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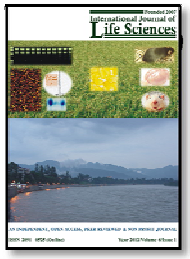

Research Article

Biodiesel Production from Oleaginous Fungi

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ABSTRACT

Biodiesel involves the mixture of fatty acyl methyl/ethyl esters, produced from transesterification neutral lipids and if the origin of the source is from oleaginous micro organisms, then it is termed as micro diesel. In the present work, aiming to exploit fungi for biodiesel production, 12 fungal isolates were screened for lipid content by Sudan Black B staining method. Among 12 isolates, lipid rich five species *viz, Mortierella alpina , M.ramanianna, M.vinancea, M.hyalina and M.verticella* have been taken for fatty acids analysis by spectrophotometry, which revealed that the amount of free fatty acids were ranged from highest in M.alpina 35 µmoles of Oleic acid , 25 µmoles of Palmitic acid and 14 µmoles of Myristic acids to lower as much as 21 µmoles of Oleic acid , 18 µmoles of Palmitic acid and 16 µmoles of Myristic acids respectively in *M.ramanianna* .

Key words: **Biodiesel; Oleaginous Fungi; Sudan Black B; Spectrophotometer assay; *Mortierella***

INTRODUCTION

The impact of drastic increase in energy consumption , concerns about greenhouse gas (GHG) emissions, the rising price of fossil fuels and the projected decrease and insecurity of their existance in the fossil fuel reserves, have led to high attention on energy production and energy security issues (Pahl 2005, Hill *et al.* 2006). Hence, Biodiesel is an alternative approach, which is ecofriendly and produced from renewable sources like vegetable oils, plant oils, or animal fats by transesterification with low molecular weight alcohols (Alcantara *et al.* 2000, Lang *et al.* 2001, Vicente *et al.* 2004). When used as fuel in diesel engines and heating systems, biodiesel has many merits, such as high energy density, more favorable combustion emission profile, improved lubricating properties and others (Tyson 2001). It is also an environmentally benign fuel compared to petroleum-based diesel, as biodiesel is, biodegradable, non-toxic and essentially carbon dioxide neutral. In brief, these merits make biodiesel a good sustainable energy carrier. But, the availability of the present plant and animal resources are very limited, demanding more time and manpower for their production, which lead to the attention of the researchers to look in to the other alternative renewable resources, such as production of Biodiesel from Bacteria, Fungi and Algae.

The fatty acid profile of microbial lipids is quite similar to that of conventional vegetable oils. Therefore, oleaginous filamentous fungi are suggested as a favorable feedstock for a sustainable biodiesel industry (Peng and Chen 2008, Zhao *et al.* 2011). . It is known that lipids accumulate in the yeast cells in the form of distinct oil globules Yeasts and fungi (especially molds) are considered as favorable oleaginous microorganisms since 1980s. Some yeast strains, such as *Rhodosporidium* sp., *Rhodotorula* sp. and *Lipomyces* sp. can accumulate intracellular lipids as high as 70% of their biomass dry weight. The most efficient oleaginous yeast *Cryptococcus curvatus* can accumulate storage lipid up to >60% on a dry weight basis. A filamentous fungus – *Mortierella alliacea* Strain YN-15, (Singh and Ward 1997) accumulated arachidonic acid (AA, C20:4n-6) mainly in the form of triglyceride in its mycelia, which yielded 46.1 g/L dry cell weight, 19.5 g/L total fatty acid, and 7.1 g/L arachidonic acid by 7-d cultivation. Hence, it is clear

that oleaginous yeasts and fungi will act as potential alternative oil resources for biodiesel production. Microscopic observation of the oleaginous yeast and fungi (M.S.Thakur *et al*., 1988) has indicated the existence of such globules. However, direct observation under the microscope may not give a correct picture unless there is a specific method to observe the lipid particles. A suitable staining technique gives this opportunity. Burdon 1946 observed fatty material in bacteria and fungi by staining dried, fixed slide preparations using Sudan Black B. In the case of both aerobic and anaerobic spore-forming bacilli, the fatty material was observed to be reduced somewhat just before active spore formation began. This limited study helped conclude that fat droplets in the cytoplasm originate at the cell periphery which presumably bears some relationship to the cytoplasmic membrane of bacterial and fungal cells. Fat staining by Sudan Black B was also used for animal tissue." Evans *et al.* (1985) used this staining technique to detect colonies that accumulate lipid in order to rapidly screen oleaginous yeasts. There is enormous interest in developing the estimations of fatty acids has been created because of their multifaceted applications. Various methods are developed for the determination of lipase activity or in turn the final products, that is fatty acid estimations. Currently, the commonly available assays include titrimetry (Pokrana 1964), turbidometry (Sakai 2002), fluorimetry (Cooper 1981) and colourimetry (Margesin 2002). But, due to low sensitivity and encountering with titrimetry , is the restricted range of pH values that can be investigated (Beisson *et al*., 2000). Moreover, titrimetry involves a long incubation period while fluorimetric assays are expensive. Hence we followed the copper soap colorimetric, which reduces the above mentioned limitations (Saisubramaniayan *et al*., 2004). Hence, in the present work, we have tried to estimate the lipid contents of the fungal isolates which is very rapid and sensitive.

