomass, a loopful of fungal mycelium was transferred to a test tube containing 10 ml of sterilized water and thoroughly mixed. One ml of this suspension was then collected using a micropipette and added to a 250 ml Erlenmeyer flask containing 100 ml of YPD media, followed by incubation at 30 ˚C for 7 days in a shaking incubator (150 rpm). Subsequently, fungal growth was harvested by filtration through Whatman filter paper (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 55–60 ˚C overnight **(Youssef *et al.*, 2021)**.

The effects of nutritional and environmental parameters on lipid production by *Fusarium verticillioides* AUMC 16026 and *Rhizopus arrhizus* AUMC 16025 were explored. The tested parameters comprised incubation periods (3, 4, 5, 6, and 7 days), temperature (20, 25, 30, and 35°C), initial medium pH (4, 5, 6, 7, and 8), carbon sources (glucose, xylose, sucrose, lactose, maltose, and cellulose), nitrogen sources (yeast extract, peptone, ammonium sulfate, and sodium nitrate), and shaking conditions (static and shaking at 150 rpm) **(Abdelhamid *et al.*, 2019; Sayeda *et al.*, 2019).** One hundred ml of YPD medium was utilized for optimizing all the parameters. All experiments were performed in triplicate.

5. Extraction of fungal lipids and determination of total lipid content

The lipids were extracted from four screened fungal isolates positive for intracellular lipid accumulation using chloroform: methanol 2:1 (v/v). The dried biomass was crushed using a mortar and pestle into a fine powder. Fifteen ml of the solvent mixture was added to a known amount of crushed dried mycelium with ultrasonication to favor cell membrane disruption for 10 minutes (twice). Using Whatman filter paper (no. 1), the solvent mixture containing the recovered lipids was filtered out of the remaining biomass. The solvent was evaporated, and then the lipids were determined gravimetrically using the following equation (the ratio of extracted lipids in comparison with the cell dry weight) **(Vicente *et al.*, 2009; Youssef *et al.*, 2021):**

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

6. Culture conditions for lipase production

Fungal cultures were inoculated into 100 ml of yeast extract peptone olive oil (YEPO) medium (8 g peptone, 4 g yeast extract, 3 g NaCl, and 30 ml olive oil dissolved in 1 L distilled water) in 250 ml Erlenmeyer flasks and incubated in an orbital shaker at 28 °C and 150 rpm for 3 days. Fungal growth was harvested by filtration and washed three times using a sterile physiological saline solution of 0.9% (9 g NaCl dissolved in 1 L water); then it was dried, weighed, and utilized as a whole-cell biocatalyst **(Elhussiny *et al.*, 2020)**.

7. Lipase assay

The lipolytic activity of the whole-cell biocatalysts was identified using the method described by **Elhussiny *et al.* (2020)**. The hydrolysis reaction involved stirring 0.5 g of dried cells and 5.5 g of oil

that was emulsified in 30 ml of Tris buffer (pH 7.5) for 2 hours at 35 °C and 150 rpm. The reaction mixture was centrifuged at 5000 rpm for 10 minutes, and the supernatant was collected. The fatty acid content was assessed by performing a titration. To start, 1 gram of the supernatant was dissolved in 25 ml of a neutralized mixed solvent consisting of diethyl ether and ethanol in a 1:1 ratio. Two drops of phenolphthalein, a color indicator prepared by dissolving 0.1 grams of phenolphthalein in 100 milliliters of either ethanol or acetone, were added. A titration was then conducted using 0.1 N NaOH until a pink color appeared, which was sustained for 15 seconds. One unit of lipase hydrolysis activity was defined as the amount of enzyme required to release 1 µmol of fatty acid per minute under the conditions described above. The experiment was performed in triplicate. Lipase activity was calculated according to the following equation:

$$Lipase\ Activity\ \left(\frac{U}{g}\right) = \frac{Volume\ of\ NaOH\ utilized\ (ml) \times Normality\ of\ NaOH \times 1000}{Weight\ of\ cells\ (g) \times\ Reaction\ time\ (min)}$$

8. Methanolic transesterification

Transesterification was carried out in a 50 ml Erlenmeyer flask containing 5 g of microbial oil, 0.75 ml of phosphate buffer (pH 7.5), and 0.5 g of the fractured dried fungal mat. The flasks were incubated in an orbital shaker at 35°C and 250 rpm. Methanol was added successively for 72 hours to a final molar ratio of 3 mol. FAMEs were detected by thin-layer chromatography (TLC) with silica gel 60 F254 (E. Merck KG). Two successive solvent systems were utilized. Solvent system A consisted of hexane/diethyl ether/acetic acid with a ratio of 70:30:1, while B had a ratio of 80:20:1, respectively. The spots were detected in an iodine chamber **(Elhussiny *et al.*, 2020)**.

9. Gas chromatography

The GC model 7890B from Agilent Technologies was equipped with a flame ionization detector at the Central Laboratories Network, National Research Centre, Cairo, Egypt. Separation was achieved using a Zebron ZB-FAME column (60 m x 0.25 mm internal diameter x 0.25 μm film thickness). The analyses were conducted utilizing hydrogen as the carrier gas, with a flow rate of 1.8 ml/minute in split-1:50 mode. The injection volume was 1 µl, and the temperature program followed the following sequence: starting at 100 °C for 3 minutes, gradually increasing at a rate of 2.5°C/min until reaching 240°C, and then maintaining this temperature for 10 minutes. The injector and detector (FID) were held at 250°C and 285°C, respectively. Quantification of the FAMEs content was conducted by comparing the retention time of FAMEs of the sample and a known concentration of FAMEs standard mixture C4–C24. The percentage of biodiesel produced from the transesterification process was determined by the following equation:

$$Biodiesel\ \%\ \left(\frac{wt}{wt}\right) = \frac{Sum\ of\ fatty\ acid\ methyl\ ester\ (FAME)}{Weight\ of\ sample\ (g)} \times 100$$

10. Statistical analysis

The data collected were analyzed by one-way analysis of variance (ANOVA) and followed by Tukey's Pairwise Comparisons with $p < 0.05$ using the software Minitab Statistics version 18.

Results

1. Screening of fungal isolates for lipid and lipase production

The source of separation for each of the 22 fungi isolates is displayed in Table 1. As per Pitt and Hocking (2022), all isolates were identified at the species level by examining microscopic features and culture parameters (growth rate, pigmentation, and colony texture). Results revealed that 22 fungal species belonged to 9 genera of mold fungi and two genera of yeast fungi.

Of the 22 strains probed, eight were found to have blue-black fat globules upon evaluation with Sudan black B stain (Table 1 and Figure 1), suggesting that these strains were capable of producing lipids. These 8 fungal strains were subjected to further quantitative screening for lipid production by determination of their lipid content. Table 2 highlights the varying lipid production capabilities of different fungal strains. Among the fungal strains listed, *Fusarium verticillioides* showed the highest lipid concentration (0.03 g/100 ml) and relatively high biomass production (0.110 g/100 ml). It also had the highest lipid content percentage (27.27%), suggesting that it was a promising candidate for lipid production. Additionally, *Rhizopus arrhizus* demonstrated relatively high lipid content percentages (25.64%), indicating its potential as a lipid producer. *Cladosporium cladosporioides* and *Trichothecium roseum* strains exhibited significant lipid content percentages (12.86 and 6.38%, respectively). The later strain *T. roseum* disclosed the maximum biomass production (0.141g/ml) denoting that lipid production was not related to biomass production. On the other hand, *Penicillium sclerotigenum*, *Talaromyces minnesotensis*, *Ulocladium oudemansii*, and yeast boasted no lipid production, as indicated by their zero-lipid concentration and lipid content percentages.

