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# A Unique Approach to Produce Lipids from Oleaginous Fungi for production of biofuel

## Abstract

Lipids produced from oleaginous fungi show great promise for biofuel production since their production neither require agricultural land nor create any unwanted competition between food/ fodder, hence oleaginous fungi became favorable feedstock for biofuel production and most suitable approach to reduce greenhouse gas emissions.
The present study deals with the feasibility for production of microbial oil from fungi. In the study, fungi strain was isolated from rotten fruits and soil sample from waste land area. The potential of these fungal isolates were evaluated, nutrient media screened to enhance biomass. Good results have been obtained for lipid accumulation when studied microscopically with 'Sudan Black B as indicator for oil/ lipid. SEM studies confirmed the channeled structure which can allow fluids and nutrients to enhance growth morphology and lipid formation. The cultivation conditions such as C/N ratio, nitrogen, temperature, pH, incubation period were optimized to maximize lipid production.
The Molecular identification by sequencing of 18S rDNA gene and BLAST search analysis revealed that it has perfect match with that of *Aspergillus Niger* with NCBI accession number HQ850370 showing 99% identity and another species, *Aspergillus Welwitschiae* with NCBI accession number KT826630 showing 100% identity.
Two potential isolates of olegineous fungi, produced maximum biomass, yield of 26.8g/l & 27.7g/l respectively and maximum lipid yield of 37.6% (w/w) & 41.9% (w/w). FTIR spectrum of extracted lipid confirmed the conversion of fatty acids to methyl esters by peaks related to carbonyl group at 1744.69 $cm^{-1}$ .Gas Chromatograph study revealed that the fatty acids oleate and linoleate were predominant along with pentadecanoic, palmitic, heptadecanoic acid, indicating the efficacy of fungal FAME towards acquiring microbial oil properties which is close to biodiesel composition. The study demonstrated that *Aspergillus Niger* and *Aspergillus Wewitschiae* are good lipid producer for microbial oil production and has more potential adaptability in the industrial production of biofuel.

## 1. Introduction

The massive global demand for crude oil, and limited supply of petroleum, has lead the world to move for alternative energy resources. Also, because of the environmental and health disadvantages resulting from the use of the unsustainable fossil fuels, finding new energy alternatives which are clean, sustainable (1), easily available and environment-friendly is the main goal of many researchers around the world. In recent years, attention has been paid to the exploration of microbial oils, which might become one of the potential sources for bio oil production in future. Oleaginous microorganisms are able to accumulate lipid, above 20% of their biomass on dry weight basis. Such microbial oil can be used for biodiesel production. Biofuel production based on biomass represents a fundamental approach to combat high energy prices and potential depletion of oil reservoirs, to reduce greenhouse gas emissions.

Microbial lipids can represent a valuable alternative feedstock for biodiesel production, and a potential solution for a bio-based economy.

In this study, the development of microbial lipids, biochemistry of lipid accumulation by oleaginous fungi and lipid production for microbial oil have been discussed.

## 2. Biochemistry of microbial lipid production

Microbes can naturally synthesize lipids for maintenance of cell membranes, storage of energy and communication. However, only a certain group of microorganisms can accumulate lipids more than 20% of their biomass and store them as triacylglycerol molecules. Biochemistry of lipid production can be divided into two aspects: (i) Nutrient deprivation; (ii) Lipid synthesis of intermediates with involvement of different enzymes.

Lipid production in oleaginous microorganisms starts when one of the growth nutrients (usually nitrogen) in the medium runs out and the carbon source is in excess. The cell accumulates excess carbon and converts it into lipids in the form of triacylglycerol. The high C/N ratio in the culturing medium is required for oleaginous microorganisms to accumulate lipids.

## 3. Materials and methods

The samples were collected from rotten fruits and garden soil, from this 1g sample was taken and added in tube containing 9ml distilled water and mixed thoroughly and serial dilutions were made to 6-fold and plated on Potato Dextrose Agar (PDA) medium. The plates were incubated at 30°C for five days in an incubator. Several fungal colonies were obtained from different plates and every single colony was purified by transferring to a new agar plate until pure cultures were confirmed.

