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Full Length Research Paper

Production of microbial oils from *Mortierella* sp for generation of biodiesel livestock

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Biodiesel is an alternative renewable fuel and its production rises rapidly. Microbial oils produced by oleaginous microorganisms can be used as a feedstock for biodiesel production so as to sustain the increasing demand for biodiesel and alleviate the competition with the food supply. This study investigated oil production by an oleaginous fungus, *Mortierella* sp., which was isolated from soils of Tamil Nadu in India. The flask culturing experiment shows that the optimal lipid production conditions were glucose as the carbon source (0.16 M), yeast extract (one percent) as the nitrogen source, temperature at 30°C and pH of 6.5. Under the optimal conditions, the oil production potential of *Mortierella* sp was examined in a three-litre pilot-scale fermentor. The fungus accumulated 44.1% of lipids in dry biomass and the biomass growth was 15.9 g l^{-1}. The fungal oil contained oleic acid of 38.2%, stearic acid of 11.5%, linolenic acid of 4.8% and palmitic acid of 19.6%, and its fatty acid composition was similar to that of vegetable oils. The crude oil had properties: density of 920 Kg/m^3 at 15°C, viscosity of 54.81 mm^2/s at 40°C, flash point of 218°C, pour point of 7.0°C, water content of 3.9%, ash content, 0.62%, carbon residue of 0.082%, acid value of 28.22 Mg KOH/g, calorific value of 32.05 MJ/Kg, free fatty acid of 14.55, and fire point of 230°C. The oil properties were much similar to Jatropha oil and rapeseed oil.

Key words: Biodiesel, microbial oil, *Mortierella* sp, oleaginous fungi.

INTRODUCTION

The increased energy demand, concerns about green-house gas (GHG) emissions, the rising price of fossil fuels and the projected decrease in the fossil fuel reserve, have led to serious concerns on energy consumption and energy security issues (Pahl, 2005; Hill et al., 2006). As a consequence, there has been a high demand for renewable energy. The European Union (EU) has published the Directive 2003/30/EC to promote the use of biofuels and other renewable energy for transport. The biofuel will be 5.75% in the transport section by 31st December, 2010; one fourth of the motor fuel consumption in EU will be replace by biofuels in 2030, which includes biodiesel (www.biomatnet.org/publications/1919 rep.pdf). Biodiesel, produced from vegetable (edible and non-edible) oils, animal fats and waste cooking oils, is one of the major renewable biofuels and can replace petroleum-based fossil fuel. Biodiesel is attractive because when it burns, there is no net CO_2 emission, leading to GHG emissions reduction (Majer et al., 2009; Krawczyk, 1996; Ma and Hanna, 1999). In addition, burning biodiesel emits less particulate matter, hydrocarbon, carbon monoxide, sulphate oxides and air toxics than petroleum-based fuels and it also cause less mutagenicity (Lotero et al., 2006). However, increasing demand for more biodiesel will require a large amount of land, which competes with the food supply. The amount of arable land in Europe is 113.8 million hectares and the amount of arable land used for biofuel crops in 2007 was

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17 million hectares, in which 4.3 million hectares alone was for biodiesel producing crops like soybean and rapeseed (Trostle, 2008). If EU wants to replace 10% of diesel with biodiesel and the feedstock for biodiesel production is still vegetable oils, then it would threaten the food security in EU so EU has to import the oils, which would threaten the food supply outside EU (Balat and Balat, 2010). Thus, to meet the increasing demand for biodiesel, alternative oil sources should be explored.

There are certain bacteria, yeast, algae and fungi that are capable of accumulating lipids in their cells of more than 20% of dry biomass under specific conditions. These microorganisms are called as oleaginous micro-organisms (Ratledge, 2002). Use of microbial oils as biodiesel feedstock has many advantages, such as similarity of fatty acid profiles with vegetable oils, short life cycle, easy culturing and growth, and no requirement for large acreage of lands. Oil production with micro algae is being carried out intensively and meso-scale demonstration projects have been implemented (Smith et al., 2009). However, the growth of micro algae needs the provision of light and also needs a large water surface. Some engineering problems are still present in micro algae oil production like the cost of collection and harvesting, and large-area growth of algae in ponds could destroy and overtake the current ecosystems (Singh and Singh, 2010). The other microorganisms for lipid production are yeast, like *R. glutinis* (Xue et al., 2010), and fungi, like *M. rouxii* (Fu et al., 2010). When applying oleaginous microorganisms for oil production, it is important to use those with high lipid accumulation capability and being safe to human beings and the environment.

