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An effective lipid-producing fungal sp. strain DGB1 and its use for biodiesel production

Gadallah Abu-Elreesh* and Desouky Abd-El-Haleem

Environmental Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), City of Scientific Research and Technological Applications (SRTA-City), Alexandria, Egypt.

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Among 30 fungal isolates screened for lipid production using Nile-red staining assay, an isolate designated DGB1 was recorded as the highest lipid producer with lipid content up to 40% (w/w). Based on morphological, biochemical and molecular analysis, DGB1 was identified as fungal sp. strain DGB1. Under the optimized time course, lipid content of DGB1 reached its maximum yield (7.2 g/L) after four days of incubation. Gas chromatography/mass spectrometry (GC/MS) analysis revealed presence of five long chain saturated fatty acids, decanoic, tridecanoic, pentadecanoic, hexadecanoic and heptadecanoic. About 50% of these fatty acids was heptadecanoic acid. However, as a maximum, ~ 20% (w/w) of the lipid contents were determined when the basal medium containing 30% glycerol was used for lipid production instead of 5% glucose. This may push us to conclude that glucose is the most suitable carbon source which could be used to produce lipids from strain DGB1. In conclusion, this work revealed the possibility of using the promising fungal strain DGB1 in biodiesel production.

Key words: Biodiesel, lipids, *Geotrichum*, heptadecanoic.

INTRODUCTION

The main resource known to obtain energy is petroleum, but the continued uses of petroleum-based fuels contribute to atmospheric pollution which is the main reason for global warming (Meng et al., 2009). Also, the scarcity of known petroleum reserves makes renewable energy resources more attractive (Anitha and Narayanan, 2012). The replacement of petroleum fuels to secure future energy supplies continues to be a major concern. The most feasible way to meet the growing demand for energy is by utilizing alternative fuels. An alternative fuel to petrodiesel must be technically feasible, economically competitive, environmentally acceptable, and easily available (Meher et al., 2006). So, biodiesel fatty acid methyl esters (FAMEs) are an alternative to petroleum-based diesel fuel.

Demand for biodiesel has increased significantly (Ong et al., 2011; Vicente et al., 2009) due to the instability of petroleum prices and the development of government measures in many countries around the world that establish a minimum proportion of biofuel for all petrol and diesel used in transport. For instance, the European Union establishes a minimum content of 5.75% of biofuel by 2010 and the United States plans to increase the amount of biodiesel to 36 billion gallons by 2022, respectively (Vicente et al., 2009).

Biodiesel constitutes a renewable fuel that is compatible

*Corresponding author. E-mail: g_abouelrish@yahoo.com. Fax: 002034593423.

Abbreviations: FAMEs, Fatty acid methyl esters; **YPD,** yeast peptone dextrose; **PBS,** phosphate buffered saline; **GC-MS,** gas chromatography/mass spectrometry.

with current commercial diesel engines and has clear benefits relative to diesel fuel including enhanced biodegradation, reduced toxicity and a lower emission profile (Vicente et al., 2009). Nonetheless, biodiesel presents some disadvantages. One of its drawbacks is the high manufacturing cost, which is mainly due to the high cost of the vegetable oil (Thiru et al., 2011). Actually, 70–90% of the biodiesel production cost corresponds to raw vegetable oil. In addition, the biodiesel industry competes with the food industry for oil crops. Therefore, it is necessary to explore new raw materials that reduce the biodiesel price without competing with food production (Vicente et al., 2009).

In this context, oils from microorganisms (also called single-cell oils) constitute a promising alternative for producing biodiesel since they present many advantages over vegetable oils from oleaginous plants. Microorganisms can accumulate high level of lipids and their production does not compete with food production since biomass residuals can be used as carbon source (Vicente et al., 2009).

