Biodiesel production from local isolate *Penicillium commune* NRC 2016

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Abstract

Twenty five local filamentous fungi were screened for their abilities to produce lipids using Nile-red staining assay. Among the tested filamentous fungi, *Penicillium commune* NRC 2016 for biodiesel production was selected and identified based on the morphological and molecular methods. Gas chromatography (GC) analysis of lipids by this fungus showed that, the resulted fatty acids are suitable for biodiesel production. The resulted fatty acids were 20% palmitic acid, 4.65% stearic acid, 13.77% oleic acid, 32% linoleic acid, 1.75% α-linolenic acid and 16.1% arachidonic acid. In conclusion, this work revealed the possibility of using the promising fungal strain *P. commune* NRC 2016 in biodiesel production.

Key words

Biodiesel - *Penicillium commune* NRC 2016 – GC analysis - Fatty acids

1-Introduction

The demand for renewable fuels has greatly increased due to limited energy resources and high oil price. Other important factors are the increased demand for energy supply and the growing concern about global warming, which is linked to the burning of fossil fuels. The use of fossil fuels accounts for about 90% of the requirement of global energy **(Gavrilescu and Chisti, 2005).** Economic and geopolitical restrictions on the use of oil allied to environmental concerns promoted the development of the biofuels market. Biodiesel is a biofuel produced from renewable sources, such as vegetable oil or animal fat, by the transesterification of triglycerides (TG) to form fatty acid alkyl esters (**Gunstone, 2001 and Rottig *et al.*, 2010).** Biodiesel is used as a partial or full substitute for petrol diesel in unmodified combustion engines for road and waterway transport, as well as engines used for power generation **(Gavrilescu and Chisti, 2005).** Biodiesel fuels are increasingly attracting attention worldwide because of its environmental benefits such as biodegradability; a decrease of sulfur and aromatic hydrocarbons content, which reduce their emission during fuel combustion and lower emission of CO, CO_2 and particulate materials **(Demirbas, 2008).** Moreover, it directly influences human health by reducing 95% of cancer risk when compared with fossil diesel **(Huang *et al.*, 2010).** The main sources of materials for biodiesel production are vegetable

oils and animal fats **(Hanna, 1991).** Fat of animal origin often contains large amounts of free fatty acids, which make its conversion into biodiesel of good quality very difficult. Moreover, the current animal fat production is not sufficient to meet the entire need for fuel consumption **(Canakci and Van Gerpen, 2001 and Demirbas, 2003**). Concerns have been expressed about the competition of vegetable oil with food supply by the use of arable land **(Brennan and Owende, 2010).** The use of microorganisms that can be easily cultivated and yield large amounts of lipids, especially triglyceride (TG), has been regarded as one of the most promising solutions to solve the negative aspects of the production of biodiesel from agricultural or animal sources **(Tao *et al*., 2006).** Oleaginous microorganisms are defined as microbial species in which the content of lipids exceeds in 20% its dry weight **(Meng *et al*., 2009).** The biological diversity of microorganisms is remarkable and specific metabolic profiles may support lipid accumulation **(Mutanda *et al*., 2011)**. The content of oil in some microorganisms can reach up to 70% of the total cellular dry weight under appropriate culture conditions **(Meng *et al*., 2009)**. Lipids accumulated in microorganisms such as fungi, bacteria, and algae are mainly in the form of triacylglycerols (TAG), used as metabolites for energy storage. Most of these lipids, which are composed of long chain fatty acids, are similar to conventional vegetable oils (**Rude and Schirmer, 2009).** Compared with vegetable oils and animal fats, the production of microbial oil has many advantages, such as: (i) the fastest growing, (ii) the recovery of cells to extract oil requires less work than the harvesting of oleaginous plants, (iii) no influence of environmental conditions in lipid accumulation when microbial growth is performed in closed systems (bioreactors) and (iv) easy growth in large scale **(Li *et al*., 2008).** This study concerned with studying the production of valuable biodiesel by *P. commune* NRC 2016, and at the same time, the harmful effects of fossil fuel in the environment will be reduced.