In this study, an indigenous oleaginous fungus was isolated and screened from the soils. The lipid production by the selected fungi was tested in shaking flask experiments and a pilot-scale fermenter. The physical and chemical properties of the microbial oil produced by this fungus was analyzed and compared with commercially available vegetable oils.

MATERIALS AND METHODS

Isolation, screening and identification of oleaginous fungi

The oleaginous fungi were isolated from soil samples taken from Tamil Nadu, India by the serial dilution and plating method using the potato dextrose agar (PDA) medium. The soil samples were serially diluted by 10^3 folds for isolation. Filtered sterilized kanamycin (35 ppm) was added into the sterilized PDA medium before plating. The plates were incubated in an environmental chamber at 30°C for 3 to 5 days. Single fungal colonies were isolated and transferred repeatedly to new plates until pure cultures were obtained, and were maintained in PDA slants at 4°C. Purified cultures were screened using the screening medium containing glucose of 30 g l^{-1} and yeast extract of 5 g l^{-1} in 1000 ml distilled water with the pH value of 5.4 (Ahmed et al., 2006). one gram of the mycelial suspension was transferred aseptically to 50 ml of the screening medium added in 250 ml conical flasks; the flasks were incubated in an incubator shaker (Innova 4320, New Brunswick, USA) at 30°C and a shaking speed of 200 rpm. High lipid producing cultures were subjected to identification based on their colony morphology observed with a microscope after dyed with Nile red. Spore characteristics were identified by the soaking plain water-agar culture method (Watanabe, 1990).

Oil production of selected oleaginous fungi in flask cultivation experiments

Identified fungi were cultivated in 250 ml conical flasks containing 50 ml of the oil production medium in the incubator shaker at 30°C and a shaking speed of 200 rpm. In order to select the optimal conditions, effects of carbon sources (glucose, fructose, sucrose and lactose), nitrogen sources (ammonium chloride – NH_4Cl, ammonium sulphate – $(NH_4)_2SO_4$, and yeast extract), temperature (20, 25, 30, 35 and 40°C) and initial pH levels (5.5, 6.5, 7.5 and 8.5) on oil production of the selected fungal species were investigated. The carbon utilization pattern and lipid production by the selected fungal species were studied under different concentrations of glucose and nitrogen. The lipid content in biomass and the biomass growth was measured every day. All the above experiments were conducted with three replications and culturing was performed in an incubator shaker at 30°C, 200 rpm for 7 days.

Oil production in a pilot-scale fermentor

The optimal conditions obtained in the flask experiments were employed in a pilot-scale 3 L fermentor (Lark innovative technologies, India) with the effective volume of 1.5 L to examine oil production by the selected fungal species at a larger scale. The reactor was shaken at 200 rpm and was provided with oxygen at an air flow rate of 0.5 to 1.0 vvm. Two-day old mycelial suspension grown in the screening medium in the shaking flasks was added in the fermentor as the inoculum at a concentration of 5%.

Analytical methods

Cell biomass was determined after harvesting mycelia from the cultures by filtration through Whatman No.1 filter paper. The harvested mycelia were thoroughly washed with sterile distilled water and then dried at 60°C in an oven for 15 h. Residual glucose in the culture filtrate was determined in accordance with the method used by Somogyi (1952). Fungal oils were extracted from the dried mycelia using the solvent choloroform: methanol (2:1) using a soxhelt extractor (Bligh and Dyer, 1959) and then the solvents were evaporated in a rotary evaporator. The amount of oils was measured using gravimetric method.