**3.1 Enrichment of oleaginous microorganisms:** The following three different compositions (g/l) of enrichment medium were used: (i) Mould seed culture medium: Glucose 70, Yeast extract 0.5, $(NH_4)_2SO_4$ 2.0, $MgSO_4.7H_2O$ 0.5, $KH_2PO_4$ 1.0, Chloramphenicol 50 mg/l. (2). (ii) Glucose 70, Ammonium sulfate 2.0, Yeast extract 0.5, $Na_2HPO_4$ 22.4, $NaPO_4$ 0.44, $MgSO_4.7H_2O$ 0.5, Chloramphenicol 50 mg/l. (3). (iii) Sucrose 30, $NaNO_3$ 3, $K_2HPO_4$ 1.0, $MgSO_4.7H_2O$ 0.5, KCl 0.5, Ferrous sulfate 0.01, NaCl 15, $ZnSO_4.7H_2O$ 6.7mg/l, $Co(NO_3)_2.6H_2O$ 1 mg/l, Chloramphenicol 50. (4)

**3.2 Screening and isolation of oleaginous microorganisms:** (a) 1 ml enriched sample was gradient diluted with sterilized water, then 0.1 ml of it was spread on screening medium. For screening Mold seed culture medium was used. The screening plates were kept in incubator at 28°C for 1-5 days. Enrichment with remaining two media does not show encouraging results.

(b) Screening for oleaginous fungi: Sudan Black B and Nile Red staining methods (2) were used for screening of oleaginous fungal strains. The Sudan Black B solution was prepared by dissolving 0.3 g Sudan Black B in 100 ml of 70% alcohol with agitation overnight. Safranin solution was prepared by dissolving 1g safranin in 100 ml distilled water. The lipids within the fungal cell took on a blue-black or light blue colour, whilst the non-lipid cellular material was stained light pink (5).

**3.3 Biomass production and determination of cell dry weight:** To screen and purify selected fungal isolates, they were cultured in Mold seed culture medium with glucose content 50, 70, 80, 90 and100 g/l respectively. Flasks were removed every 24 hours for five days and microbial cells were harvested. The fungal biomass of screened isolates, mycelia were harvested from the incubated flasks by suction filtration through Whatman No 1 filter paper and thoroughly washed with distilled water. Then filtered mycelia were dried at 60°C in an oven till a constant weight was achieved.

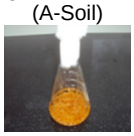
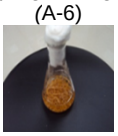
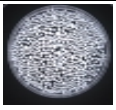
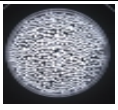
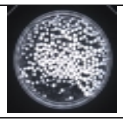
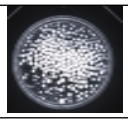
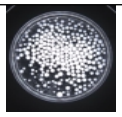
**3.4 Determination of growth rate:** The optical densities (OD) for growth study were carried out at 600 nm (UV-VIS Spectrophotometer) in time-based study for oleaginous fungi culture. The Schuett colony Quant automated colony counter was used to determined size of fungal pellet during cultivation.

**3.5 Scanning Electron Microscopy (SEM) analysis:** Culture was washed thrice with normal saline, then immersed completely in 2.5% glutaraldehyde solution. The solution was dispersed by an oscillator and then allowed to stand for more than 2 hours so that the cells were fixed with glutaraldehyde. Finally, the culture suspension was fixed into slide and gold-coated for imaging by SEM.

**3.6 Gas Chromatograph analysis:** The lipids were weighed and analyzed using a Gas Chromatography unit equipped with capillary column (Id: 0.25mm, length: 100m) and FID as detector. The carrier gas Helium was maintained at flow rate of 1.0 ml/min. The injection volume was 1ul. The injector and detector temperatures were set at 220°C and 250°C, respectively. Identification of peaks was performed by comparisons with Sigma Aldrich FAME standards and identification had been done on the basis of their specific peaks.

**3.7 Lipid extraction:** Lipid extraction from dried biomass was performed according to the Folch et al. method. Prior to lipid extraction, mycelia were freeze–dried at -50°C for 24 hours. The mycelia were grounded into fine powder, added into 150 ml of chloroform and methanol mixture (2:1; v/v). The suspension was then left overnight at room temperature. The homogenate was filtered and washed with 150 ml of 0.1% NaCl solution. The mixture was shaken and allowed to settle into two phases and then the organic layer was transferred into a rotary bottle and evaporated to obtain the microbial oil. The trans-esterification reaction was carried out by refluxing the microbial oil with 50 ml mixture of 6% KOH in methanol. The aqueous phase was washed two times with n-Hexane, subsequently acidified with 6N HCl to make the solution acidic (pH 2-3). The acidic solution was extracted with n-Hexane to extract fatty acid. Extracted fatty acid from oil was methylated with 14% $BF_3$–methanol mixture. Fatty acid methyl esters (FAME) were analyzed by Thermo Scientific make FTIR spectrometer (Model No: Nicolet iS5) to confirm the conversion of fatty acid to methyl esters, observing peak around 1740 $cm^{-1}$.