2-Materials and Methods

2.1 Samples collection and fungi isolation

Five soil samples gave codes: A, B, C, D, and E were collected in October 2014 from several districts in Egypt: Giza (2 samples), Assiut (2 samples) and El-Menia (1 sample) governorates. The sample placed in clean plastic bags and stored at 4ºC until used. Basal medium was used for isolation and purification of oleaginous fungi according to **Li *et al*. (2011)**. The typical formula was (g/L): xylose 100.0; yeast extract 1.0; KH_2PO_4 2.0; $MgSO_4.7H_2O$ 0.75; Na_2HPO_4 1.0; $CaCl_2.2H_2O$ 0.2; $FeCl_3$ 0.01, and $ZnCl_2$ 0.1 then the pH was adjusted at 7.0 (±1), 10ml/L of Rose Bengal (4, 5, 6-Tetrachlorofluorescein) was added at concentration 50 mg/L. One gram of soil particles was placed in a graduated cylinder and 50

ml of the sterilized medium was added. The mixture was incubated for 24 h. at 28°C under shaking (180 rpm). One milliliter of the desired dilution was transferred directly into each of sterilized agar medium cooled to just above solidifying temperature then added to petri dishes and incubated at 28^{o}C for 7 days. The developing colonies were isolated and sub-cultured again until purification. For further investigation, growing fungal isolates were sub-cultured on slants of Czapek-Dox medium according to **Robinson *et al*. (1998)** as follows (g/L): glucose 30.0; $NaNO_3$ 3.0; K_2HPO_4 1.0; $MgSO_4.7H_2O$ 0.5; KCl 0.5; $FeSO_4.7H_2O$ 0.01, agar 15, at pH 6.0 (±1) and kept in a refrigerator at 4^{o}C until used.

2.2 Screening for oleaginous fungi isolates

2.2.1 Qualitative screening by using Nile Red Stain

For visualization of intracellular lipids, one ml of the culture broth was rinsed with *n*-hexane and then with phosphate buffered solution (PBS) (0.2 M, at pH 7). After the oil drops attached on mycelia were removed, the mycelia were used for visualization of intracellular lipids. The mycelia were mixed with 0.5 ml PBS solution and 0.05 ml Nile red solution at concentration 10 mg/ 1000 ml acetone **(Greenspan *et al*., 1985**). According to **Lim *et al*., (2003**) the Nile red was used for staining intracellular lipid droplets after the storage of the mixture of Nile red and mycelia for 30 min in a dark place, stained lipid bodies were photographed using fluorescence microscope (HBO50/ac Axiostar plus, Zeiss, Jena, Germany) equipped with a CCD camera (105 colar Axiocam, Zeiss, Jena, Germany)**.**

2.2.2 Quantitative screening by biodiesel production

For selection the highest lipid producer among the obtained isolates, they were cultured in a basal medium according to **Abu-Elreesh and Abd-El-Haleem, (2013)**. The typical formula was **(**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 glucose 50, with initial pH 6.0 (±1) the cultures were incubated at 28°C for 7 days, then the fungal biomass were obtained by centrifugation using sigma 3-18 KS centrifuge (5000 rpm/4°C/10 min) and washed three times with distilled water. Exacted weight of the biomass was taken, and frozen over night at –80°C. Direct transformation of frozen biomass was carried out according to the method of **(Lewis *et al*., 2000)** in a single step, 12 ml of methanol, hydrochloric acid, and chloroform at ratio10:1:1 respectively was added to the dried fungal biomass (200 mg) in screw-cap test tube and subjected to sonication (5 min; 20 kHz) to facilitate cell membrane disruption, pre-sonicated cells were suspended in the solution, vortexed and immediately placed at 90°C under stirring for 8 h in water bath at the end of reaction, the mixture was diluted with distilled water and then extracted with ethyl acetate (100 ml × 2). The two immiscible layers of distilled water and ethyl acetate were

separated using separating funnel and the ethyl acetate mixture was dried over anhydrous sodium sulfate and evaporated. The residual fatty acid methyl esters (FAMEs) were weighed to calculate the yield.

2.3 Gas Chromatographic analysis

Gas Chromatographic analysis for of fatty acids methyl esters (FAMEs) for the high producer isolates was performed in Central Laboratories in National Research Centre (NRC) using Agilent Technologies 6890 N (Net Work GC system) the USA. The oven was held at initial temperature 50ºC and maintained for 2 min. At rate 10, 8, 5, 6ºC/min, raised to 70, 170, 200 and 240ºC, at the rate of 2, 9, 5, 15 min and run time 60.17 min. Injector temp was held at 250ºC splitless. A capillary column HP-5MS (5% phenyl methyl siloxane) has a dimension of 30 m× 320 µm, film thickness 0.25 µm. The flame ionization detector temperature was 280°C and the flow rate was 1.5 ml/min. The carrier gas was nitrogen, with a flow rate of 30 ml/min. hydrogen flow rate was 30 ml/min and air flow rate 300 ml/min.