Additionally, Table 1 presents the results of the qualitative screening of 22 fungal isolates for lipase production using the phenol red agar plate method (refer to Section 2, page 4). The results explained the ability of fungal isolates to produce lipase ranging between producer (8 strains), weak producer

(6 strains), and nonproducer (8 strains) based on the change of color from red to yellow as shown in Figure 2. Further quantitative screening of the strains reported as lipase producers by measuring the lipolytic activity of the whole-cell biocatalysts was carried out. Figure 3 illustrates the lipase activity, measured in units per gram of cells, for different fungal strains. *R. arrhizus* demonstrates the highest lipase activity among the listed fungal strains, with a value of 4.67 U/g. Moreover, *P. crustosum* and *A. niger* expressed relatively high lipase activities (3.83 U/g and 2.5 U/g, respectively), while *U. oudemansii* exhibited moderate lipase activity (1.5 U/g). On the other hand, *T. roseum* and *P. chrysogenum* have relatively lower lipase activities compared to the other strains. Data suggested that *R. arrhizus* and *P. crustosum* were the most promising fungal strains for lipase production, based on their higher lipase activities. Consequently, they were selected as whole-cell biocatalysts for transesterification of the produced fungal oil into biodiesel.

Table 1. Screening of oleaginous fungal isolates for lipid droplet (fat globule) production and lipase production.

Isolation source	Fungal isolates	Lipid droplet (fat globule) production	Lipase production*
Morta	*Penicillium crustosum*	-	++
Jam	*Penicillium sclerotigenum*	+	+
Rotten sugarcane	*Fusarium verticillioides*	+	+
	Talaromyces minnestotensis	+	-
Spoiled margarine	*Epicoccum nigrum*	-	+
	Yeast	+	+
Grapes	*Trichothecium roseum*	+	++
Polluted soil by oil Generator 1	*Aspergillus niger*	-	++
	Penicillium chrysogenum	-	++
	Ulocladium tubercuilum	-	+
Polluted soil by oil Generator 2	*Aspergillus niger*	-	++
	Yeast	-	-
Oil-rich soil from a gas station	*Aspergillus asperescens*	-	-
	Aspergillus niger	-	++
	Ulocladium alternaria	-	-
Agriculture soil	*Aspergillus versicolor*	-	-
	Aspergillus violacea	-	-
	Cladosporium cladosporioides	+	-
	Penicillium glabrum	-	-
	Penicillium longicatenatum	-	+
	Rhizopus arrhizus	+	++
	Ulocladium oudemansii	+	++

**Lipase production: (++) producer, (+) weak producer, and (-) nonproducer.*

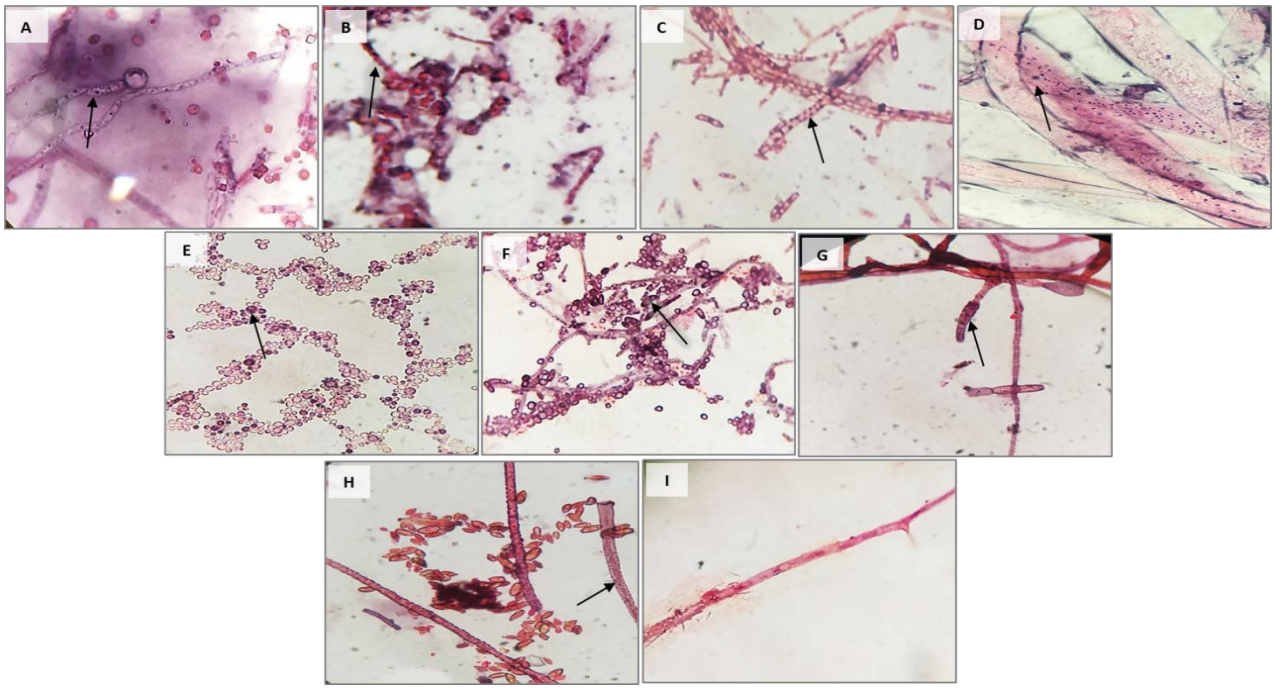

Fig. 1. Positive results (blue-black lipid droplet or fat globule) of oleaginous fungi stained by Sudan black B as seen under the light microscope (100X) with oil immersion length. (A) *P. sclerotigenum.* (B) *T. minnestotensis.* (C) *F. verticillioides.* (D) *R. arrhizus.* (E) Yeast. (F) *T. roseum.* (G) *U. oudemansii.* (H) *C. cladosporioides.* (I) Negative result.

Table 2. Quantitative screening of oleaginous fungal isolates for lipid production.