The principal oleaginous microbial species are microalgae, bacteria, fungi and yeasts. Some works have dealt with the use of oleaginous microorganisms for biodiesel production. However, little information has been reported so far on the use of lipids from fungi and bacteria for biodiesel production (Li et al., 2008; Meng et al., 2009). Fungi are an attractive source of lipids for use in biodiesel synthesis (Ratledge, 1991, 2004). Several species of fungi are able to accumulate significant amounts of intracellular lipid (Takeno et al., 2005); this lipid production can be optimized by adding supplementary nutrients to culture media and/or by altering culture conditions during growth (Somasekhar et al., 2003). In comparison to the microalgae, the growth of fungi can be carried out in conventional microbial bioreactors, which will improve the biomass yield and will reduce the costs of biomass and oil productions (Vicente et al., 2009).

Consequently, there is a need to identify new microorganisms with a high lipid-producing ability and improve upon the biodiesel production efficiency of the known microbial producers. Therefore, the main objectives of the present study were: (i) isolation and identification of fungal isolates capable of yielding high amount of storage lipids, (ii) characterization and comparative analysis of fatty acid profiles of novel isolated fungus, and (iii) testing the ability of the selected isolate on the growth on glycerol as a sole carbon source and test their potential utilities as biodiesel feedstock.

MATERIALS AND METHODS

Isolation, screening of oleaginous fungi and total lipids determination

Soil samples were collected from different sites in Egypt; 1 g of each soil sample was individually suspended in 1 mL of sterile distilled water, serially diluted to 10 fold and plated on yeast peptone dextrose (YPD) agar plates (in g/L: glucose 10, yeast extract 5 and peptone 10 with the initial pH=6). The plates were incubated at 30°C for 3 days in an incubator. Several fungal colonies were obtained from different plates and it was purified by single colony, transferred repeatedly to a new YPD plate until pure cultures were confirmed. Subsequently, lipid accumulation was screened in pure isolates on YPD agar plates containing 25 µg Nile-red (Sigma) per liter (Spiekermann et al., 1999). After incubation at 30°C for 48 h, YPD plates were visualized on an UV box and the lighted isolates were recorded positives. To select the highest lipid producer among them, isolates were cultured in basal medium (in g/L: yeast extract 0.5, $MgSO_4.7H_2O$ 0.4, KH_2PO_4 2.0, $CaCl_2$ 0.5, $CuSO_4$ $5H_2O$ 0.05 and 5% glucose (w/v), with initial pH 6.0). As well, the basal medium containing 30% glycerol was used to check the ability of the selected isolates to consume cheap carbon sources instead of glucose. Flasks were removed after incubation and microbial cells were harvested from the media by centrifugation and washed with distilled water three times, then freeze dried at –50°C. Exacted weight was taken, and then total lipids were extracted from the dried biomass with chloroform: methanol, volume ratio of 2:1. Ultrasonication to favor cell membrane disruption during extraction was done. The mixture containing extracted lipids was separated from residual biomass by centrifugation and the solvent fraction was transferred to a new tube. Then, the residual of solvent was removed in a rotary evaporator followed by lyophilization to determine the ratio of extracted lipids in comparism to the cell dry weight.

To visualize the intracellular lipids inside cells of DGB1, cells were stored in the dark with 0.5 mL phosphate buffered saline (PBS) solution and 0.05 mL Nile-red solution (Nile red 25 µg Nile-red/ acetone 1000 mL) for 30 min (Lim et al., 2003). Then, stained lipid bodies were photographed using fluorescence microscope (IX-70, Olympus, Tokyo, Japan) equipped with a CCD camera (U-CMT, Olympus, Tokyo, Japan). Molecular identification of strain DGB1 was performed by the amplification of 28S rDNA primers (Sandhu et al., 1995). Sequencing was performed using ABI PRISM dye terminator cycle sequencing kit with AmpliTaq DNA polymerase and an Applied Biosystems 373 DNA sequencer (Perkin-Elmer, Foster City, Calif.). The sequences were analyzed using the CHECK CHIMERA and the SIMILARITY RANK programs of the Ribosomal Database Project (Altschul et al., 1990). Also analyzed using the BLAST program (National Centre for Biotechnology Information) to determine the closest available database sequences, selected rDNA sequences were aligned using the Clustal W program (Shingler, 1996). Published sequences were obtained from GenBank. A phylogenetic tree was constructed using Clustal W by distance matrix analysis and the neighbour-joining method (Saitou and Nei, 1987). Phylogenetic trees were displayed using TREEVIEW (Page, 1996).