2.4 Classical and molecular identification of the promising fungal isolate

The promising biodiesel producer isolate (C1) was identified by morphological and molecular methods.

2.4.1 Morphological identification of the promising fungal isolate

Identification of the isolated fungi during our investigation was carried out using the morphological characteristics as colony diameter, a color of conidia, extracellular exudates, pigmentation and the color of reverse mycelium. Microscopic features were examined also as conidial heads, fruiting bodies, the degree of sporulation, and the homogeneity characters of conidiogenous cells by optical light microscope (10×90) Olympus CH40 according to **Ainsworth, (1971) and Pitt, (1985).** Fungal isolates were grown on Czapek-Dox medium (CYA) and malt extract-agar medium (MEA) at 28°C for 7 days. The cultures were then kept at 4°C.

2.4.2 Molecular identification of the best-producing fungal isolate

The molecular identification of the promising fungal isolate including the preparation of the fungal biomass, then extraction of fungal genomic DNA, and finally PCR amplification for fungal DNA.

2.4.2.1 Preparation of the fungal biomass:

The spores of 4 days old culture of tested fungi were collected by addition of 5 ml sterile saline solution into the slant and the obtained suspension was inoculated into 100 ml of Czapek Dox's medium in 250 ml Erlenmeyer flask. After incubation at 28ºC for 4 days, the

cultures were filtered and the biomass was collected and washed with distilled water three times.

2.4.2.2 Genomic DNA extraction

Genomic DNA of fungi was extracted according to **Sharma *et al.* (2007)** as the following; 50 to 100 mg fresh fungal mycelia were homogenized by mortar. Five hundred microliter of DNA extraction buffer (200 mM Tris-HCl pH 8, 240 mM NaCl, 25 mM EDTA, and 1% SDS) were then added to the homogenized fungal DNA. One ml of phenol/$CHCl_3$, in the ratio of 1:1 (v/v), was added and mixed gently for 10 min on a shaker at (150 rpm) followed by centrifugation at 15000 xg for 10 min. The upper phase was transferred to a new tube and 0.1 ml of 3 M sodium- acetate buffer (pH 5.2) and 2 ml of ethanol (96%) were added and mixed well, incubated for 30 min at -20°C followed by centrifugation (15000 xg/4°C/20 min). The resulting pellets were washed with 700 µl of 70% ethanol, air dried and re-suspended in 100 µl of sterile bi-distilled water.

2.4.2.3 PCR amplification

The primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used for the PCR. The PCR reaction was performed with 20 nanograms of genomic DNA as the template in a 30 µl reaction mixture by using an *EF-Taq* (Sol Gent, Korea) as follows: Activation of Taq polymerase at 95°C for 2 min, 35 cycles of 95°C for 1 min, 55°C, and 72°C for 1 minutes each were performed, finishing with a 10 minutes step at 72°C. The amplification products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing reaction was performed using a PRISM Big Dye Terminator v3.1 Cycle sequencing Kit. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95°C for 5 min, followed by 5 min on ice and then analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA).

3- Results

3.1 Qualitative screening for fungal isolates

The present study was carried out on 25 fungal isolates from locally soil samples collected from five locations in Egypt. The isolates were obtained using standard serial dilution technique from the original samples. The obtained results revealed that the majority of isolates were found in Assiut II while the minimum isolates were detected in Assiut I (Figure 1).