Fungal strain	Biomass (g/100 ml)	Lipid concentration (g/100 ml)	Lipid content (%)
C. cladosporioides	0.070^{c}	0.009^{b}	12.86^{c}
***F. verticillioides* AUMC 16026**	0.110^{b}	0.03^{a}	27.27^{a}
P. sclerotigenum	0.057^{c}	0^{c}	0^{e}
***R. arrhizus* AUMC 16025**	0.039^{d}	0.01^{b}	25.64^{b}
T. minnestotensis	0.062^{c}	0^{c}	0^{e}
T. roseum	0.141^{a}	0.009^{b}	6.38^{d}
U. oudemansii	0.034^{d}	0^{c}	0^{e}
Yeast	0.015^{e}	0^{c}	0^{e}
LSD (0.05)	0.0173	0.0071	1.22

*a, b, c, d, and e: each letter is produced very differently from the other. Values followed by the same letter do not differ significantly ($p < 0.05$)

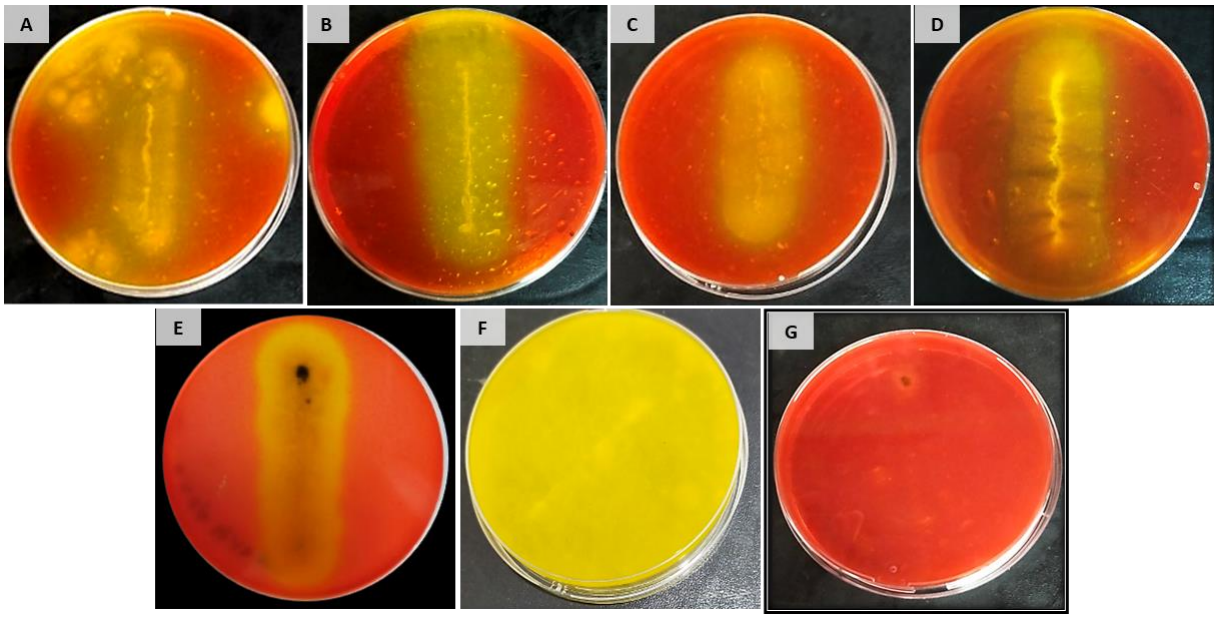

Fig. 2. Positive results of lipase production. (A) *P. crustosum.* (B) *A. niger.* (C) *P. chrysogenum.* (D) *T. roseum.* (E) *U. oudemansii.* (F) *R. arrhizus.* (G) Negative result.

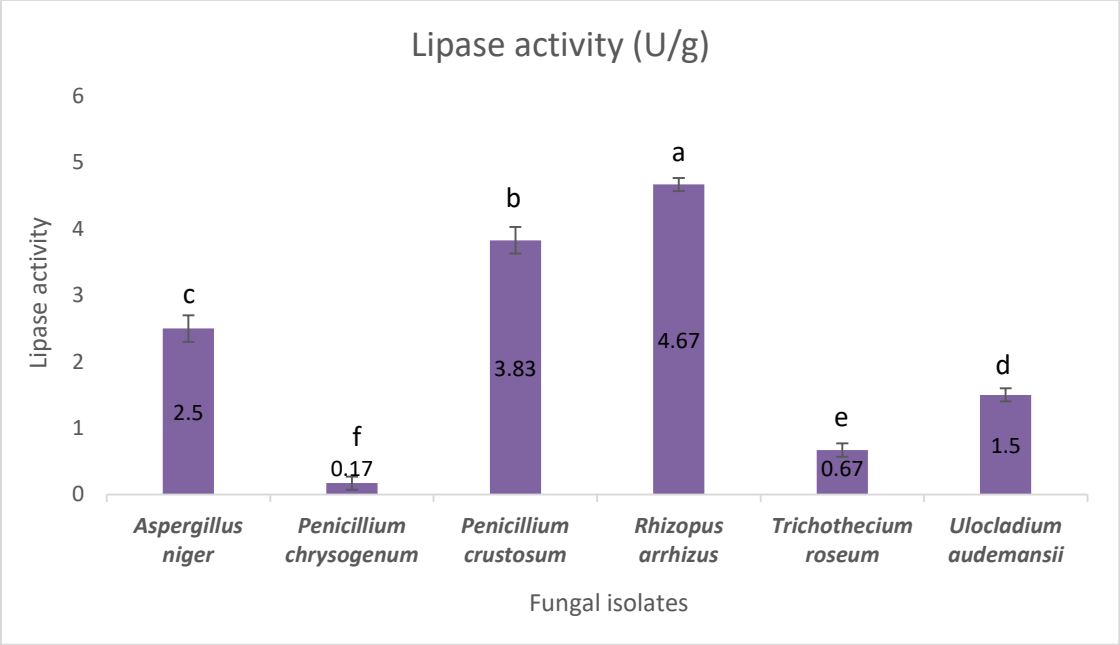

Fig. 3. Quantitative screening of fungal isolates for lipase production.

*a, b, c, d, e, and f: each letter is produced very differently from the other. Values followed by the same letter do not differ significantly ($p < 0.05$). Error bars represent the standard deviation of the mean.

2. Molecular identification of the most potent fungal isolates

The most potent fungal isolates for lipid production were identified molecularly as *F. verticillioides* and *R. arrhizus* (27.27% and 25.64%, respectively, as shown in Table 2), as well as *P. polonicum* which exhibited a relatively high lipase activity of 3.83 U/g (as depicted in Figure 3). Briefly, DNA amplification was performed using a universal fungal primer set internal transcribed spacer ITS1 and ITS4. Following genomic DNA amplification, the PCR product underwent sequencing, processing, and subsequent submission to GenBank.

BLAST analysis was conducted to process the sequences, revealing an ancestral association of the rRNA gene sequences of *F. verticillioides* and *R.*

arrhizus with other strains of the same species. Phylogenetic trees were also constructed for each strain (Figures 4 and 5). The sequence data of *F. verticillioides* were submitted to GenBank with accession number OR910640 and designated as *F. verticillioides* AUMC 16026. Additionally, the rRNA gene sequence of *R. arrhizus* was designated as *R. arrhizus* AUMC16025 (accession number OR910641), and that of P. polonicum was designated as *P. polonicum* Pmor (accession number PP263626).