Biodiesel production

Direct transformation of lyophilized biomass was carried out according to the method of Lewis et al. (2000). In a single step, methanol: chloroform 10:1 (v/v) mixture was used as a reagent-solvent system where the appropriate amount of sulfuric acid catalyst was used. After 8 h in the reaction vessel immersed in a thermostatic bath the FAME was obtained.

Gas chromatographic (GC) analysis

Characterization of biodiesel using gas Chromatography /mass spectrometry (GC-MS) was performed with Agilent 6890N Gas Chromatograph connected to Agilent 5973 Mass Spectrometer at 70 eV (m/z 50–550; source at

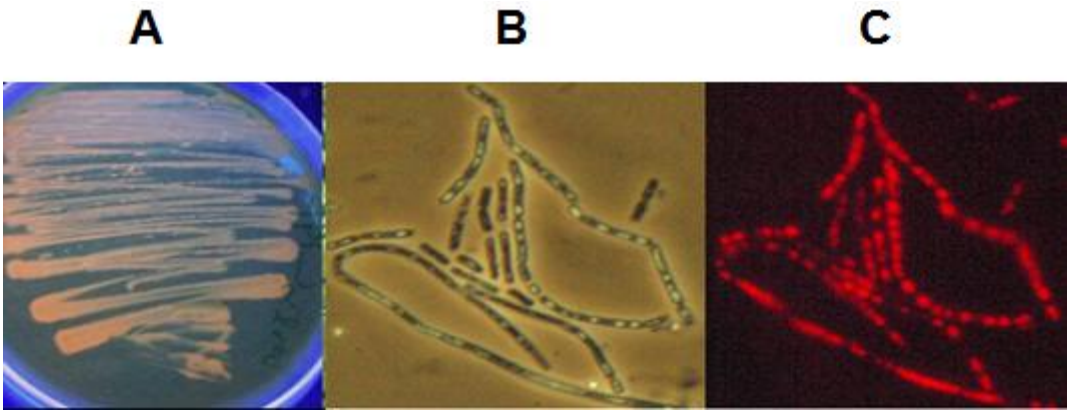

Figure 1. Screening for lipid production among fungal isolates using Nile-red staining assay on YPD agar plates (A), light microscopic phase contrast of strain DGB1 (B), and fluorescence microscopy of Nile-red stained strain DGB1 (C).

230°C and quadruple at 150°C) in the EI mode with an HP-5ms capillary column (30 m ´ 0.25 mmi.d., 0.25 mm film thickness; J & W Scientific, USA). The carrier gas, helium, was maintained at a flow rate of 1.0 mL/min. The inlet temperature was maintained at 300°C and the oven was programmed for 2 min at 150°C, then increased to 300°C at 4°C/min, and maintained for 20 min at 300°C. The injection volume was 1 µL, with a split ratio of 50:1. Structural assignments were based on interpretation of mass spectrometric fragmentation and confirmed by comparison of retention times as well as fragmentation pattern of authentic compounds and the spectral data obtained from the Wiley and NIST libraries.

RESULTS

Screening of microbial isolates for lipids production

Under the UV transilluminor, nine fungal isolates exhibited strong fluorescence signals in comparison to other isolates. To confirm the Nile-red assay, cells of each isolate were grown in basal media. All grown flasks were exposed to the same conditions, and three separate flasks were inoculated from the same culture in each case. Data from shaken flasks indicated that five isolates out of nine did not contain measurable lipids (data not shown); while isolate DGB1 contained > 40% lipids of its cell dry weight. Therefore, isolate DGB1 was used in further studies.