The fatty acids present in the fungal oils were determined after conversion into fatty acid methyl esters (FAMEs). The method was used by Morison and Smith (1964). After addition of 2 ml of 0.5 M KOH in methanol to the oils along with two standards (penta-deconoic acid and ribitol), the mixture was boiled in a water bath at 90°C for 30 min. After cooling down to ambient temperature, 2 ml of 14% BF_3 in methanol was added and the mixture was boiled at 90°C for 30 min. After cooling down, 2 ml of water and 1 ml of hexane was added and the mixture was centrifuged at 5000 rpm at 28°C. The top liquid layer was filtered through 0.22 µm cellulose acetate membrane filter paper and then analyzed with gas chromatography (GC) (ASTM American standard for testing of materials). The GC conditions were 70 ev (m/Z) 50-550, source temperature at 230°C and quadruple temperature at 150°C in the EI

mode with an HP-5ms capillary column (30 m × 0.25 mm i.d., 0.25 mm film thickness; J&W scientific, USA). Helium was the carrier gas at a flowrate of 1.0 ml/min. The inlet temperature was 300°C and the oven temperature was programmed at 150°C for 2 min, increase at 4°C /min up to 300°C and then at 300°C for 20 min. Samples were injected at 1 µl with a split ratio of 50:1. The extracted crude oil samples were subjected to the analysis of the physical and chemical properties, including kinetic viscosity, specific gravity, flash point, fire point, cloud point, pour point, calorific value, carbon residue, free fatty acids, acid value and ash content, according to the procedures of American Standards for Testing of Material.

RESULTS AND DISCUSSION

Isolation, screening and identification of oleaginous fungi

The present study allowed selection of hyper oleaginous fungi from soil samples to provide indigenous fungal species for lipid production without threatening the existing ecosystem and human beings. Several oleaginous fungi were isolated from soils samples taken from various locations in Tamil Nadu, India. Colonies showing good mycelial growth on Petri plates were selected and screened for lipid production. Five high lipid producing isolates were selected, and evaluated for morphological and spore producing characteristics (data not shown). The isolate KK1 that had the highest lipid content in biomass (28%) and the highest biomass growth (10 g l^{-1}) in the screening medium among the five isolates was identified as *Mortierella* sp., and was used in future studies. The lipid content in this fungal species was much higher than that of fungus *M. circinellorides* (15-19% lipid content) (Ratledge, 2002). It has been known that microorganisms have the potential to accumulate intracellular lipids, but such intracellular lipid contents are usually less than approximately 10 % of the dry biomass. However, the oleaginous microorganisms have the potential to accumulate lipids in their bodies' at least equivalent to about 20% of the dry biomass. The morphological characteristics of the isolate KK1 included: lobbed (petal shaped) mycelium, white colour, and thick growth (Figure 1). Spores produced were intercalary chlamydospores. Watanabe has observed that *Mortierella* sp. has a morphological characteristic of wavy mycelium and is capable of producing different spores like chlamydospores and zygospores, but no sporangia; the typical characteristic of *Mortierella* is lack of garlic-like odour. The isolated fungi, KK1, had all properties of *Mortierella* sp. given by Watanabe, such as white wavy mycelium and intercalary chalymadospores.

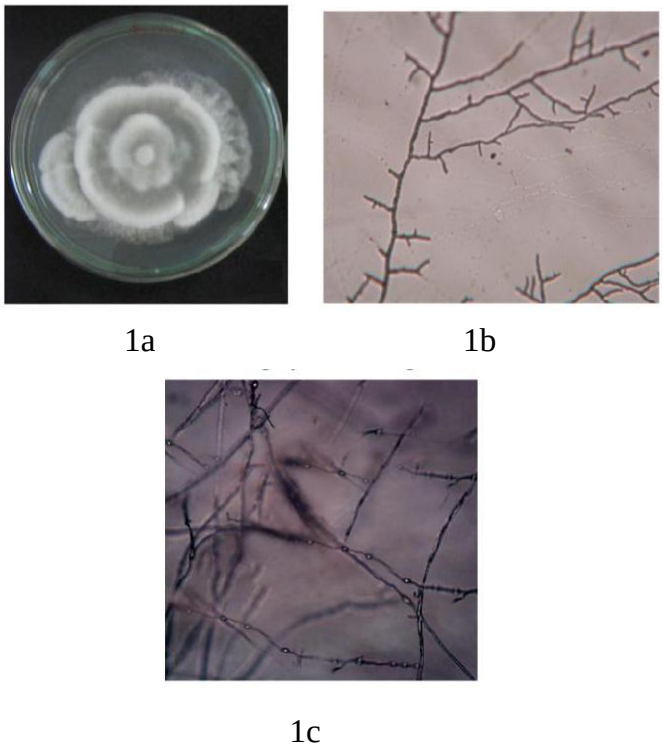

Figure 1. Morphological characteristics of the fungal isolate KK1. (a) Growth of KK1 on PDA medium. (b) Microscopic view showing mycelium KK1. (c) Microscopic view showing spores of KK1.