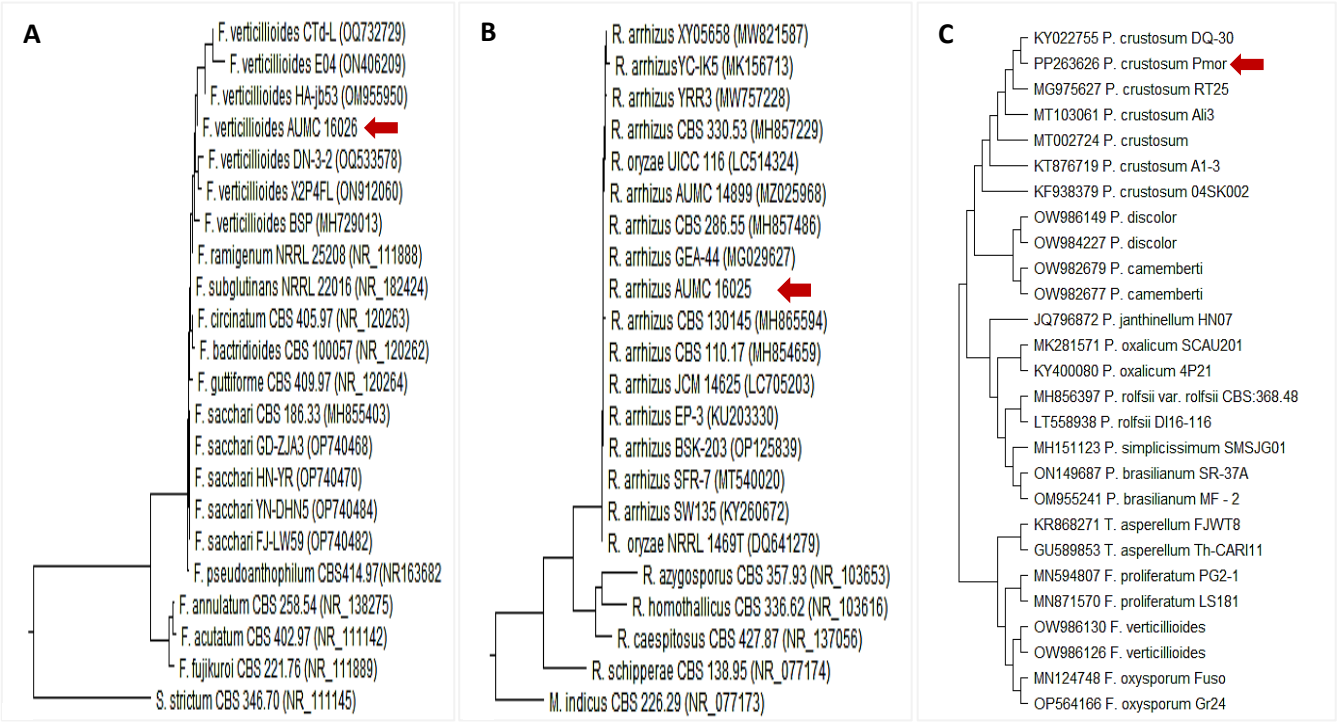

Fig. 4. Phylogenetic tree based on ITS sequences of rDNA of (arrowed) (A) *Fusarium verticillioides* AUMC 16026, (B) *Rhizopus arrhizus* AUMC 16025, and (C) *Penicillium crustosum* Pmor aligned with sequences of closely related strains accessed from GenBank. Phylogenetic analyses were done using MEGA6.

3. Optimization of culture conditions for lipid production

Two fungal isolates were selected for optimization of lipid production, specifically *F. verticillioides* AUMC 16026 and *R. arrhizus* AUMC 16025 due to their clear and abundant blue-black droplets, as well as their lipid content percentages (27.27 and 25.64, respectively).

Effect of incubation period on fungal lipid production

F. verticillioides AUMC 16026 and *R. arrhizus* AUMC 16025 were grown at different tested periods. The effect of different incubation periods on the lipid production by the two tested fungal genera was displayed in Figure 5A. The data indicated that the highest lipid production for the two fungal genera *F. verticillioides* AUMC 16026 and *R. arrhizus* AUMC 16025 was recorded after three days of incubation (28.6% and 30%, respectively). As for the biomass of *F. verticillioides* AUMC 16026 and *R. arrhizus* AUMC 16025, it gradually increased during the first 7 days of incubation, reaching maximum values on the seventh day of incubation (0.25g/100 ml and 0.064 g/100 ml, respectively), as shown in Figure 5B.

Effect of incubation temperature on fungal lipid production

F. verticillioides AUMC 16026 and *R. arrhizus* AUMC 16025 were incubated at different incubation temperatures for 3 days. At 25°C, *R. arrhizus* AUMC 16025 demonstrated the highest fungal biomass and lipid production, as depicted in Figures 5C and 5D, with values of 29.9% and 0.087 g/100 ml, respectively. Conversely, *F. verticillioides* AUMC 16026 achieved its peak fungal biomass and lipid production at 30°C, with values of 29.6% and 0.125 g/100 ml, as described in the same figures.

Effect of initial medium pH on fungal lipid production

F. verticillioides AUMC 16026 was incubated for 3 days at a temperature of 30°C with different initial medium pH values. The results disclosed that the

highest percentage of lipid production (31.7%, as described in Figure 5E) was achieved when the initial medium pH value was 4.0. On the other hand, the optimum initial medium pH value for biomass production was 7 (0.175 g/100 ml, as shown in Figure 5F). For *R. arrhizus* AUMC 16025, the incubation was conducted for 3 days at a temperature of 25°C with different initial medium pH values. The greatest percentage of lipid accumulation (30.3%, as shown in Figure 5E) was accomplished when the initial pH was 4.0. Contrarily, the optimum initial medium pH value for biomass production was 5 (0.142 g/100 ml, as explained in Figure 5F).

Effect of different carbon sources on lipid production

F. verticillioides AUMC 16026 was incubated for three days at 30°C, with six different carbon sources and a medium pH of 4.0. As seen in Figures 6A and 6B, the highest biomass and lipid production was fulfilled when sucrose was employed as the sole carbon source (31.92% and 0.26 g/100ml, respectively). Similarly, *R. arrhizus* AUMC 16025 was also incubated for three days at 25°C, with six different carbon sources and a medium pH of 4.0. The results unveiled that glucose was the best carbon source for lipid production (30.49%, Figure 6A) while sucrose was found to be the ideal carbon source for biomass production (0.157 g/100 ml, Figure 6B).

Effect of different nitrogen sources on lipid production

Four distinct nitrogen sources were utilized for *F. verticillioides* AUMC 16026, along with sucrose as a carbon source for each. the culture was incubated for three days at 30°C and pH 4.0.

The nitrogen sources included two organic and two inorganic sources. As depicted in Figure 6C, the maximum lipid production (32.67%) was fulfilled when an inorganic source, sodium nitrate, was employed. However, the optimal nitrogen source for biomass production was inorganic ammonium sulfate (0.344 g/100 ml, Figure 6D). In general, inorganic nitrogen sources were found to be better for lipid production and growth of *F. verticillioides* AUMC 16026. To cultivate *R. arrhizus* AUMC 16025, four different nitrogen sources were employed separately with glucose as a carbon source. The fungus was cultured for three days at pH 4.0 and 25°C. The nitrogen sources enclosed two organic and two inorganic sources. As seen in Figure 6C, the highest lipid production for *R. arrhizus* AUMC 16025 (31.62%) was recorded when an organic source, peptone, was used. However, the optimal nitrogen source for the growth of *R. arrhizus* AUMC 16025 was inorganic ammonium sulfate (0.188 g/100 ml, Figure 6D).

