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Optimization of culture conditions for biodiesel production from Egyptian isolate *Penicillium commune* NRC2016

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Abstract

Background: Biodiesel is a type of renewable energy, an ideal substitute for petroleum diesel fuel. The present study concerns about optimization of culture conditions for biodiesel production by *Penicillium commune* NRC2016.

Results: The maximum lipid production from *P. commune* NRC2016 was investigated using basal liquid medium with initial pH 7.0, incubation temperature 20 °C, and after 5 days of incubation time at static condition. Six types of agro-industrial by-products (broken rice, rice straw, wheat bran, corn stalk, sweet sorghum, and bagasse) were separately used as components of semi-solid fermentation media. The highest lipid accumulation was recorded with sweet sorghum 99.1 mg/g as compared with the other by-products. Biodiesel obtained from *P. commune* NRC2016 was blended "B5" and the physical properties were determined and found to be as follows: density 0.8 g/ml, viscosity 2.1 mm^2/s, flash point 77.0 °C, cloud point − 1.5 °C, iodine value 42.3 g I_2/100 g, acid value 2.1 mg/g, pour point − 1.7 °C, and cetane number 47.8 min.

Conclusions: This work revealed the optimization of culture conditions for biodiesel production from Egyptian fungal strain *P. commune* NRC2016.

Keywords: Biodiesel, *Penicillium commune* NRC2016, Optimization, Agro-industrial by-products, Physical properties

Introduction

Consumption of fossil fuel accounted for about 90% of global energy requirement (Gavrilescu and Chisti 2005). Economic and geopolitical restrictions on utilizing of petro fuels allied to environmental concerns promoted the development of the biofuels market (Rottig et al. 2010). Biofuels are solid, liquid, or gaseous fuels that are produced from biomass, the liquid biofuels such as ethyl alcohol and biodiesel (Giampietro et al. 1997 and Pimentel and Patzek 2005). Ethanol was the primary liquid biofuel used in the transport sector on a commercial scale, made from the fermentation of sugary (starchy) crops such as corn switchgrass, wood wheat straw, and rice straw (Pimentel and Patzek 2005; Tsegaye et al. 2017; Tsegaye et al., 2018a and b). Biodiesel (fatty acid methyl- or ethyl-esters) is a promising renewable source of energy, which is currently produced by transesterification of triacylglycerol of biomass origin viewed as an attractive substitution to diesel fuel due to its positive environmental characteristics. It is nontoxic, biodegradable, has a favorable emissions profile, and is produced from a variety of renewable resources including soybean, palm, sunflower, rapeseed, Jatropha, animal fat, and waste oils (Al-Widyan and Al-Shyoukh 2002; and Agarwal and Agarwal 2007). Attention had shifted to sources of non-edible oil like those produced from oleaginous microorganisms (those microorganisms that have lipid content in excess of 20%). Lipids from microorganisms are viewed as a possible alternative for industrial production due to their fatty acid composition which is like that of vegetable oils. The microbial lipids are rich in some valuable polyunsaturated fatty acids, such as -linoleic acid, which was often utilized in dietary supplements and for infant nutrition (Huang et al. 2009). The oil content in some microorganisms could reach 70% of the total cellular dry weight under appropriate culture conditions (Meng et al. 2009). Oleaginous fungi are most commonly utilized for this purpose, because they can be

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used as substrates in a wide range of raw materials (Papanikolaou et al. 2004a; Fakas et al. 2009; and Ochsenreither et al. 2016). This work aimed to study the effects of physical and nutritional parameters and investigated for the optimum lipid production from the Egyptian oleaginous fungus strain, *P. commune* NRC2016.

Materials and methods

Microorganism

Penicillium commune NRC2016 with accession number KU752217 was isolated from an enriched soil sample from Assiut government in Egypt. This strain was chosen and identified according to Hussein et al. (2017). It indicated that it had high lipid content and the best fatty acids composition compared with other 36 comparing isolates.