Identification of isolate DGB1

As shown in Figure 1, the results ensure that strain DGB1 is indeed a fungus with cream-colored on agar plates with homothallic spore production (hyphae splitting at the septum to form individual cells called arthrospores). Under fluorescence microscope, the stained lipid bodies obviously appeared. Subsequently, strain DGB1 was subjected to biochemical identification using API 20C Aux system. The results placed it under the yeast genus *Geotrichum* and/or *Candida* but *Candida* were excluded according to the pure cell morphology that was closely related to *Geotrichum* rather than *Candida*. As well, partial 28S ribosomal RNA nucleotide sequence was determined on the strain, DGB1. Using the alignment search tools (http://blast.ncbi.nlm.nih.gov), the 28S rDNA homology value of strain DGB1 showed similarity (>99%) with the soil fungal strains DM2-295, DM2-240, DM2-243 and DM070 which have the following GenBank accession numbers;AB438803,AB438762,AB438765andAB438433, respectively. The GenBank accession number for strain DGB1 nucleotide sequence is JX565425. However, based on both morphological and biochemical characterization, strain DGB1 was identified as fungal sp. strain DGB1. The constructed phylogenetic tree is presented in Figure 2.

Biomass production and lipid accumulation

To determine the optimum time course of the highest biomass and lipid yield, strain DGB1 was grown on YPD agar plates followed by seeding in a basal medium containing 5% glucose. Figure 3 shows how the lipid production varied with the growth of strain DGB1. The biomass yield that may represent the cell growth shows a sharp increment in the first 4 days, then tends to be constant over the remaining studied culturing period. However, the lipid yield during this period increased and reached its maximum value of 40% (18 g/L biomass and 7.2 g/L lipid yield) after 4 days then decreased slightly until 5 days, after this culturing period the lipid yield dramatically decreased. Concerning lipid production and the cell growth profiles, it is evident that the total lipids production was almost in parallel with cell growth till the first 4 days studied. As shown in Figure 4, the same growth pattern was observed when strain DGB1 grown

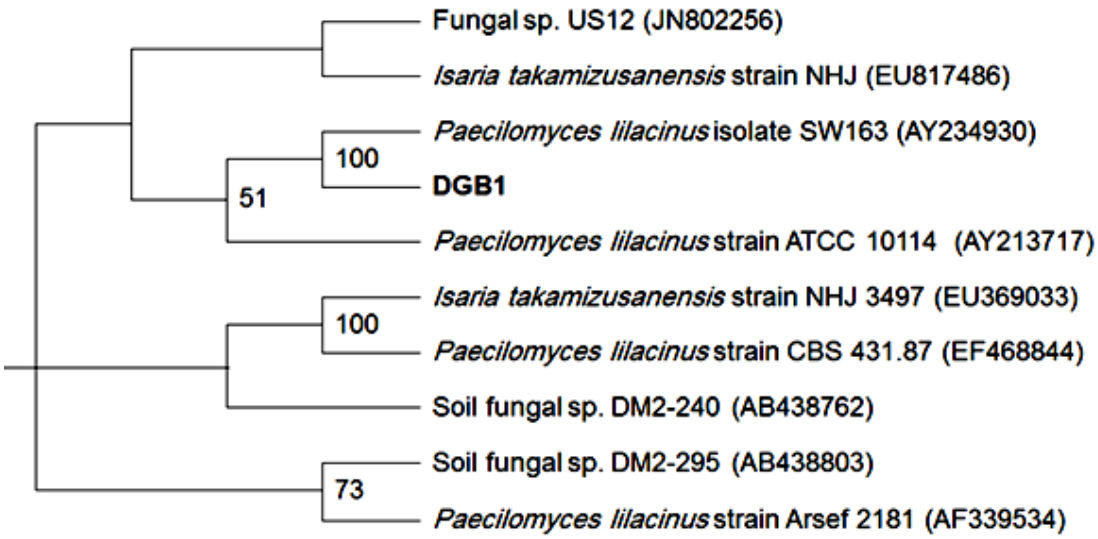

Figure 2. Phylogenetic tree showing the relationships among the selected isolate DGB1 and other closely related sequences collected from the Gene Bank.