Effect of static and shaking conditions on fungal lipid production

The lipid and biomass production of *F. verticillioides* AUMC 16026 and *R. arrhizus* AUMC 16025 strains were evaluated under static and shaking conditions (at 150 rpm) at the previously optimized conditions. For *F. verticillioides* AUMC 16026, the optimal conditions were found to be a 3-day incubation time, an incubation temperature of 30°C, a pH of 4.0, sucrose used as the carbon source, and sodium nitrate utilized as the nitrogen source. On the other hand, *R. arrhizus* AUMC 16025 exhibited optimal conditions with a 3-day incubation time, an incubation temperature of 25°C, a pH of 4.0, glucose employed as the carbon source, and peptone serving as the nitrogen source. The results in Figure 6E unveiled that the highest values of lipid production for *F. verticillioides* AUMC 16026 and *R. arrhizus* AUMC 16025 were documented when the two fungal genera were grown under static conditions (44.44% and 36.9%, respectively). Contrarily, the best growth for the two tested fungi was noticed under shaking conditions (0.085 g/100 ml and 0.08 g/100 ml, respectively, Figure 6F).

Optimization results unearthed that *F. verticillioides* AUMC 16026 was a more potent lipid producer (44.44%) than *R. arrhizus* AUMC 16025 (36.9%, Figures 6 E and 6 F). Consequently, *F. verticillioides* AUMC 16026 oil was selected as a feedstock for biodiesel synthesis.

4. Methanolic transesterification

The transesterification process was carried out using the whole-cell lipase of *R. arrhizus* AUMC16025 and *Penicillium crustosum* Pmor as a biocatalyst to convert the extracted lipid from *F. verticillioides* AUMC 16026 into biodiesel. Finally, gas chromatography analysis was performed on the produced biodiesel. The percentage of biodiesel generated from *F. verticillioides* AUMC 16026 oil was 36.74% and 35.77% using *R. arrhizus* 16025 and *P. crustosum* Pmor whole-cell lipase, respectively.

5. Analysis of biodiesel by gas chromatography

The composition of fatty acid methyl esters of biodiesel produced from *F. verticillioides* AUMC 16026 oil transesterified using whole-cell lipase of *R. arrhizus AUMC* 16025 and *P. crustosum* Pmor was analyzed by GC/FID. As illustrated in Table 3 and Figure 7, FAMEs profiles with corresponding retention time (RT) confirmed the presence of linoleic acid (27.28%) and oleic acid (25.46%) mostly for *F. verticillioides* AUMC 16026 oil transesterified using *R. arrhizus AUMC* 16025 whole-cell lipase. Oppositely, oleic (23.53%), palmitoleic (21.28 %), linoleic (18.88%), and palmitic acids (11.84%) were the most abundant FAMEs for *F. verticillioides* AUMC 16026 oil transesterified using *P. crustosum* Pmor whole-cell lipase.

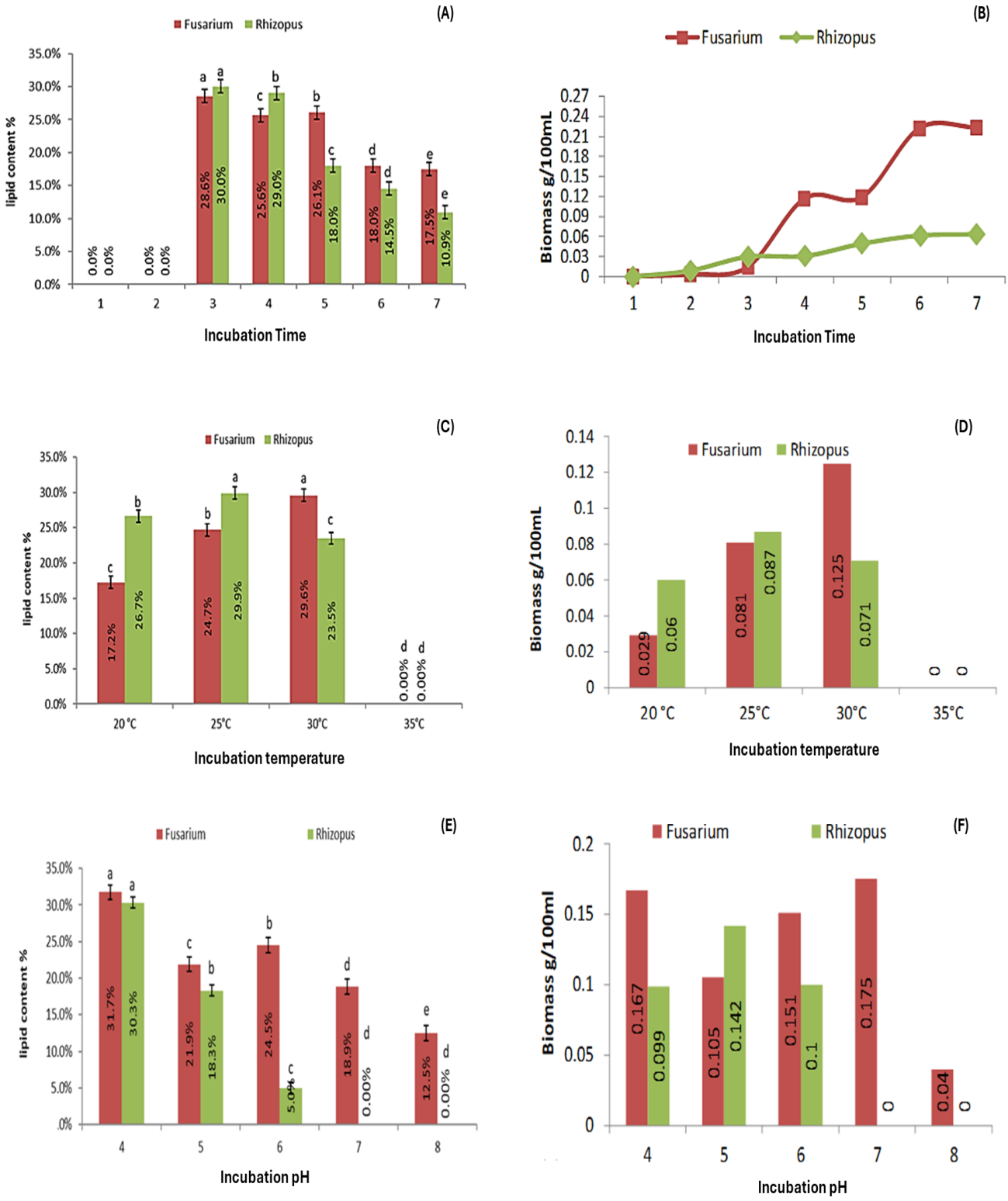

Fig. 5. The effect of the incubation period (A, B), incubation temperature (C, D), and incubation pH (E, F) on lipid production and biomass production.

*a, b, c, d, and e: each letter is produced very differently from the other. Values followed by the same letter do not differ significantly ($p < 0.05$). Error bars represent the standard deviation of the mean.