Optimization of culture conditions on lipid production

Effect of nutritional and environmental parameters on lipid production of *P. commune* NRC2016 was carried out. Basal medium used for the optimization according to Sergeeva et al. (2008) was obtained (g/l): glucose 5.0; yeast extract 2.0; KH_2PO_4 0.05; K_2HPO_4 0.05; $MgSO_4.7H_2O$ 0.05; $MnSO_4$ 0.1; $CaCl_2$ 10; $FeCl_3$ 0.2; asparagine 0.05; leucine 0.05; glycine 0.05; and glutamic acid 0.05; then, the pH was adjusted at 7.0 ± 0.1. The prepared inoculum size of *P. commune* NRC2016 was 6.4×10^5 spores/ml for all the following experiments.

Effect of initial medium pH on lipid production

This experiment was carried out to evaluate the effect of initial pH on lipid production of *P. commune* NRC2016. The pH of the culture medium was adjusted with 1 N HCl or 1 N KOH after autoclaving at different values (4.0, 4.5, 5.0, 5.5, 6.0, 7.0, and 8.0).

Effect of incubation temperature on lipid production

This experiment was carried out to evaluate the effect of different incubation temperatures on lipid production of *P. commune* NRC2016 in the range of 20–40 °C.

Effect of incubation period on lipid production

This experiment was carried out to evaluate the effect of different incubation period on lipid production of *P. commune* NRC2016 ranging between 1 and 11 days.

Effect of inoculum size on lipid production

This experiment was carried out to evaluate the effect of inoculum size on lipid production of *P. commune* NRC2016. Spore suspension of the culture medium was adjusted at different values of inoculum size (0.50, 0.75, 1.00, 1.25, and 1.50 ml) and individually added into 100 ml of liquid basal production medium.

Effect of static and shaking conditions on lipid production

This experiment was carried out to evaluate the effect of shaking at 80 rpm and static condition on lipid production of *P. commune* NRC2016.

Effect of different carbon sources on lipid production

This experiment was carried out to evaluate the effect of different carbon sources on lipid production of *P. commune* NRC2016. An equimolar amount of five different carbon sources as xylose, glucose, sucrose, lactose, and raffinose was individually added to the production medium.

Effect of different nitrogen sources on lipid production

This experiment was carried out to evaluate the effect of different nitrogen sources on the lipid production of *P. commune* NRC2016. An equimolar amount of six different nitrogen sources as yeast extract, peptone, casein, ammonium sulphate, sodium nitrate, and ammonium nitrate was individually added to the production medium.

Effect of agro-industrial by-products on lipid production

Six agro-industrial by-products such as broken rice, wheat bran, rice straw, corn stalk, sweet sorghum, and bagasse were used as feedstock by *P. commune* NRC2016 as semi-solid fermentation media for lipid production.

Lipid extraction and determination

The amount of *P. commune* NRC2016 lipid was increased by using the optimum environmental conditions. Indirect transesterification procedure by initial extraction of lipids from biomass followed by transesterification was carried out according to Vicente et al. (2010). In the common lipid extraction method, the culture biomass was collected by using Sigma 3-18 KS centrifuge (5000 rpm/4 °C/10 min) then washed three times with distilled water to remove the medium residues. The mycelia were dried in an oven at 60 °C until a constant dry weight was obtained. Then, the lipid extraction and determination in the biomass were estimated by the methods of Bligh and Dyer (1959) and Lewis et al. (2000).

Extraction of lipid

Lipid was extracted from the fungal biomass with a mixture of chloroform and methanol solvent (2:1 *v*/*v*). Two hundred milligrams of the dried biomass was washed three times with 10 ml of the solvent mixture for 10 min each together with ultrasonication to favor cell membrane disruption Folch et al. (1957) and Vicente et al. (2009). The solvent mixture containing extracted lipids was separated from residual biomass by centrifugation using Sigma 3-18 KS centrifuge (5000 rpm/4 °C/10 min),

and all the fractions from each stage were collected and the solvent was evaporated.