Oil production in flask culturing experiments

The highest lipid producing isolates *Mortierella* sp. KK1 was subjected to the optimization studies so as to find the optimal conditions for oil production of this isolate. Effects of different carbon sources (glucose, fructose, sucrose and lactose) and nitrogen sources (yeast extract, NH_4Cl and $(NH_4)_2SO_4$), on oil production of isolate, KK1 are presented in Figure 2. Among the four carbon sources tested, glucose exhibited the highest lipids content in biomass (28.2±0.2%) and the highest biomass growth (10 g l^{-1} ± 0.5), followed by fructose (the lipid content of 26.5±0.3%). The least lipids content (10.8±0.1%) and biomass growth (1.9 g l^{-1}±0.1) was recorded when the organic carbon source was lactose. All the carbon sources except lactose gave relatively high lipid contents, over 20%. This indicates that *Mortierella* sp. KK1, can utilize a wide range of carbon sources for oil production, which is contrary to most of fungi that prefer to utilize simplest carbon sources, like glucose. Among the nitrogen sources tested, yeast extract supported the maximum lipid content (26.3±0.3 %) and the biomass growth (6.3 g l^{-1} ±0.1) than NH_4Cl and $(NH_4)_2SO_4$; this indicates that organic nitrogen sources are better suitable for lipid production of Isolate, KK1 than inorganic nitrogen sources. This finding has also been observed by (Jang et al., 2005; Bajpai et al., 1991), who reported that 1%t yeast extract supported the maximum lipid content and

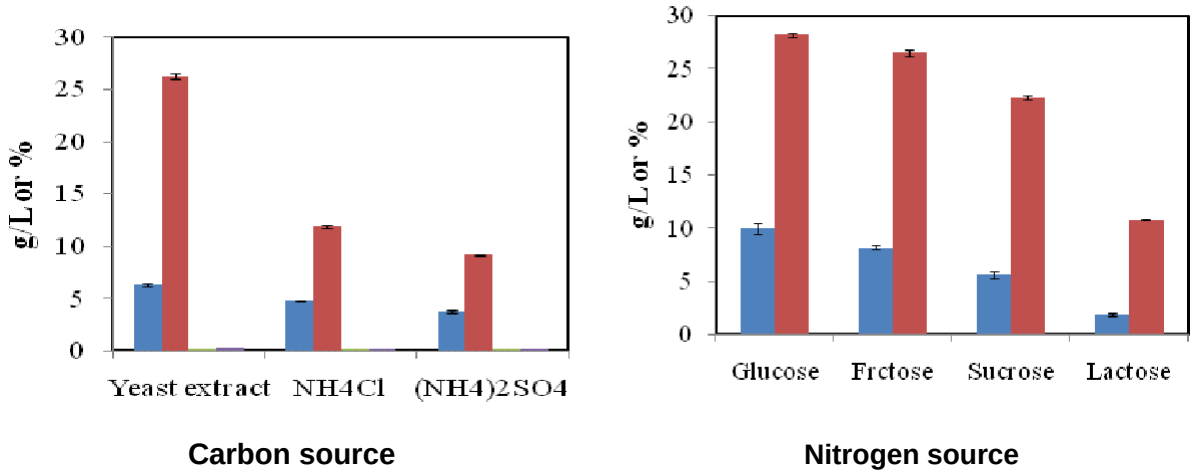

Figure 2. Standardization of growth parameters for maximum lipid production by *Mortierella* sp. KK1. Effect of carbon and nitrogen sources.