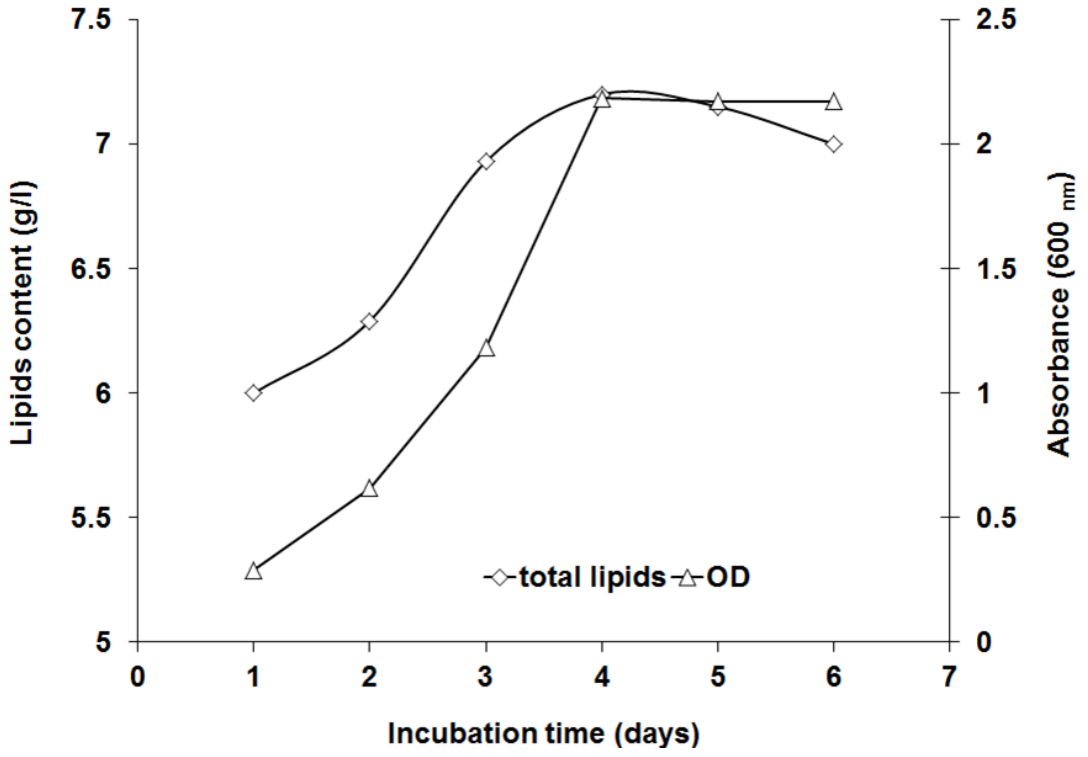

Figure 3. Time course of both growth and lipid yield of strain DGB1 grown in basal medium containing 5% glucose.

on a basal medium containing 30% of glycerol. However, the maximum cell dry weight and lipid contents (3.5 and 0.7 g/L) were obtained at the 5th day of incubation. In addition, no measurable lipids were recorded in the first four days of incubation.

Fatty acid profiles

In order to compare their potential utilities as biodiesel feedstock, fatty acid composition (FAME) of strain DGB1 was extracted by acid methanolysis (Lewis et al., 2000).