Determination of lipid

Lipid was determined according to Mishra et al. (2014) method using sulfo-phospho-vanillin (SPV) reagent. It was prepared by dissolving 0.6 g vanillin in 10 ml absolute ethanol then 90 ml deionized water. Subsequently, 400 ml of concentrated H_3PO_4 was added to the mixture, and the resulting reagent was stored in a dark bottle. For the fungal lipid quantification SPV reaction, a known amount of fungal lipid was used, 2.0 ml of concentrated H_2SO_4 (98%) was added to the fungal lipid sample and heated for 10 min at 100 °C, then cooled for 5 min in an ice bath. Five milliliters of freshly prepared SPV reagent was added, and the sample was incubated for 15 min at 37 °C in an incubator shaker (200 rpm); the absorbency was measured at 530 nm by using JASCO V-630 spectrophotometer in order to quantify the lipid concentration within the fungal lipid samples.

Standard curve for lipid determination

The standard lipid stocks were prepared using commercial radish oil at 20 mg in 10 ml chloroform (2 mg/ml), which was subsequently stored in a refrigerator at − 4 °C before use. Different amount of lipid in microliters of standard oil solution was added in the empty tube. The tubes were kept in a water bath at 60 °C for 10 min to evaporate the solvent, and 100 μl of water was added to the lipid standard. Further samples were prepared and determined according to Mishra et al. (2014) by SPV reaction method as described previously.

Biodiesel production

Biodiesel production according to Vicente et al. (2009) experiment was planned to use HCl as an acid catalyst and the reaction temperature of 25 °C. Reactions of extracted microbial lipids were performed in 15 ml glass closed vessels with magnetic stirring (900 rpm) using a methanol to oil ratio of 60:1 M and a catalyst concentration of 8 wt% relative to microbial oil. The reactor was then immersed in a thermostatic bath at the reaction temperature for 8 h. The biodiesel layer was collected, and the crude glycerol was washed five times with petroleum ether:diethyl ether (80:20%) as well as with the same volume of water. The upper organic layers were put together with the first biodiesel layer, and the solvent was removed on a rotary evaporator leaving the residue containing the biodiesel, which was used in measuring the reaction yield relative to the dry microbial biomass.

Blend biodiesel preparation

Oil methyl ester of the selected fungal isolates was added to diesel at concentration of 5% with low stirring rate. The mixture was stirred for 20 min and left to reach equilibrium before analysis; the test methods were used as follows.

Physical properties of biodiesel

The physical properties analysis for biodiesel was performed at the Engineering Development and Consulting Unit in National Research Centre (NRC). Biodiesel was blended with petroleum diesel at a concentration of 5% and determined according to the following methods: density (ASTM D1298), viscosity (ASTM D445), cloud point (ASTM D2500), pour point (ASTM D97), iodine value (ASTM D5768), and acid value (ASTM D664).

Results

Optimization of the culture conditions on lipid production

The effect of nutritional and environmental parameters for the best lipid production of the identified strain *P. commune* NRC2016 was indicated as follows.

Effect of initial medium pH on lipid production

In the present work, the medium pH was adjusted for *P. commune* NRC2016 at different values. Figure 1a shows the maximum lipid accumulation reaches 33.16% at initial pH 7.0 while the minimum value reaches to 17.80% at initial pH 4.0.

Effect of incubation temperature on lipid production

P. commune NRC2016 was incubated at different incubation temperatures at pH 7.0. Figure 1b indicates the maximum lipid production reached 41.18% at optimum incubation temperature 20 °C while the minimum value reaches to 6.98% at incubation temperature 40 °C.

Effect of incubation period on P. commune NRC2016 lipid production

P. commune NRC2016 was grown at pH 7.0 and a temperature of 20 °C for different tested periods. Figure 1c shows the maximum lipid production reached 46.36% after an incubation period of 5 days while the minimum value reaches 2.85% after the first day.