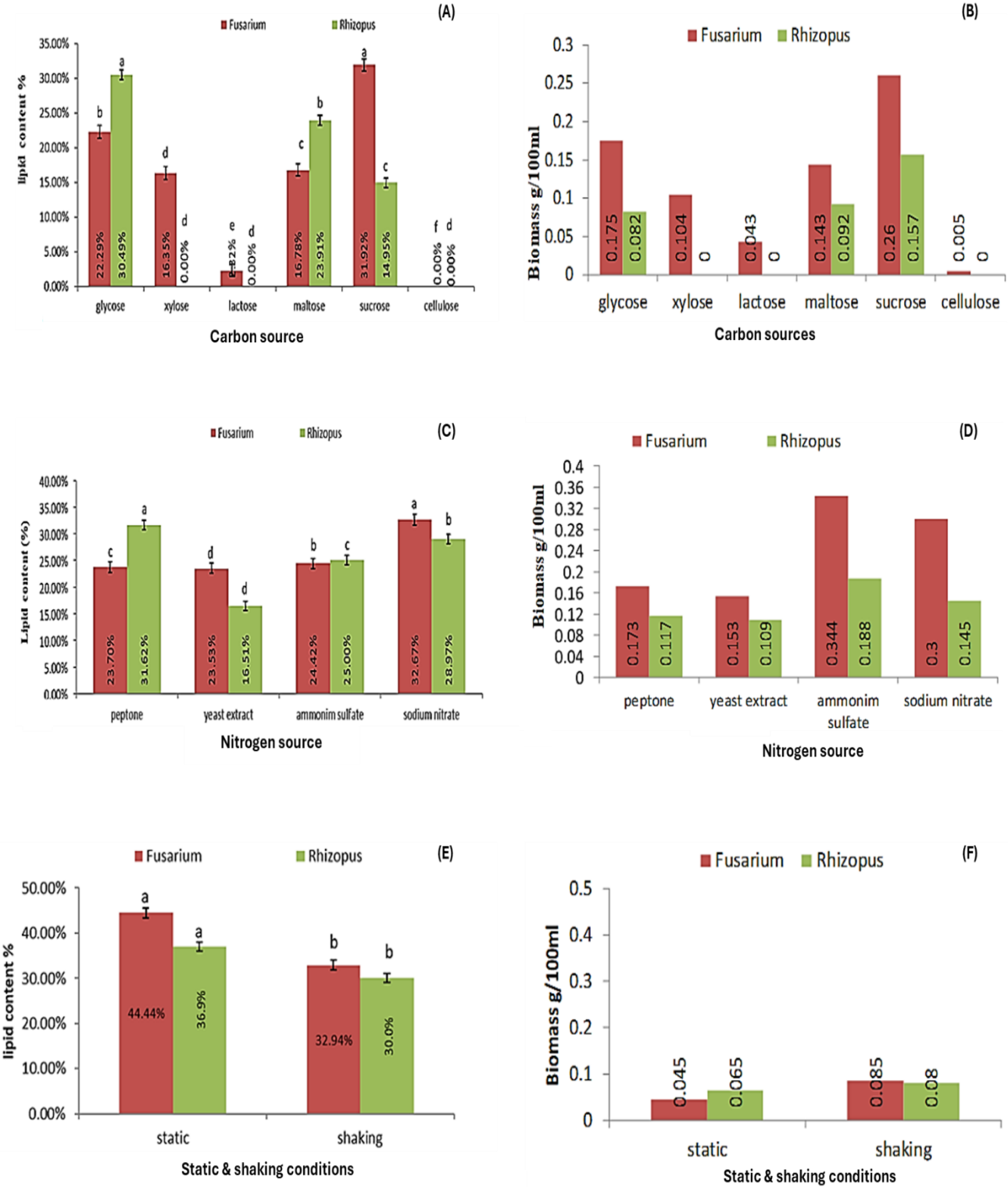

Fig. 6. The effect of carbon source (A, B), nitrogen source (C, D), and static and shaking condition (E, F) on lipid production and biomass production.

*a, b, c, d, e, and f: each letter is produced very differently from the other. Values followed by the same letter do not differ significantly ($p < 0.05$). Error bars represent the standard deviation of the mean.

Table 3. Fatty acid composition of biodiesel produced from *F. verticillioides* AUMC 16026 oil transesterified by *R. arrhizus* AUMC 16025 and *P. crustosum* Pmor whole-cell lipase.

Peak	RT	Name	C	Area sum% *a*	Area sum% *b*
1	16.072	Lauric acid	(C12:0)	0.17	0.27
2	18.73	Tridecanoic acid	(C13:0)	0.06	0.38
3	21.58	Myristic acid	(C14:0)	0.23	0.33
4	26.299	cis-10-Pentadecenoic acid	(C15:1)	0.08	0.32
5	27.337	Palmitic acid	(C16:0)	15.32	11.84
6	28.574	Palmitoleic acid	(C16:1)	9.56	21.28
7	31.207	Margaric acid	(C17:0)	6.75	0.6
8	32.812	Stearic acid	(C18:0)	7.77	7.72
9	33.667	Oleic acid	(C18:1)	25.46	23.53
10	35.395	Linoleic acid	(C18:2)	27.28	18.88
11	37.512	Linolenic acid	(C18:3)	0.87	0.84
12	37.955	Arachidic acid	(C20:0)	0.66	0.76
13	41.395	Homo-γ-linolenic acid	(C20:3)	2.28	3.53
14	42.796	Behenic acid	(C22:2)	0.25	1.3
15	44.237	EPA	(C20:5)	0.72	0.86
16	44.773	*cis*-13,16-Docosadienoic acid	(C22:2)	0.33	0.51
17	47.367	Lignoceric acid	(C24:0)	0.21	3.15
18	49.323	DHA	(C22:6)	1.99	3.9

* *a:* biodiesel produced using *R. arrhizus* AUMC 16025 whole-cell lipase; b: biodiesel produced using *P. crustosum* Pmor whole-cell lipase

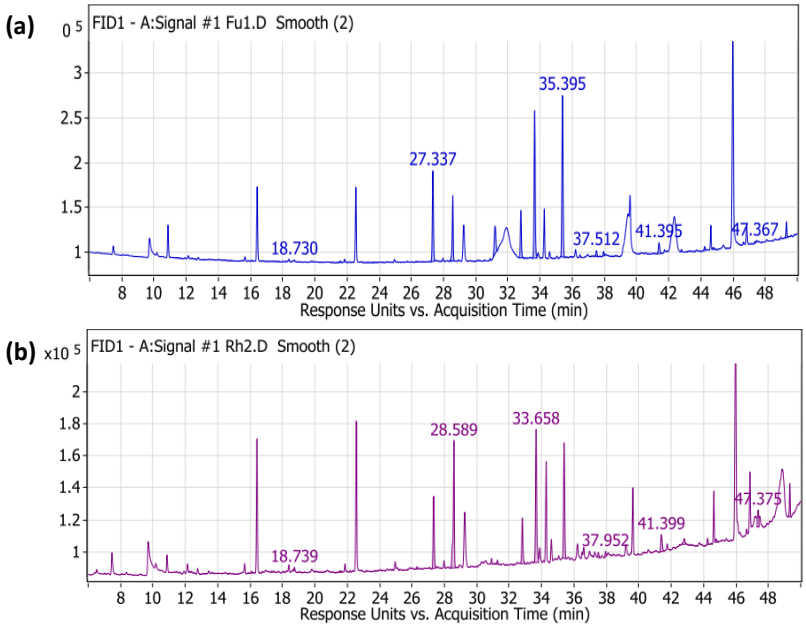

Fig. 7. Gas chromatography-profile analysis of biodiesel produced from *F. verticillioides* AUMC 16026 oil using (a) *R. arrhizus* AUMC 16025 whole-cell lipase and (b) *P. crustosum* Pmor whole-cell lipase.