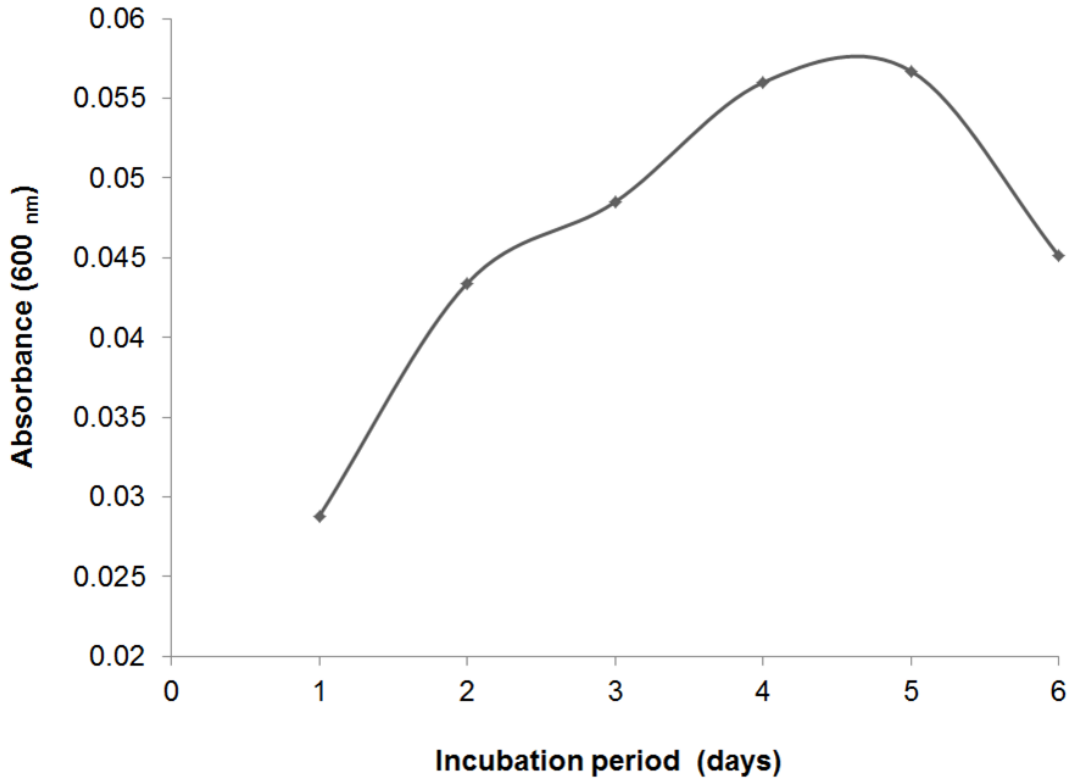

Figure 4. Time course of growth of strain DGB1 grown in basal medium containing 30% glycerol.

Table 1. Fatty acid composition of extracted total lipids from strain DGB1 by GC/Mass.

Fatty acid	Retention time	Peak area	Height of peak	Percentage (%) of each fatty acids in the total lipids extracted
Decanoic	21.959	382147	99826	21.1
Tridecanoic	19.552	119046	32598	6.7
Pentadecanoic	29.149	205458	54481	10.9
Hexadecanoic	25.627	100097	27579	6.3
Heptadecanoic	33.765	1087013	252098	50.5

As presented in Table 1, fatty acid profiles by GC/MS showed that decanoic, pentadecanoic and heptadecanoic acids are present in major quantities while tridecanoic and hexadecanoic acids were present in lower amounts.

DISCUSSION

This work described screening, isolation and characterization of oleaginous fungi for biodiesel production. Fungi as hosts for synthesis of lipids have certain advantages over bacteria: 1) The comparative larger volume of fungal cells compared to bacteria is of great value to allow more synthesis and storage capacity of the lipids production; 2) the industrial fungal are used for production of valuable products and the amount of down-stream processes in biotechnology using fungi or plants.

It is known that conventional methods of lipid determination have many complicated steps, that is, extraction, purification, concentration and determination, which are time-consuming. A spectrophotometric methods using Sudan black B, fluorescence spectrometric using Luminor 490PT and Nile-red (Thakur et al., 1989; Cooksey et al., 1987; Lee et al., 1998) were reported to determine the lipid content of yeasts, algae, and ciliates. Among several dyes, Nile-red seems preferable for the intracellular lipid determination. It emitted strongly positive red fluorescence signals only with hydrophobic compounds like lipids and intended to show any lipid particles inside the cells and it did not react with any tissue constituent except by solution and could be detected by fluorescence spectroscopy (Gorenflo et al., 1999; Spiekermann et al., 1999). As shown in Figure 1A, Nile-red staining was

obviously able to recognize among lipid-producing and non producing isolates. The fluorescence intensity of stained cells under UV light depends on the lipid concentration. Microorganisms that do not produce lipids are only lightly fluorescent because they contain almost no lipids (Schlegel, 1990). In the same direction, fluorescence microscopic examination of the selected isolate DGB1 confirmed the presence of intracellular lipidic granules (Figure 1C).