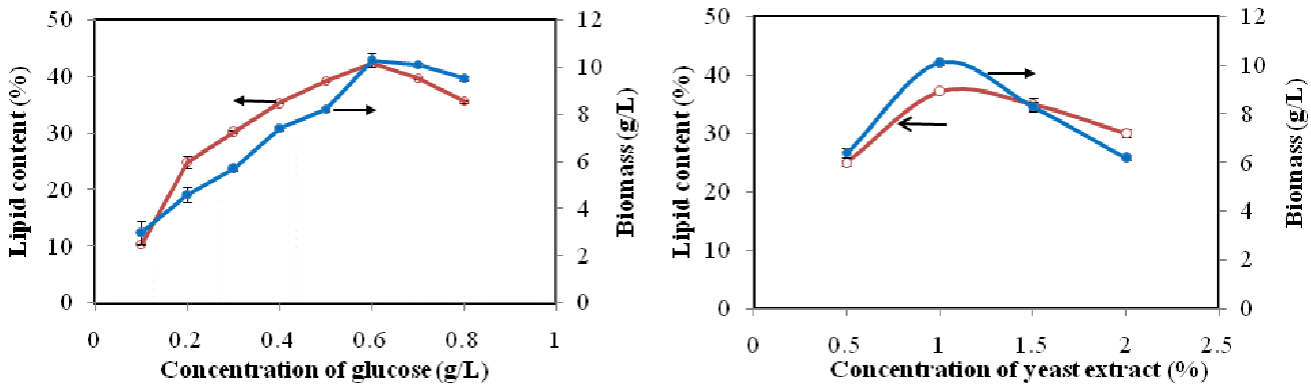

Figure 3. Standardization of growth parameters for maximum lipid production by *Mortierella* sp. KK1. Effect of concentrations of glucose and yeast extracts.

cell growth in Mortierella alpine and Mortierella elongate. Since glucose and yeast extract gave better results than other organic carbon sources and nitrogen sources, respectively, their optimum concentrations for the maximum lipid production by isolate, KK1 were investigated and presented in Figure 3. When the lipid production medium was prepared with glucose concentrations ranging from 0.1 to 0.8 M and a constant yeast extract concentration of 5 g/L, the lipid content and the biomass growth gradually increased with the increase in the glucose concentration from 0.1 M to 0.5 M and reached the maximum levels of 42.3±0.2% and 10.3 g l^{-1} ±0.3 at 0.6 M of glucose, respectively. Thereafter, the lipid content declined to 35.6±0.2% at 0.8 M of the glucose concentration. When the culturing medium was made with yeast concentrations ranging from 0.5 to 2% and a constant glucose concentration of 0.16 M, the maximum lipid content (37.2±0.2%) was observed at the yeast extract concentration of 1%. According to Ratledge (1989), high carbon and low nitrogen levels, for instance, C: N = 60:1, support lipid accumulation in cells of oleaginous microorganisms; this is consistent with the results obtained in the present study. While, further increase in the glucose concentration (above 0.6 M) resulted in the decreased biomass growth and lipid accumulation. The reason might be intolerance of fungi to high glucose concentrations, which can lead to a high osmotic pressure and cell lysis.

Effects of temperature and initial pH on the biomass growth and lipid content in biomass of *Mortierella* sp. KK1 were also investigated (Figure 4). Both the biomass growth (10.2 ±0.1g l^{-1}) and the lipid content (43.2 ±0.2%) reached the maximum at 30°C and other temperatures were not suitable for lipid production of this fungal

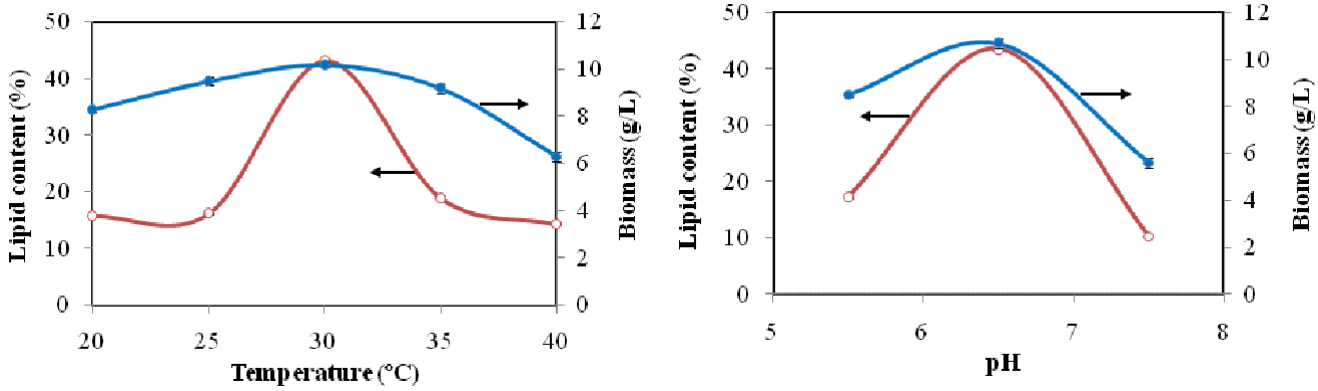

Figure 4. Standardization of growth parameters for maximum lipid production by *Mortierella* sp. KK1. Effect of temperature and pH.