Effect of inoculum size on lipid production

The inoculum size of *P. commune* NRC2016 was adjusted at different values. The media pH was adjusted at 7.0 then incubated for 5 days at a temperature of 20 °C. Figure 2a indicates the maximum lipid production reached 30.37% and was obtained by using inoculum size 0.75% while the minimum value reaches to 28.50% by using inoculum size 0.50%.

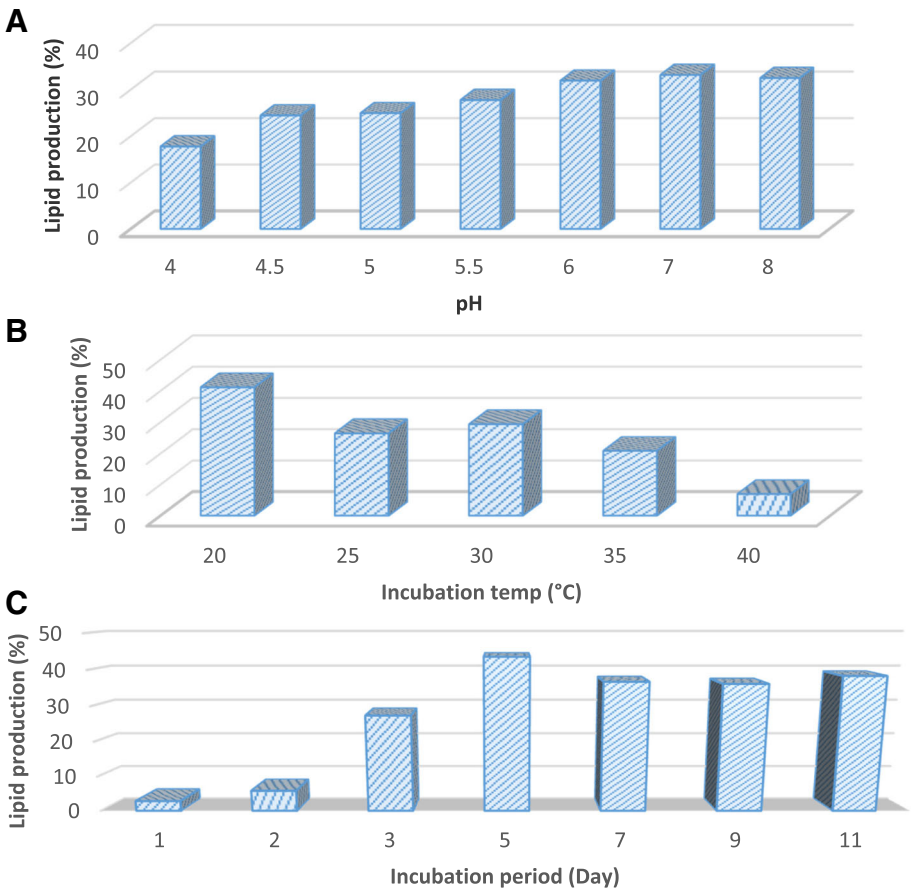

Fig. 1 Effect of initial pH (**a**), incubation temperatures (**b**), and incubation period (**c**) on *P. commune* NRC2016 lipid production

Effect of static and shaking conditions on lipid production

Static and shaking condition at 80 rpm was evaluated for growth of *P. commune* NRC2016. The inoculum size was adjusted to 1% of the medium. The medium pH was adjusted at 7.0 then incubated for 5 days at temperature 20 °C as optimum conditions. Figure 2b indicates the maximum lipid production reaches 36.75% under static while the minimum value reaches to 24.23% under shaking condition.

Effect of different carbon sources on lipid production

An equimolar amount of five different carbon sources was used under inoculum size of 1% of broth medium, pH 7.0, incubation time of 5 days, and incubation temperature of 20 °C under static condition. The results in Fig. 2c indicate that the maximum lipid accumulation reaches 34.92% in the presence of xylose while the minimum value reaches 9.17% in the presence of lactose.