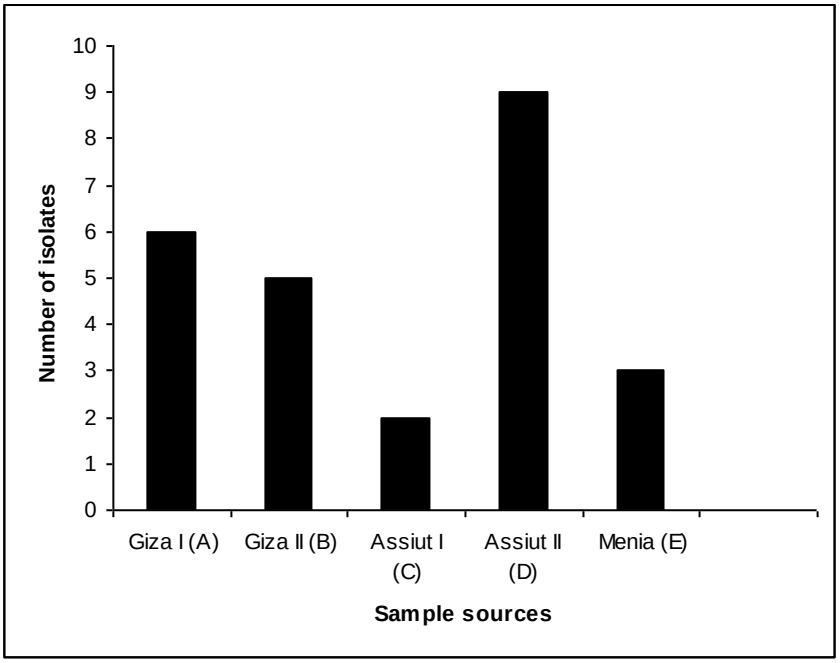


Figure (1) The number of isolated fungi from different locations in Egypt

All fungal isolates were exhibited strong fluorescence signals when using Nile-red assay. Figure (2) indicated the lipid particles under a fluorescence microscope. Nile-red viable colony staining assay was used to screen the biodiesel producers.

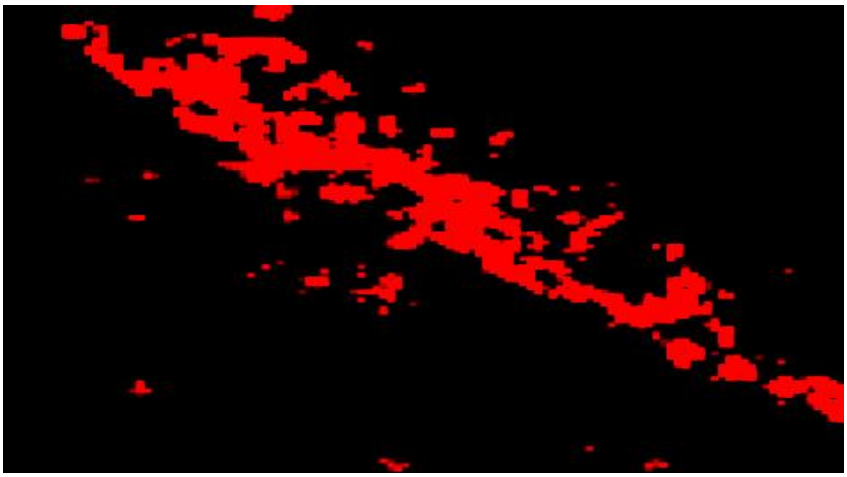


Figure (2) Fluorescence microscopy of Nile-red stained fungal strains

3.2 Biomass and biodiesel production

Fungal cells were grown in basal medium containing glucose and yeast extract as a carbon and nitrogen sources, respectively. Table (1) shows fungal biomass and biodiesel yield of all isolates. The highest biodiesel yield was obtained from the isolates A4, B4, C1, D10 and E1 of all locations respectively.

Table (1) Biomass and biodiesel production of the tested fungal isolates obtained from different locations (A, B, C, D, and E)

Isolates	Biomass (g/L)	Biodiesel (g/L)	Biodiesel (%)
A1	2.384	0.188	7.880
A2	1.460	0.100	6.800
A3	1.152	0.304	26.400
A4	**0.568**	**0.244**	**42.950**
A5	1.168	0.125	10.950
A6	4.000	0.108	2.700
B1	2.000	0.300	15.000
B2	0.400	0.012	3.000
B3	0.200	0.044	5.000
B4	**1.200**	**0.232**	**19.300**
B5	1.200	0.160	13.300
C1	**1.600**	**0.3568**	**22.300**
C2	2.268	0.168	7.400
D1	24.632	0.036	5.700
D3	1.248	0.024	1.900
D4	6.820	1.152	16.800
D6	1.424	0.044	3.080
D7	1.208	0.052	4.300
D8	0.868	0.040	4.600
D9	0.704	0.068	9.650
D10	**0.824**	**0.260**	**31.600**
D11	0.925	0.012	1.200
E1	**0.800**	**0.252**	**31.500**
E2	2.00	0.352	17.600
E3	2.264	0.148	6.500