MATERIALS AND METHODS

Sources of oleaginous fungi from selected plants:

Oleaginous plants like *Jatropa curcas* (Neves *et al.* 2009), *Pongemia pinnata*, *Madhuca longifolia, Ricinus communis* and *Helianthus annus* were selected.

Collection of Plant samples and sampling:

Jathropa curcas and *Pongemia pinnata* were collected from Dhanvantri vana, Jnanabharathi. *Madhuca longifolia* and *Ricinus communis* had been collected from the field of Bangalore University campus and *Helianthus annus* from Agriculture University campus, Bangalore. The plant parts like leaves, flowers and seeds were excised using a sterile knife and brought to the laboratories in sterile polythene bags.

Different parts of fresh healthy plant samples will be cut into small pieces (5mm × 2mm) using sterile blade, then the smaller pieces will be surface sterilized by immersing in 4 % sodium hypochlorite solution for 90 Seconds followed by 70% ethanol treatment for 5 Seconds and thoroughly washed with sterile distilled water (Suryanarayanan and Thennarasan , 2004). The surface sterilized samples were placed on sterile potato dextrose agar (PDA) plates amended with 50mg/l tetracycline and Incubated in BOD cum humidity incubator at 28^0 to 30^0C for 2 to 3 days. The hyphal tip of oleaginous fungi growing out from the plant tissue were transferred to PDA plates amended with 50mg/l tetracycline to suppress the bacterial growth. After Incubation at 30^0C for 7 to 14 days, purity of the culture will be determined by colony morphology. (Suthep wiyakrutta, *et al,* 2007). The isolated fungi were identified by their morphological characteristics, based on standard manuals (Gilman 1957, Barnett and Hunter 1986). The *Mortierella* species, like *M.alpina* (accession no: 6344), *M.ramanniana* (a.c. no: 6320), *M.vinanceae* (a.c.no: 6302), *M. hyalina* (a.c.no 6301), *M.verticella* (a.c.no: 6304) Cultures were procured from MTCC (Microbial Type Culture Centre) Chandigarh and are revived as per the instructions of the culture centre.

Fermentation and microbial biodiesel production optimization studies:

For the Secondary level of screening of oleaginous fungi , about 2.1grams of Potato Dextrose Broth dissolved in 50ml of distilled water has taken in a 250 ml conical flask and autoclaved for 15 mins at 121^0C and 15 lbs pressure. The inoculum was prepared by hyphal suspension of three to seven days old slant by transferring to 10ml of sterile distilled water and about one ml the so prepared inoculum was used to inoculate 50 ml of autoclaved PDB media. The inoculated flasks kept for incubation at 150 rpm at 30^0C for 72 hours to 96 hours.

Lipid Analysis:

The lipid analysis is carried out in two stages, due to the large number of isolates, they were screened by Sudan Black B staining method which reduced the number of efficient strains to five isolates and are further subjected to spectrophotometric analysis for fatty acid estimation.

Qualitative and Quantitative analysis of Lipids and Fatty acids:

Staining with Sudan Black B:

In view of the necessity to develop a rapid and sensitive method (Thakur *et al.* 1988, Kenneth L Burdon 1946) for qualitative estimations of oil accumulated in microbial cells, the potentiality of using Sudan Black B stain is utilized. In view of the necessity to develop a rapid and sensitive method (M.S.Thakur et al., 1988) for qualitative estimations of oil accumulated in microbial cells, the potentiality of using Sudan Black b stain is utilized. Sudan black b has been successfully reported for using to detect the microbial fat by Burdon 1946 and Evens *et al,* 1985.