Effect of different nitrogen sources on lipid production

An equimolar amount of six different nitrogen sources was used in the presence of xylose as carbon source with inoculum size of 1%, pH 7.0, incubation time of 5 days, and incubation temperature of 20 °C under static condition as the optimum conditions. The results in Fig. 2d indicate the maximum lipids production reaches to 43.06% in the presence of peptone while ammonium sulphate appears the lowest value 19.34%.

Effect of agro-industrial by-products as feedstock on lipid production

The ability of *P. commune* NRC2016 for growing on agro-industrial as feedstock to produce fungal lipid was examined under optimum conditions. The results in Fig. 3 indicate the highest lipid accumulation reaches 99.1 mg/g in the presence of sweet sorghum followed by wheat bran 68.9 mg/g then broken rice 43.2 mg/g and it could not grow on other waste-products.

Standard curve for lipid determination

Fungal lipid was estimated by sulfo-phospho-vanillin (SPV) reaction against standard lipid stock. The standard lipid stock was prepared using commercial radish oil at 20 mg in 10 ml chloroform (final concentration, 2 mg/ml). Figure 4 indicates the standard curve when the

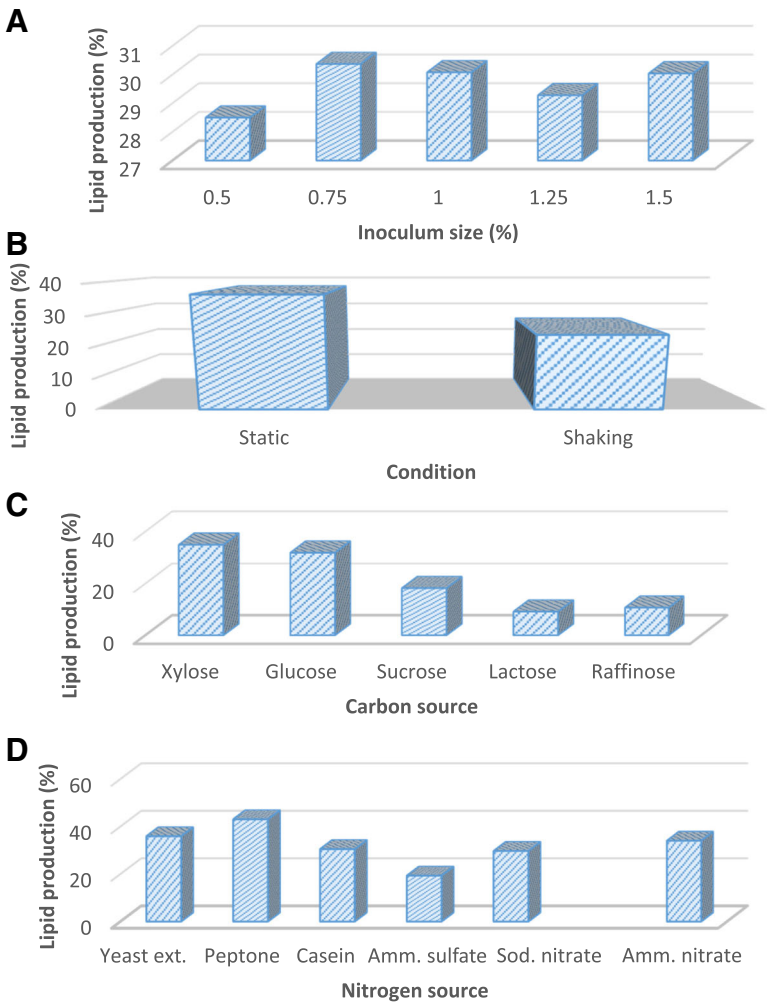

Fig. 2 Effect of inoculum size (**a**), static and shaking conditions (**b**), different carbon sources (**c**), and different nitrogen sources (**d**) on *P. commune* NRC2016 lipid production