3.3 Analysis of biodiesel by Gas Chromatography

The composition of fatty acid methyl esters with the profiles which are cited for the high producer isolates for each location (A4, B4, C1, D10, and E1) was in Table (2). According to that, the best producer isolate is C1. The single cell oils in C1 were found to contain a high fraction of mono and polyunsaturated fatty acids mainly 13.8% oleic acid (C18:1), 32.1% linoleic acid (C18:2), 1.8% α-linoleic acid (C18:3) and 18.4% arachidonic acid (C20:4), and a limited percentage of saturated fatty acids mainly 19.3% palmitic acid (C16) and 4.7% stearic (C18).

Table (2) Fatty acid composition of the high producer fungal isolates for each location (A4, B4, C1, D10 and E1)

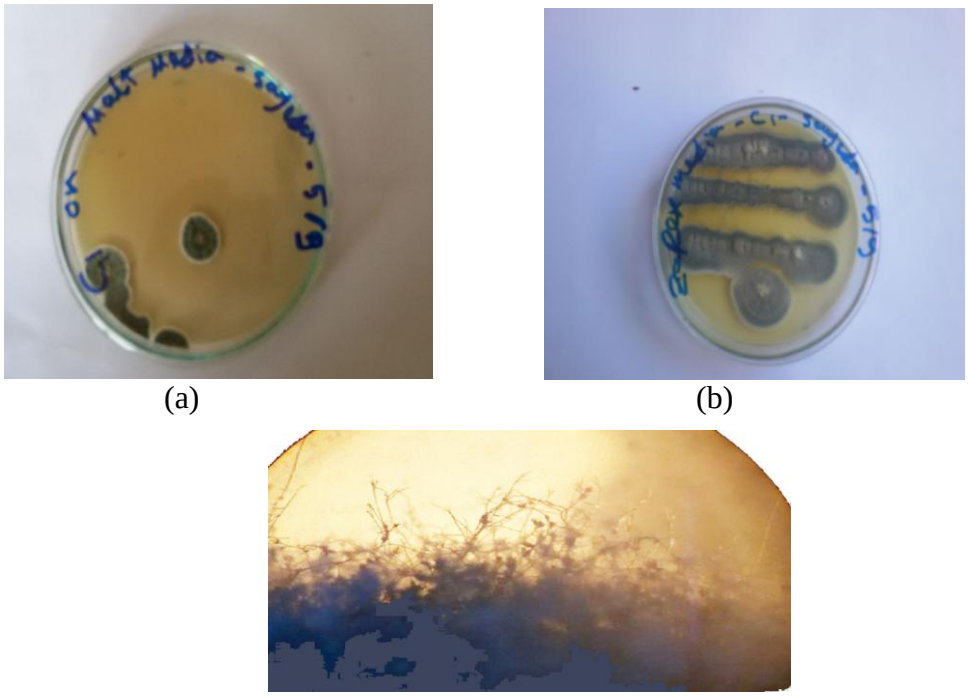
Isolates	Fatty Acid %						
	Palmitic acid (C16:0)	**Palmitoleic acid (C16:1)**	**Stearic acid (C18:0)**	**Oleic acid (C18:1)**	**Linoleic acid (C18:2)**	**α-Linolenic acid (C18:3)**	**Arachidonic acid (C20:4)**
A4	0.0000	0.1285	0.0000	0.4659	0.0000	0.0724	0.0000
B4	0.0000	0.1949	0.0000	2.1503	0.0000	0.3142	0.0000
C1	**19.3323**	**0.0000**	**4.6572**	**13.7730**	**32.0887**	**1.7569**	**18.3961**
D10	0.0000	2.0262	0.3286	11.1266	5.5675	3.0356	0.0000
E1	0.0000	0.8988	0.0000	6.4547	1.7298	0.0000	0.0000

3.4 Classical and molecular identification of the fungal isolate

Fungal taxonomy is traditionally based on comparative morphological features and molecular identification.