Discussion

Biodiesel (Greek, bio, life + diesel from Rudolf Diesel) refers to a diesel-equivalent, processed fuel derived from biological sources. Chemically, biodiesel is defined as the mono-alkyl esters of long-chain fatty acids derived from renewable biolipids. It is an environmentally friendly alternative fuel to Petro-diesel because it is renewable, nontoxic, and biodegradable and reduces emissions.

Microorganisms that accumulate lipids need a medium that is deficient in other nutrients, particularly nitrogen, and abundant in sugars or polysaccharides. Previous researchers have provided evidence for the capacity of microorganisms to produce lipids in a medium with minimal nitrogen **(Gema *et al.*, 2002)**. The limitation of nitrogen in the medium prevents cell proliferation and the already-formed lipids are stored in the cells, resulting in lipid accumulation. Low media levels cause nicotinamide adenine dinucleotide isocitrate dehydrogenase (NAD-IDH) activity to decline. This results in the suppression of the tricarboxylic acid circulation (TCA) cycle, which stops protein synthesis and causes lipid accumulation because excess carbon is converted to triglycerides (TAG). Two enzymes, malate enzyme and ATP citrate lyase, have been found to affect lipid accumulation.

The recovered strains were cultivated on a nitrogen-limited medium containing glucose as a carbon source (high C/N ratio corresponding to 80). Multiple studies reported that a high carbon-to-nitrogen ratio (C/N) improved lipid accumulation by 18% to 46% when increased from 20 to 70 **(Kamoun *et al*., 2018).**

The higher lipid accumulation by oleaginous microorganisms demands optimizing the medium cultivation conditions and nutritional factors **(Ageitos *et al.*, 2011)**. Lipid content varied according to the nature of the microorganism and culture conditions such as incubation period, incubation temperature, medium pH, carbon sources, nitrogen sources, and static and shaking conditions **(Abdelhamid *et al.*, 2019).** *F. verticillioides* AUMC 16026 and *R. arrhizus* AUMC 16025 were selected for optimization of lipid accumulation, as they were oleaginous fungi with a total lipid content of 20% or more of their dry weight **(Ratledge, 2013)**.

The incubation period has an observable effect on lipid production. The lipid content of each strain differs based on its specific growth rate. In the current study, the third day of incubation was the optimum incubation period for *F. verticillioides* AUMC 16026 and *R. arrhizus* AUMC 16025 yielding maximum lipid production of 28.6% and 30%, respectively. These results agree with those reported by **Kumar *et al.* (2011)** who investigated that the accumulation of lipids by *Mortierella* sp. started 24 h after the depletion of the nitrogen sources and reached 42% until the carbon source exhaustion. Afterward, the lipid yield decreased.

The temperatures of 25 °C and 30 °C were identified as the optimal incubation temperatures for lipid production. These temperatures were determined based on the high activity levels exhibited by all fungal enzymes involved in the process **(Carlile *et al.*, 2001)**. **Patel and Desai (2019)** estimated that the incubation temperature of 30ºC was the optimum value for extracellular enzyme production for polysaccharide degradation and lipid accumulation by fungi. **Venkata and Venkata (2014)** reported that the optimum temperature for *Aspergillus awamori* was 30°C. However, lipid production was drastically reduced gradually when the fungus was incubated at a higher temperature (35–40°C).

The original pH of the medium was discovered to be a crucial environmental factor influencing lipid accumulation. This is because the concentration of hydrogen ions in the medium directly impacts the permeability of the plasma membrane, which regulates the transportation of substances into and out of the cells. The pH level also plays a role in metabolic activity, cell growth, sporulation, and the formation of products **(Minhas *et al.*, 2016)**.

In contrast with the findings of **Ali *et al.* (2017); Jiru *et al.* (2017); and Sayeda *et al.* (2019),** the present data exhibited that the optimum pH was 4. They claimed that pH values ranging between 5 and 6 were determined to be the optimal pH range for the growth and lipid production by most oleaginous fungi.

In this study, six different carbon sources were tested (glucose, xylose, sucrose, lactose, maltose, and cellulose). The highest lipid accumulation was obtained with glucose for *R. arrhizus* AUMC 16025 where lipid production percentages reached 30.49%. Glucose is known to serve as the best source of carbon utilized by oil-producing microorganisms to yield maximum lipid production. This is because glucose is an easily obtainable and inexpensive carbon source, and every cell has the necessary machinery to metabolize it. It does not need to synthesize extra enzymes to isomerize alternative carbon sources of glucose, which requires time and energy **(Li *et al.*, 2007**, **Carvalho *et al.*, 2018,** and **Assawah *et al.*, 2020)**. Interestingly, for *F. verticillioides* AUMC 16026, the highest biomass and lipid production was recorded when sucrose was used as a carbon source (31.92%, 0.26 g/100 ml).

The latest findings support earlier research showing that the type of fungus used to produce lipids determines the appropriate source of nitrogen. For example, **Abdelhamid *et al.* (2019)** proclaimed that using peptone as a nitrogen source induced the highest lipid accumulation (43.06%) for *Penicillium commune* NRC 2016. **Youssef *et al.* (2021)** declared that yeast extract was the best nitrogen source for *Aspergillus niger*. Furthermore, **Ramírez-Castrillón *et al.* (2017)** found that inorganic ammonium sulfate was the most suitable nitrogen

source. The present study proved that inorganic nitrogen sources such as ammonium sulfate were more effective for cell growth compared to organic nitrogen sources such as peptone.

The results of the present study highlighted that the highest lipid contents were produced in static conditions. This finding was consistent with the results of previous studies conducted by **Abdelhamid *et al.* (2019)**, **Sayeda *et al.* (2019)**, and **Ali and El-Ghonemy (2014)**, who reported that lipid accumulation was greater in static conditions compared to shaking conditions. **Shafiq and Ali (2017)** explained that many species of oleaginous fungi could accumulate significant amounts of intracellular lipids in static conditions. In shaking conditions, the stored intracellular lipid content was utilized to maintain generations of cells resulting in the production of lipid-free biomass **(Subhash and Mohan, 2014)**. This phenomenon was known as lipid turnover **(Wu *et al.*, 2010)**. Oppositely, shaking conditions led to optimal growth due to increased cell biomass along with dissolved oxygen **(El-Fadaly *et al.*, 2009)**. Moreover, this suggested that the fungal biomass did not correspond to their lipid content.

Microbial lipases are currently receiving much attention due to their biotechnological potential, including broad substrate specificity, high yield, and low-cost production. Therefore, they have been widely utilized in industrial applications, such as biodiesel production, organic synthesis, food, pharmaceutical, and detergent chemistry. Lipases are enzymes capable of hydrolyzing triacylglycerol **(Elhussiny *et al.*, 2020)**.