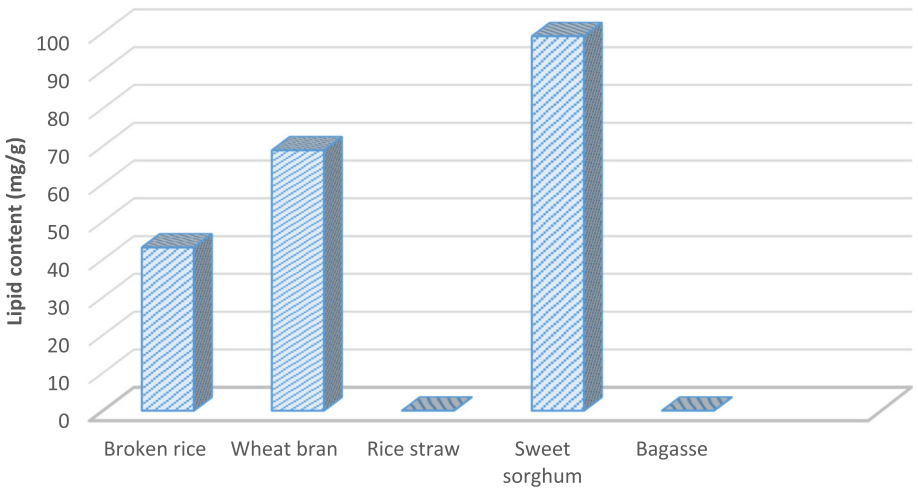

Fig. 3 Lipid production *P. commune* NRC2016 using different agro-industrial by-products as semi-solid state sources

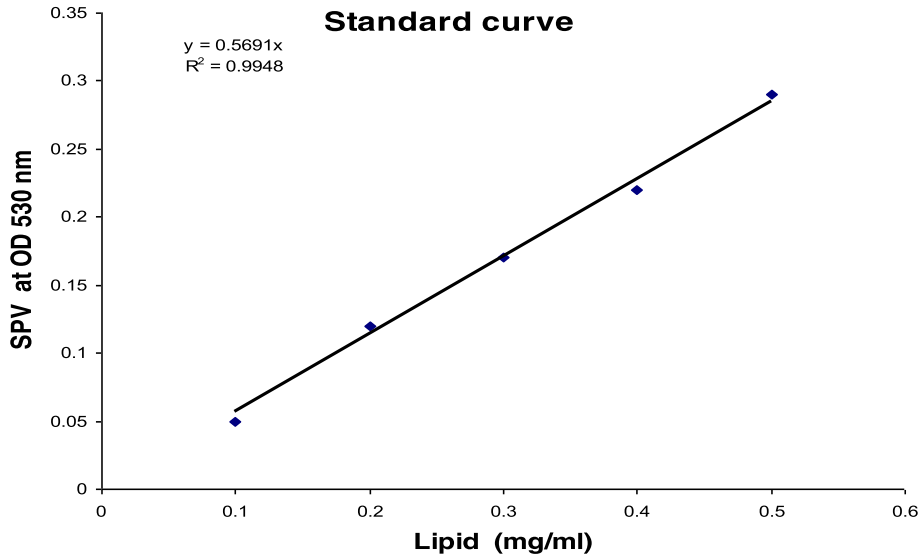

Fig. 4 Standard curve of radish oil using sulfo-phospho-vanillin reagent

absorbance was plotted against known volumes of radish oil; a linear relationship with very strong linear correlation ($R^2 > 0.99$) was obtained.

Biodiesel production

The extracted lipid obtained from *P. commune* NRC2016 was estimated by sulfo-phospho-vanillin reagent (SPV) technique. After lipid extraction, indirect transesterification process using acid as a catalyst was used for transferring the extracted lipid into biodiesel.