3.4.1 Morphological identification of the promising fungal isolate

The promising isolate characteristically formed colonies of the medium size, texture velutinous to floccose, conidia bluish grey to dull green on Czapek-Dox medium (CYA), dull green on malt extract-agar MEA) media and exudates clear to pale brown and reverse usually pale, sometimes yellow or brown. Stipes finely to roughen- walled on both CYA and MEA media, 100-200 µm in long. Pencilli terverticillate, metulae 8-15 µm in long, in whorls of 2-5 phialides flask – shaped, tapering in a narrow neck 7-9 µm in long. Conidia spherical to sub spherical with smooth walled and 3-4 µm in long (**Ainsworth, 1971 and Pitt, 1985**) that was showed in Figure (3).



(a) (b)

(c)

Figure (3) Morphological identification of the promising isolate ((a) Growth of the promising isolate on malt extract-agar medium, (b) Growth of the promising isolate on Czapek-Dox medium, and (c) Light microscopic phase contrast of the promising isolate)

3.4.2 Sequencing of 18S rRNA gene of the promising isolate

To portray the strain, the nucleotide sequences of 18S rRNA of the strain were detected. The phylogenetic tree was structure by the method based on 18S rRNA sequences as showen in Figure (4). The 18S rRNA gene from the genomic DNA of the *Penicillium commune* NRC 2016 was enzymatically amplified by Taq DNA polymerase by using a universal fungal primer. From the phylogenetic analysis of a sequence of *P. commune* NRC 2016 with which closely related strains from the database (http://www.ncbi.nlm.nih.gov/). It appears a distinguished identity with *P. commune* NRC 2016. The rRNA sequence of *P. commune* NRC 2016 was deposited to Gene Bank under accession number KU752217.

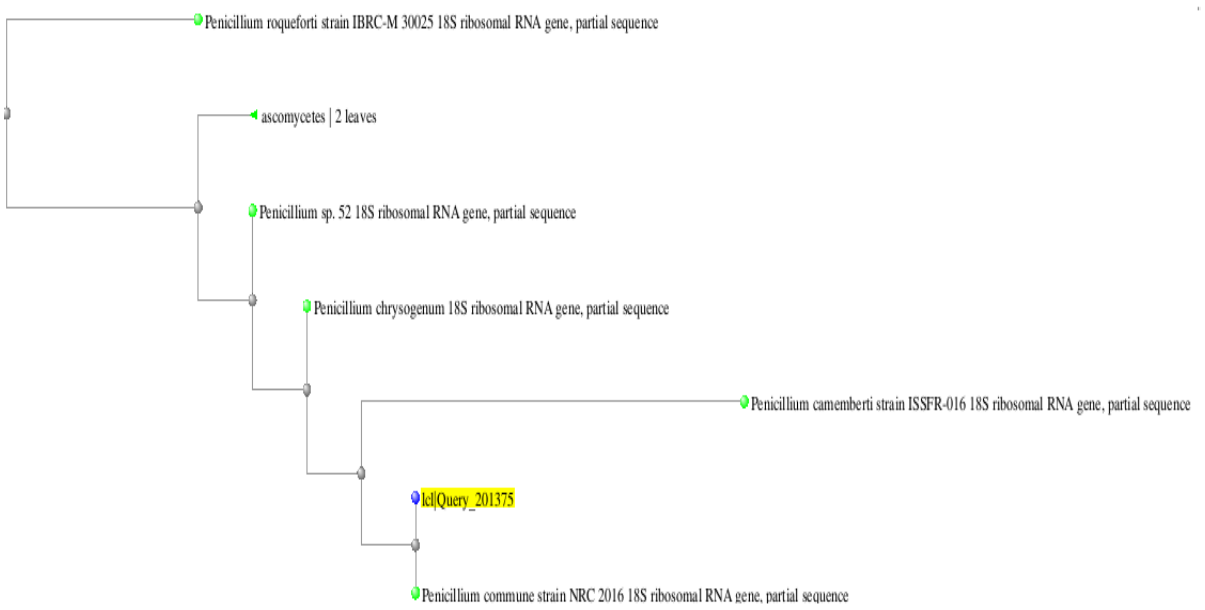


Figure (4) Phylogenetic tree showing the relationships among the selected isolate *P. commune* NRC 2016 and other closely related sequences collected from the Gene Bank

Discussion

Fungi are the most important biotechnological useful organisms **(Bennett, 1999).** In the present study, Nile-red staining assay was used to screen the biodiesel producers. It is known that conventional methods of lipid determination have many complicated steps that are extraction, purification, concentration, and determination. A spectrophotometer methods were safe the time consuming and Nile-red seems preferable for the intracellular lipid determination, that stain is emitting strongly positive red fluorescence signals only with hydrophobic compounds like any lipid particles inside the cells and could be detected by fluorescence spectroscopy **(Lim *et al.*, 2003 and Beopoulos *et al.*, 2008)**.