**3.8 Molecular Identification:** The genetic identification of the isolates were carried out following standard methodology. i) DNA were isolated from the pure culture ii) The ITS region of rDNA was amplified using fungal universal primers ITS4 and ITS5, iii) The sequencing PCR was set up with ABI-Big Dye Terminator v3.1 cycle sequencing kit, iv) The sequence data was aligned with publicly available sequences and analyzed to reach identity. The sequence data generated were subjected to homology search through Basic Local Alignment Search Tool (BLAST) of National Centre for Biotechnological Information (NCBI).

| | *Aspergillus welwitschiae* (A-Soil) | *Aspergillus niger* (A-6) |
|---|---|---|
| Shake flask fermentation for 5 days |  |  |
| Temp: 30°C, RPM: 180 |  |  |
| Diameter | 4.1-7.1mm | 4.0-6.9mm |
| Temp: 25°C RPM: 180 |  |  |
| Diameter | 3.0-5.3mm | 3.2-5.5mm |
| Temp: 35°C RPM: 180 |  |  |
| Diameter | 2.8-4.2mm | 2.9 -4.9mm |

## 4.0 Results and Discussion

**Fig. 1: Fungal pallets formation analysis**

Two potential filamentous fungal strains were evaluated on glucose as carbon source after secondary screening (Table 1). These fungal isolates, namely A6 and A-soil, showed positive results for lipid accumulation when studies with Sudan Black B (Table 1). These lipids were accumulated within cell bodies, as indicated by blue colour lipid/ oil droplet in the fungi hyphae when observed under microscope (Table 1).

During shake flask fermentation, the cultivated fungal cells formed pallets, such cell palletisation has advantage for easier separation by simple filtration than centrifugation. Also, the agitation rate influenced the size distribution of the pellets, with an average pellet diameter range of 4-7 mm at 180 rpm with temperature 30°C (Fig. 1). Cell palletisations have been observed during cultivation at their maximum level at 30°C temperatures, 120 hours. Studies also revealed that difference in temperature of ±5°C has significantly decreased the size and yields (Fig. 1).

**Table 1. Morphological depiction of isolates**

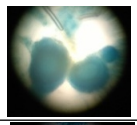
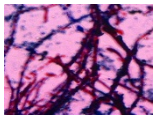
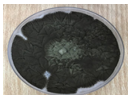
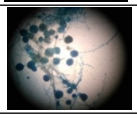
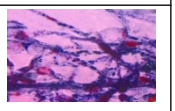
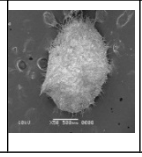
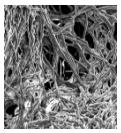
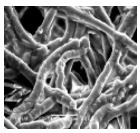
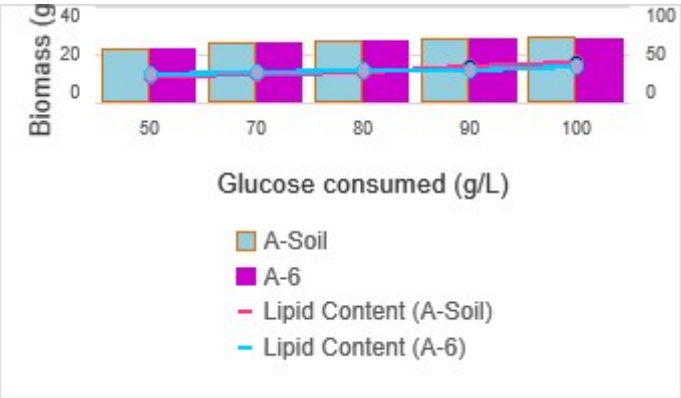
| Fungal isolates | Colony morphology | Microscopic analysis | |
|---|---|---|---|
| | | Lactophenol blue | Sudan Black B |
| A-6 |  |  |  |
| A-soil |  |  |  |

**Fig. 2: Optical density of fungal strains at 600 nm**

**4.1 Study of Optical density:** Growth of fungi was screened by measuring optical density (OD) of the broth at 600 nm wavelength. 5 ml of broth culture was transferred to the cuvette and initial OD was determined using a UV-Visible spectrophotometer.5 ml of the liquid culture from inoculated culture flask was transferred, using cotton plug filter to a cuvette after every 24 hours of incubation and OD was determined. This process was repeated for a period of 120 hours (Fig. 2). Plot of OD values against the hours indicated that fungal strains have typical 'S' shaped curve. A lag phase with slow growth was initially observed followed by sharp increase in growth and very little change in growth was seen at later stages of growth, which possibly indicated that the stationary phase had been achieved in 120 hours.