Biodiesel physical properties

In this work, the amount of biodiesel produced by the tested fungal identified isolate was blended with diesel at ratio 5%. Table 1 shows the biodiesel physical properties obtained from blending biodiesel of *P. commune* NRC2016 as compared with the standard diesel ASTM, standard biodiesel ASTM, and ASTM D975 (standard biodiesel for B5). The result indicated that density was 0.83 g/ml, viscosity was 2.05 mm^2/s, flash point was 77.0 °C, cloud point was − 1.50 °C, pour point was − 1.70 °C, iodine value was 42.30 $gI_2/100$ g, cetane number was 47.86 min, and acid value was 2.14 mg/g.

Discussion

Fungi are the most important and useful biotechnological microorganisms (Bennett 1998). The higher lipid accumulation achievement by oleaginous microorganisms demanded the optimization of medium cultivation conditions and nutritional factors (Ageitos et al. 2011). Lipid content varied according to the nature of microorganism and culture

Table 1 Physical properties of biodiesel obtained from blending biodiesel of *P. commune* NRC2016 as compared with standard diesel ASTM, standard biodiesel ASTM, and ASTM D975 (standard biodiesel for B5)

Physical properties	Test method ASTM	*P. commune* NRC 2016 biodiesel B5	Standard diesel ASTM	Standard biodiesel ASTM	ASTM D975 for B5
Fuel composition	–	Mix	C10-C21 HC	C12-C22 FAME	Mix
Density (g/ml at 20 °C)	D1298	0.83	0.85	0.87 to 0.90	0.85
Kin. viscosity at 40 °C, (mm^2/s)	D445	2.05	1.30 to 4.10	1.90 to 6.00	1.90 to 4.10
Flash point (°C)	D93	77.00	60.00 to 80.00	100.00 to 170.00	60.00 to 80.00
Cloud point (°C)	D2500	− 1.50	− 15.00 to − 5.00	− 3.00 to − 12.00	− 35.00 to 5.00
Pour point (°C)	D97	− 1.70	−20.00 to −6.00	− 15.00 to 10.00	− 35.00 to − 15.00
Iodine value ($gI_2/100$ g))	D5768	42.30	–	< 25.00	–
Cetane no. (min)	D613	47.86	40.00	< 47.00	40.00 to 55.00
Acid value (mg/g)	D664	2.14	0.03	0.80 max	–