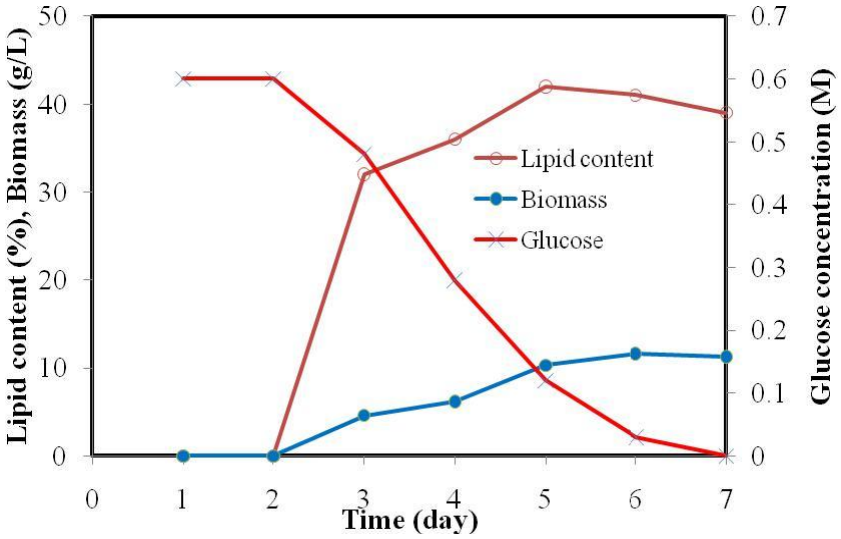

Figure 5. Lipid accumulation and glucose utilization by isolate *Mortierella* sp. KK1.

species. Xue et al. (2010) observed that the lipid content was almost not affected by temperature in the range of 30 to 37°C. It was observed that several hypae of fungi were swollen with a tendency towards lysis on the 6th day during inoculation at temperature of above 30°C. The optimum pH for the biomass growth and lipid production of *Mortierella* sp. KK1 was 6.5, at which, the maximum biomass growth was 10.7±0.2 g l^{-1} and the maximum lipid content was 43.5±0.2. When pH was 8.5, there was no detection of biomass growth in the medium. The best pH for the biomass growth and lipid production together with a high proportion of poly unsaturated fatty acids in oleaginous fungi *Mortierella alpina* and *Mortierella elongata* was around 6.0 and fungi preferably grew well below neutral pH (Ratledge et al., 1989). The carbon utilization pattern and lipid accumulation of *Mortierella* sp. throughout a seven-day fermentation period at an initial glucose concentration of 0.6 M and an initial yeast extract concentration of one per cent was studied (Figure 5). The results show that glucose consumption commenced from the second day but there was no obvious lipid accumulation and biomass growth detected. The lipid accumulation initiated on Day 3 with a biomass concentration of up to 4.6 g l^{-1} by consuming 0.32 M concentration of glucose, and then it rose to 36% on Day 4 after utilizing 0.48 M of glucose. The maximum lipid content (42%) was obtained on Day 5 with consumption of 0.57 M of glucose and thereafter declined. The similar changes were found by (Papanikolaou et al., 2004) when *Mortierella isabellina* was grown on nitrogen-limiting medium with the organic carbon source of glucose, the lipid production commenced after depletion of nitrogen, increased further until a maximum of biomass growth of 4.5 g l^{-1} was obtained at the 120th h of fermentation, and

Table 1. Fatty acid profile of crude *Mortierella sp.*KK1 oil.

Fatty acid	Structure	Wt (%)
Palmitic	16:0	19.63
Palmitoleic	16:1	0.26
Stearic	18: 0	11.45
Oleic	18: 1	38.22
Linoleic	18: 2	4.78
Linolenic	18: 3	0.52
Mystric	14: 0	0.45
Arachidic	20: 0	3.29
Homo gamma linolenic acid	20:3	8.10
Behenic	22: 0	0.22

Table 2. Fuel properties of the crude *Mortierella* sp. KK1 oil compared with commercial plant oils.