In the present investigation, the synthesis of biodiesel from fungal oil and methanol by whole-cell lipase as a catalyst has been examined to overcome issues of using chemical catalysts. Thus, lipase activity was screened qualitatively by phenol red agar medium screening plates that contained lipids as a carbon and energy source for fungi. Eight strains were reported to have lipolytic activity specifically *P. crustosum* Pmor, *A. niger*, *P. chrysogenum*, *T.* roseum., *U. oudemansii*, and *Rhizopus arrhizus* AUMC 16025. Most of these strains were isolated from sources containing oils. The results of the current study stipulate that oily isolation sources are the most favorable sources for isolating lipase-producing fungi.

Isolates showing good lipid hydrolysis activity were further subjected to quantitative screening. From the observation, *R. arrhizus* AUMC 16025 exhibited the highest lipase activities followed by *P. crustosum* Pmor.

Numerous studies have utilized *Rhizopus* sp. whole-cell lipase to produce biodiesel. **Athalye *et al.* (2013)** used *R. oryzae* as a biocatalyst to produce biodiesel from cottonseed oil. Similarly, **He *et al.* (2016a)** employed *R. chinensis* to produce biodiesel from soybean oil. Additionally, **Elhussiny *et al.* (2020, 2023)** adopted *R. americanus* 2aNRC11 and *R. stolonifer* 1aNRC11 whole-cell lipase to produce biodiesel from waste frying oil.

Conversely, only a few studies have utilized *Penicillium* sp. whole-cell lipase. **Lima *et al.* (2019)** manipulated *P. citrinum* as a biocatalyst for the hydrolysis of vegetable oils, making *P. crustosum* Pmor a new promising strain in whole-cell lipase production. To our knowledge, there were no reports of using *Penicillium* sp. whole-cell lipase for biodiesel biosynthesis.

Consequently, the two strains *R. arrhizus* AUMC 16025 and *P. crustosum* Pmor were selected to be applied in the transesterification of *F. verticillioides* AUMC 16026 oil to compare their results.

A higher percentage of monounsaturated and saturated FAs (66.28% and 70.18%) than unsaturated FAs (33.72% and 29.82%) were estimated for biodiesel synthesized by whole-cell lipase of *R. arrhizus* AUMC 16025 and *P. crustosum* Pmor, respectively. These results agreed with those of **Ramos *et al.* (2009)** and **Youssef *et al.* (2021)** who noticed that good biodiesel quality should have low levels of polyunsaturated fatty acids and high levels of saturated and monounsaturated fatty acids. Furthermore, based on the above remark, it could be concluded that *P. crustosum* Pmor produced biodiesel that was slightly better than *R. arrhizus* 16025. This implied that *P. crustosum* Pmor whole-cell lipase exhibited promising potential as an effective biocatalyst for biodiesel synthesis, surpassing the capabilities of *R. arrhizus* 16025. It was worth noting that this marked the initial documentation of *P. crustosum* Pmor whole-cell lipase being employed as a biocatalyst for biodiesel synthesis.

Conclusion

It has been concluded, based on the optimization results, that *F. verticillioides* AUMC 16026 was a more potent lipid producer (44.44%) than *R. arrhizus* AUMC 16025 (36.9%). Therefore, *F. verticillioides* AUMC 16026 has been selected to produce oil that would be used as a feedstock for ecofriendly biodiesel production.

During the study, it was found that the percentage of *F. verticillioides* AUMC 16026 oil conversion to biodiesel using *R. arrhizus* AUMC 16025 and *P. crustosum* Pmor whole-cell lipase as a biocatalyst was 36.74% and 35.77%, respectively. Further work is needed to optimize the conditions of the transesterification process to increase the percentage of oil conversion to biodiesel by *P. crustosum* Pmor whole-cell lipase. Both the biodiesel produced by the two lipases and the biodiesel made from leftover frying oil and vegetable oil have a quality that is reasonably comparable to one another.

R. arrhizus AUMC 16025 and *P. crustosum* Pmor whole-cell lipase exhibit promising potential as an effective biocatalyst for biodiesel synthesis.

Additionally, this is the first record of using *P. crustosum* Pmor whole-cell lipase as a biocatalyst for biodiesel synthesis.

Authors' contributions:
Ereny Atef Wadee: Methodology, Visualization, Writing - original draft. Saadia Mohamed Easa: Supervision. Abd El Hamid Aly Hamdy: supervision. Hala A. Amin: Supervision, Conceptualization, Writing - review & editing. Adel A. Al-Mehallawy: Supervision, Writing - review & editing. Nadia A. Elkady: Supervision, Processing and interpretation of experimental data, Writing – review& editing.

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تقييم وقود الديزل الحيوي المنتج من بعض زيت الفطريات الدهنية باستخدام الخلية الكاملة المنتجة لإنزيم الليبيز

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يعتبر الديزل الحيوي، أسترات ميثيل الأحماض الدهنية، هو بديل مستدام وصديق للبيئة. تهدف هذه الدراسة إلى إنتاج الديزل الحيوي باستخدام الزيت المنتج من الفطريات الدهنية، تحديدًا، فطر *فيوزاريم فيرتسلويد* كمادة أولية والخلية الفطرية الكاملة المنتجة لليبيز لسلالتي *ريزوبس أريزيس* و*بنسليم كريستوسم* كعامل حيوى محفز. تم عزل سلالات فطرية مختلفة واختبار قدراتها على إنتاج الدهون وإنتاج الليبيز. أظهر *فيوزاريم فيرتسلويد* أعلى إنتاج من الدهون عند زراعته في وجود السكروز ونترات الصوديوم (الأس الهيدروجيني ٤) لمدة ٣ أيام عند ٣٠ درجة مئوية في ظل ظروف ثابتة. تميز فطر *ريزوبس أريزيس* أن لديه أعلى نشاط لليبيز بقيمة ٤,٦٧ U/g يليه *بنسليم كريستوسم* ٣,٨٣ U/g. لذلك، تم اختيارهم لأسترة الزيت الفطري. تم استخدام كروماتوغرافيا الغاز لتحليل أسترات ميثيل الأحماض الدهنية المنتجة. كشف تحليل الديزل الحيوي المنتج من زيت فطر *فيوزاريم فيرتسلويد* باستخدام ليبيز خلية *ريزوبس أريزيس* الكاملة أن حمض اللينوليك (٢٧,٢٨٪) والأوليك (٢٥,٤٦٪) كانت أسترات الميثيل الأكثر وفرة. بينما أظهرت تلك التي تم إنتاجها باستخدام ليبيز خلية *بنسليم كريستوسم* الكاملة أن حمض الأوليك (٢٣,٥٣٪) والبالميتوليك (٢١,٢٨٪) الأكثر وفرة. إن جودة الديزل الحيوي المنتج مشابهة لجودة الديزل الحيوى المنتج من الزيت النباتي ونفايات زيت القلي. هذا يشير إلى أن الزيت المستخرج من فطر *فيوزاريم فيرتسلويد* هو مادة أولية جيدة لإنتاج الديزل الحيوي.