Microorganisms that can accumulate oils in lipid form more than 20% of their biomass are defined as an oleaginous species, **Zhu *et al.* (2008) and Kitcha & Cheirsilp (2011)** reported that yeast strains such as *Rhodosporidium* sp., *Rhodotorula* sp. and *Lipomyces* sp. can accumulate intracellular lipids from 30 - 70% of their biomass dry weight. In oleaginous microorganisms the carbon substrate continues to be taken up by cells and is channeled into the lipid biosynthesis pathway, increasing triacylglycerol (TAG) production and its storage in lipid bodies (**Beopoulos *et al.*, 2009, Meng *et al.*, 2009 and Ageitos *et al.*, 2011**).

The composition of fatty acid methyl esters with the profiles which are cited in this study for the high producer isolates were in agreement with (**Papanikolaou and Aggelis,**

2002, Subramaniam *et al*., 2010 and Fei *et al*., 2011) those found in plants. These include palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3). According to that the best producer isolate is C1 that produced high content of fatty acid methyl esters, which could use in the biodiesel production while the other isolates couldn't use them. The high degree of unsaturation inherent to the FAMEs derived from the obtained fatty acids would evidence lower oxidative stability, but excellent fuel properties at low temperatures, which are an advantage in winter operation, are similar to **Vicente *et al.* (2004).**

Fungal taxonomy is traditionally based on comparative morphological features according to **(Zhang *et al.*, 2008).** However, special caution should be taken when closely related or morphologically similar endophytes are identified, because the morphological characteristics of some fungi are dependent on medium and cultural conditions can substantially affect vegetative and sexual compatibility **(Zhang *et al.*, 2006)**. In contrast, molecular techniques exhibit high sensitivity and specificity for identifying microorganisms and can be used for classifying microbial strains at diverse hierarchical taxonomic levels **(Sette *et al.*, 2006).** Regardless of the morphological identification and depending on the molecular analysis of 18S rRNA sequence of the selected fungal isolate, was identified as *Penicillium commune* NRC 2016 strain and was deposited to gene bank under accession number KU752217.

4-Conclusion

A promising fungal strain designated *P. commune* NRC 2016 for biodiesel production was isolated from an Egyptian soil. It was the best biodiesel productivity strain among the tested isolates. In addition, GC study revealed that the fatty acids, palmitic, oleic, linoleic and arachidonic acid were predominant in the biodiesel sample. The results suggest that *P. commune* NRC 2016 could be useful for biodiesel production**.**

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الملخص العربى

انتاج وقود الديزل الحيوي باستخدام فطرال *Penicillium commune* NRC 2016 المحلى

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اختبار 25 نوعا من الفطريات الخيطية المحلية وكشف قدراتها على إنتاج الدهون باستخدام طريقة صبغة ال -Nile red ومن خلال ذلك تم اختيار افضل فطر. واعتمادا على كلا من التحليل المورفولوجي و التحليل البيولوجي الجزيئي تم تعريف هذا الفطر باسم *Penicillium commune* NRC 2016. وبتحليل الدهون الناتجة من هذا الفطر باستخدام جهاز الكروماتوجرافى الغازي اظهرت النتائج ان الأحماض الدهنية الناتجة مناسبة لإنتاج الديزل الحيوي. وناتج كشف التحليل الكروماتو جرافى الغاز ي للاحماض الدهنية الناتجة من هذا الفطر كالتالى حمض البالم يتك 20٪، وحمض الستريك 4.65٪، وحمض الأوليك 13.77٪، وحمض اللينوليك 32٪، وحمض الألفا لينولنيك 1.75٪ وحمض الأر اشيدونيك 16.1٪. نستخلص من ه ذه الدراسة إمكانية استخدام السلالة الفطرية *Penicillium commune* NRC 2016 كسلالة فطرية واعدة فى انتاج الديزل الحيوي.