Property	Unit	Crude Fungal oil	Jatropha oil	Rapeseed oil	Diesel	Biodiesel standard	
						ASTM D 6751-02	DIN EN 14214
Density at 15°C	Kg/m^3	920	940	911	850	-	860-900
Viscosity at 40°C	mm^2/s	54.81	24.54	37.3	2.60	1.9-6.0	3.5-5.0
Flash point	°C	218	225	246	68	>130	>120
Pour point	°C	7.0	7.0	-31.7	-20	-	-
Water content	%	3.9	1.40	-	0.02	<0.03	<0.05
Ash content	%	0.62	0.80	0.006	0.01	<0.02	<0.02
Carbon residue	%	0.082	1.0	0.31	0.17	-	<0.3
Acid value	Mg KOH/g	28.22	28.00	197.07			
Calorific value	MJ/Kg	32.05	38.65	39.7	42	-	-
Free Fatty acid	%	14.55	14.9	-	-	-	-
Fire point	°C	230	-	275-290	-	-	-

thereafter declined. The decrease in the lipid content might be due to the mechanism used by microorganisms that intercellular lipids were used for cellular maintenance or as a carbon source for biomass synthesis in carbon limited conditions (Fu et al., 2010; Kusdiana and Saka, 2005).

Oil production in the pilot-scale fermentor

Oil production by the fungal isolate *Mortierella sp.* KK1 was scaled up in a 3 L bench scale fermentor with the working volume of 1.5 L under the optimal conditions obtained in the flask culturing experiments (the glucose concentration of 0.6 M, the yeast extract concentration of 1%, temperature of 30°C and pH of 6.5). The reactor was stirred at 200 rpm and was operated in a batch mode. After operation for five days, the lipid content of 44.1% and the biomass growth of 15.9 g l^{-1} were obtained. Two repetitive experiments yielded similar results, indicating the reproducibility and stability of the strain.

The composition of crude *Mortierella sp.*KK1 oil is presented in Table 1. The oil contained mysteric acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, arachidic acid, gamma linolenic acid and behenic acid. For biodiesel production, long chain fatty acids like oleic, linoleic, stearic and palmitic acids are favourable for engine performance (Kumar et al., 2007) all the four fatty acids were found in *Mortierella* sp. crude oil with the composition of 38.2, 4.8, 11.5 and 19.6%, respectively. The crude fungal oil contained ɣ-linolenic acid with a fraction of 8.1% in total fatty acids, which can be used as a dietary supplement for treating inflammation and auto-immune diseases.

Physical and chemical properties of the crude *Morierella* sp. KK1 oil were analysed and were compared with properties of two commercial plant oils – Jatropha oil and rapeseed oil (Table 2). In addition, the obtained values were compared with the ASTM and DIN EN standards for biodiesel. The kinetic viscosity (54.81

mm^2/s at 40°C) and the water content (3.9%) of the crude *Mortierella* sp.KK1 oil were higher than those of the two crop oils. The high kinetic viscosity can be reduced when converting the fungal oil into biodiesel by trans-esterification. The slightly higher water might be reduced to a minimum level by further optimization studies. The yield of methyl esters during the alkaline catalytic transesterification process is not affected by such water content (Demirabs, 2003). The fungal oil had a free taffy acid content of 14.6%, which was similar to that of Jatropha oil but higher than rapeseed oil. According to Kusdiana and Saka's study, such free fatty acid content does not significantly affect the efficiency of the transesterrification process (Demirabs, 2003). The other properties were more or less similar with the two crop oils.

Conclusions

This study investigated oil production of an oleaginous fungus *Mortierella sp.* KK1 which was isolated from soils in Tamil Nadu, India. The fungus can utilize a wide range of organic carbon sources and yeast extract as the nitrogen source to produce lipids. The highest lipid content in biomass was 43.5% and the highest biomass growth was 10.7 g/L. The fatty acid composition of the crude *Mortierella sp.* oil was similar to that of vegetable oils. Its physical and chemical properties are suitable for biodiesel production through transesterification. In consideration with various advantages of microbial oils over the crop oils, the fungal oil can be a potential feedstock for bio diesel production.

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