– Not determined

conditions like medium pH, incubation temperature, incubation period, static and shaking condition, carbon sources, and nitrogen sources (Alvarez and Steinbuchel 2002 and Papanikolaou et al. 2004b). The initial medium pH was found to be a significant factor for lipid accumulation. Lilly and Barnett (1951) recorded that the hydrogen ion concentration in the medium was an influential factor for growth and other life processes like sporulation. It was known that the function of plasma membrane was to regulate the transport of substances from in and out the cells. Previous studies recorded the influence of pH value on the microorganism's growth kinetics and concluded that the medium pH was an important environmental factor affecting cell growth and products formation (Amanullah et al. 2001). Comparable results were obtained by Ruan et al. (2014), Ali and El-Ghonemy (2014), and Jiru et al. (2017); they recorded that pH values between 5 and 6 were found to be the suitable pH for most fungal growth. Incubation temperature shows that the maximum *P. commune* NRC2016 lipid accumulation reached 41.18% at 20 °C similar to that of Carlile et al. (2001) who investigated that all fungal enzymes exhibited high activity at a temperature 20–30 °C. Incubation time of the *P. commune* NRC2016 lipid production reached 46.36% after the fifth day. Our result was similar to Ali and El-Ghonemy (2014) for *Aspergillus* sp. and *Trichoderma viride* NRC314 and reported that maximum lipid production was obtained after incubation time of 5 days. The *P. commune* NRC2016 lipid production reached 36.75% under static condition which is higher than that produced under shaking condition. This result was congruent with Kirrolia et al. (2012) and Ali and El-Ghonemy (2014). Among different tested carbon sources in this study, the highest lipid accumulation was obtained with xylose dependent for *P. commune* NRC2016 where lipid production percentage reached to 34.92%. These results were congruent with Li et al. (2011). They studied the oleaginous fungi lived in Qinghai plateau and reported that fungal isolates were able to use xylose in the industrial application of biodiesel. While the majority of oil-producing microorganisms are known to use glucose to produce oil (Papanikolaou et al. 2004b; Loffhagen et al. 2006; and Li et al. 2007) because it is available and cheap carbon source. Among different tested nitrogen sources in this study, the highest lipid accumulation was obtained with peptone and reached 43.06% while inorganic nitrogen sources were not suitable for lipid accumulation. This result was almost on the same line with Huang et al. (1998); they recorded that inorganic nitrogen sources were good for cell growth but not suitable for lipid production, while organic nitrogen sources like peptone are good for oil production but not good for cell growth. Moreover, Xing et al. (2012) reported that among inorganic nitrogen sources, ammonium sulfate was shown to be the favorable nitrogen source. In the present study, the highest *P. commune* NRC2016 lipid accumulation reached to 99.1 mg/g in the presence of sweet sorghum as feedstock compared with the other agro-industrial by-products. These results were almost accepted with Pandey et al. (2000) who reported that fungi could grow well under solid state conditions. Also, Economou et al. (2010) investigated the use of sweet sorghum in the fungal biotechnology production of single cell oil by using the oleaginous *Mortierella isabellin*a and yielded oil content of 11.0% of the dry substrate. Sufficient amount of lipid was produced after *P. commune* NRC2016 was grown under the best conditions and extracted with chloroform:-methanol (2:1 *v*/*v*). These fungal lipids showed high lipid content that reached > 40%. Many researchers (Vicente et al. 2009; Ochsenreither et al. 2016; and Ren et al. 2017) used similar methods for fungal lipid production. The extracted lipid obtained from *P. commune* NRC2016 was estimated by sulfo-phospho-vanillin reagent (SPV) technique. Also, Mishra et al. (2014) used SPV reaction to rapidly obtain an overview of lipid accumulation in microalgae. After lipid extraction, indirect transesterification process using a catalyst was used to convert the extracted lipid into biodiesel. This technique was confirmed by Lewis et al. (2000), Vicente et al. (2010), and Ren et al. (2017); they reported that indirect transesterification was the popular method for lipid extraction using acid as a catalyst used for transferring the extracted lipid into biodiesel. In this work, the amount of biodiesel produced by the tested fungal identified isolate was blended with diesel at a ratio of 5% which was accepted by Pimentel et al. (2006) and Knothe (2006); they reported that all engine manufacturers provided certain warranties on the engines powered with biodiesel while B5 was accepted by all engine manufacturers. The biodiesel physical properties obtained from blending biodiesel of *P. commune* NRC2016 as density, viscosity, flash point, cloud point, pour point, iodine value, cetane number, and acid value were determined and compared with standard diesel ASTM, standard biodiesel ASTM, and ASTM D975 (standard biodiesel for B5).

Conclusion

The identified fungal isolate *P. commune* NRC2016 designated for biodiesel production was isolated from Egyptian soil. Optimum conditions for maximum lipid production of 43.6% were obtained when the basal liquid medium was used with xylose as a carbon source and peptone as a nitrogen source, with initial pH 7.0 after 5 days at static condition. When agro-industrial by-products were used as components of semi-solid fermentation media, the highest lipid accumulation for *P. commune* NRC2016 was 99.1 mg/g in the presence of sweet sorghum. The physical properties of blending biodiesel produced from the tested identified fungal isolate were in accordance with biodiesel standards. Therefore,

this study revealed the possibility of using *P. commune* NRC2016 for biodiesel production.

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Authors' contributions
All the participant researchers contribute to do this work, and this research was from the thesis of Abdelhamid, S.A. (2018), Biochemical studies on the production of biodiesel from some species of fungi, MSc of Science, Ain Shams University, Egypt. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable

Consent for publication
All the participant researchers are consent for publication

Competing interests
The authors declare that they have no competing interests.

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