**4.2 Biomass and Total Lipid yields:** The biomass generations were carried out with strains on different glucose and nitrogen concentrations (6). The fungal strains A6 and A-soil showed significant biomass yields of 26.9 g/l and 27.7 g/l respectively. The total lipid from the cell mass was extracted and estimated by Bligh and Dyer (1959). The fungal Isolate A-soil, A-6 yielded maximum lipid content in the range of 26.9%-41.9% w/w and 29.8%-37.3%w/w. The graphical representation of the biomass and lipid content percentage in the cell is depicted in Fig. 3.

| Fungal isolate | Rasolution | | |
|---|---|---|---|
| | 500um | 50 um | 10 um |
| A-Soil |  |  |  |



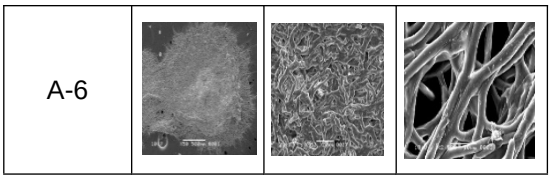




**Fig. 3: Biomass & lipid content (%) in isolates**

**Fig. 4: SEM micrograph**

**4.3 SEM analysis:** SEM micrograph at 500um, 50um, 10um, showed that the mycelia of fungi were cylindrical and pipe shaped. Lesser interstitial voids and more structured channel were observed (Fig. 4), which provide conditions for improved mass transfer. Moreover, a channelled structure allowed fluids and nutrients to pass through easily to ultimately enhance growth morphology and lipid formation.

**4.4 FTIR analysis:** Fatty acids consist of a long chain of carbons with a carboxyl group at one end. Depending on their structure, fatty acids can be saturated or unsaturated. Lipids consist of three fatty acids bonded to one glycerol molecule, known as triglycerides. Fatty acids methyl esters (FAME) were analysed by Fourier transformed infra-red (FTIR) spectroscopy to confirm the conversion of fatty acids to methyl esters by observing peak (Fig. 5).
The esterified lipid shows the stretching vibrations at 1744 $cm^{-1}$ thereby confirming C=O (carbonyl group) functional group in organic matter from fungal isolates A Soil, A-6. (Fig 5)

**Fig. 5: FTIR spectra of A-Soil and A-6 isolates FAME**

**4.5 Gas Chromatography analysis:** FAME chromatogram (Fig. 6) from the samples of A-soil, shows peaks for methyl ester of Pentadecanoic acid (C15:0), Palmitic acid (C16:0), heptadecanoic acid (C17:0), oleic acid (C18:1) and linolenic acid (C18:2).
The peak as observed form sample A-6, methyl ester of Pentadecanoic acid (C15:0), heptadecanoic acid (C17:0), oleic acid (C18:1) and linolenic acid (C18:2).

**Fig. 6: Gas Chromatograms of A-Soil and A-6 isolates FAME**

Fatty acid analysis indicated that the lipids of isolates were rich in five fatty acids, among them there was maximum percentage presence of oleic acid (C18:1) and linolenic acid (C18:2), with minimal presence of pentadecanoic acid (C15:0), palmitoleic acid (C16:1) and heptadecanoic acid (C17:0) in the fatty acid samples. Microbial oils are composed of 14-18 carbon chains (7), close to biodiesel composition and have more potential adaptability in the industrial production of biofuel. The presence of higher carbon number fatty acid indicates the efficacy of fungal isolates towards acquiring biofuel properties.

**4.6 Identifications of fungal isolates:** The filamentous fungal isolates were genetically identified through molecular approaches. The tested fungal strain A-Soil showed 99% sequence similarity with *Aspergillus niger* with NCBI accession number HQ850375, while strain A-6 showed 100% sequence similarity with *Aspergillus welwitschiae* (NCBI accession number KT826630) (Table 2).

**Table 2: Molecular identification analysis of isolated Oleaginous Fungi**

| Isolate | Closest match in NCBI database | Gene bank accession no | Sequence similarity |
|---|---|---|---|
| A-Soil | *Aspergillus Niger* | HQ 850370 | 99% |
| A-6 | *Aspergillus Welwitschiae* | KT826630 | 100% |

## 5.0 Conclusion

Two potential isolates of olegineous fungi, namely A-6 and A-soil, produced maximum biomass, yield of 26.8g/l & 27.7g/l respectively and maximum lipid yield of 37.6% (w/w) & 41.9% (w/w).

FTIR spectrum of extracted lipid confirmed the conversion of fatty acids to methyl esters by peaks of carbonyl group at 1744 $cm^{-1}$. Gas Chromatograph study revealed that the fatty acids oleate and linoleate were predominant along with pentadecanoic, palmitic, heptadecanoic acid, indicating the efficacy of fungal FAME towards acquiring biofuel properties and potential adaptability in the industrial production of biofuel.

By sequencing of 18S rDNA gene and BLAST search analysis, the isolates were identified as Aspergillus Niger with NCBI accession number HQ850370 and Aspergillus Welwitschiae with NCBI accession number KT826630. It can be concluded that Aspergillus Niger and Aspergillus Welwitschiae are good lipid producer for microbial oil production.

## 6.0 References:

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