Figure 3 shows time course of cell growth and lipid yield of selected strain DGB1 that gave the highest lipid yield. Microorganisms that can accumulate oils in lipid form more than 20% of their biomass are defined as oleaginous species. Some yeast strains, such as Rhodosporidium sp., Rhodotorula sp. and Lipomyces sp. can accumulate intracellular lipids from 30 - 70% of their biomass dry weight (Kitcha and Cheirsilp, 2011; Zhu et al., 2008).

Fungal taxonomy is traditionally based on comparative morphological features (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 medium dependent 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). In the present study, regardless of the morphological identification and depending on the molecular analysis of 28S rDNA sequence of strain DGB1, we identify it as a fungal sp. Strain DGB1. This was inconsistent with that recorded by Tsuge et al. (2008) where a mucor isolate had 99% homology with both *Mucor* sp. and *Penicillium* sp. But the morphology was apparently different from *Penicillium* and hence it was identified as *Mucor* with regards to morphology.

Biomass production and lipid accumulation

It was known from previous studies that the best carbon source for lipid accumulation in fungi is glucose under limitation of nitrogen (Syed et al., 2006; PrabuddhaDey et al., 2011). In the present study, the maximum yield of lipids in strain DGB1 was obtained after 5 days of incubation. This might be due to the fact that in the case of high carbon and nitrogen ratio, glucose did not get exhausted, and biomass accumulation continued throughout the first four days (PrabuddhaDey et al., 2011). However, relatively low biomass amount was achieved when strain DGB1 grew on 30% of glycerol. As general consideration, biomass and single cell oil production from fungi cultivated on glycerol-based media is relatively restricted (Fakas et al., 2009). Also, besides the somehow low biomass production achieved by DGB1, lipid yield did not exceed the value of 0.7 g/L. This presumably is due to the relatively poor regulation of the enzymes involved in the primary metabolic steps of glycerol assimilation (Fakas et al., 2009).

Fatty acid profiles

Fatty acid profiles of the lipids produced by strain DGB1 showed that decanoic, pentadecanoic and heptadecanoic acids are present in major quantities, while tridecanoic and hexadecanoic acids were present in lower amounts (Table 1). This is in agreement with the results of Losel (1988) and Ziino et al. (1999) who recorded the extraction of decanoic, hexadecanoic and stearic acids from Geotrichum. DGB1 strain showed the presence of heptadecanoic acid for the first time in a high amount up to 50%. Previously, heptadecanoic acid was extracted from various microbial sources in a minute amount not exceeding 2% (Cao et al., 2010; Saenge et al., 2011; Cheirsilp et al., 2011). It is well known that heptadecanoic is used as internal standard in all assays of biodiesel production (Persson et al., 2010). However, the identified five fatty acids are saturated which provide an advantage, since the presence of unsaturated fatty acids, show low oxidative stability especially under long periods in storage conditions above ambient temperatures with exposure to air and/or light (Knothe, 2007). This result also agree with the previous biodiesel derived from oleaginous yeast and microalgal lipids where it tend to be more saturated and tend to give more favorable properties of biodiesel. These include an increased cetane number (CN), decreased nitrogen oxides (NOx) emissions, shorter ignition delay time, and oxidation stability (Saenge et al., 2011).

Conclusion

In the present study, a promising fungal sp. strain designated DGB1 for biodiesel production was isolated from an Egyptian soil. It was found that DGB1 strain has a high lipid productivity that reach 40 and 20% on a basal media containing 5% glucose or 30% glycerol, respectively. In addition, the fungal isolate showed fatty acid profile with decanoic acid that act as the most powerful short chain fatty acid in biodiesel and the presence of heptadecanoic acid in a high amount that reached 50%. The results suggest that biodiesel produced from strain DGB1 might be having valuable stability properties and high cetane number which give future industrial applications.

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