The procedure includes the a 24 hours to 48 hours old slant is taken and its thin film is made on clean glass slide and kept for drying in the air, and later it is heat fixed . Then the slide is flood with Sudan black B stain(prepared about 0.3% in 70% ethanol) and the slide is allowed to remain stand for 20 mins at room temperature(Usually less than 10 minutes for staining period is sufficient where as for good amount total cellular lipid staining to result , much time is needed). No further staining apparently occurs after the stain turns greenish or brownish colour, even if it turns complete drying, then the sample will not be get affected. The excess stain is removed and the slide is blotted to complete drying. Finally the slide is counter stained with Safranin, (0.5 % aqueous solution) for 5 to 8 seconds and over staining with counter stain is avoided. The counter stained slide is washed with distilled water, blot dried and observed under high magnification in light microscope. Since the cellular lipid content in most of the fungal slide takes up the characteristic blue – black colour almost at once, it is possible to screen more isolates fungi, depending on the proportionate developed color to their lipid content in a short duration of time intervals.

Extraction of Lipids:

The Extraction of the lipids from the screened fungal isolates was done by the method of Bligh and Dyer method 1959. The fungal tissue is extracted in chloroform: methanol mixture (1:2, v/v) filtered and re-extracted with chloroform. The chloroform layer containing total lipids is evaporated to dryness and the percentage of total lipids is calculated. First, the biomass of the fungal tissue is filtered in whatmann no.1 filter paper and washed two to three times with distilled water to remove unwanted media and other debris. It is then kept for drying in an oven at 70^0C for 5 to 6 hours, until it achieves a constant weight. Later the tissue is homogenized in a pestle and mortar for 2 minutes in a chloroform and methanol mixture. Next again 10 ml of chloroform is added to the homogenizing mixture and grinded for one more minute. Then about 10 ml of sterile distilled water is added and homogenized for one more minute. Then the homogenate has been filtered in Buchner funnel using whatmann no .1 filter paper and the filtrate is transferred to graduated measuring cylinder. The upper aqueous methanolic layer is removed by aspirating with Pasteur pipette (along with small volume of upper chloroform layer to ensure complete removal of the upper layer) and the remaining chloroform layer is collected and dried in rotary flash evaporator until it achieves a constant weight.

Spectrophotometric analysis:

To Estimate the Fatty acids, which are the chief components required to synthesize biodiesel, the extracted lipids from the screened fungi were quantified by Spectrophotometric methods followed as described by Kwon Y Dae and Joon S Rhee 1986, Saisuburamaniyan *et al.* 2004. In this method, the copper colourimetry measures colour after the fatty acids are converted to copper soaps with colour reagents and this method is a simplified version of the method described by Lowry and tinsley. Copper reagent is prepared by dissolving 5% (w/v) copper acetate in distilled water, later it is filtered and the pH is being adjusted with pyridine reagent to 6.1. The standard curves of the free fatty acids from 10 micro moles to fifty micromoles such ac oleic acid, palmitic acid, Myristic acid are dissolved in 5ml of isooctane in respective test tubes. Slight warming is necessary to make solution for solid palmitic and myristic acids. Then 1.0 ml of copper acetate-pyridine reagent is added and the two phases formed were mixed vigorously for 90 seconds using vortex mixer. The mixture was allowed to stand for 20 to 30 seconds

until the aqueous phase was sedemented clearly from the solution of isooctane and fatty acids. The standard curves of free fatty acids vs. absorbancy determined by measuring the absorbance of iso octane solution at 715nm against the control which doesn't contain the free fatty acids.

RESULTS

Isolation of Fungi from different source:

There were about seven different isolates of the fungal strains were isolated from different samples and the result are tabulated in (Table -1).

Qualitative Lipid Analysis:

Sudan black B Staining:

In the preliminary screening of 12 fungal cultures shows different intensity in color uptake of dye, based on the lipid content which are as follows (Table -2). Out of this, five fungi species are high lipid accumulating and the remaining isolates are of insignificant in intracellular lipid accumulation and are not considered.

Quantitative Lipid Analysis:

Out of the above twelve isolates, only the procured fungi from culture center shown high lipid content where the other which are isolated from different sources were not significant in intra cellular lipid accumulation so only five fungal species of high lipid content were extracted for their dry biomass and lipid content and their lipid co efficient was calculated as follows and which resulted in *Mortierella alpina* as having highest yield of about 20 g/L, 37% and 1.78 g/L respectively and the remaining four fungal species *M.ramanniana, M.vinancea ,M. hyalina and M.verticella* were resulted in 13.8 , 9.8 , 12.3 and 8.4

Table 1. Different types of fungi isolated from different sources

Sl.No	Plant Name	Leaves	Flower	Seeds
1	*Jatropa curcas*,	*Aspergillus* sp	*Chaetomium* sp	*Fusarium* sp
2	*Pongemia pinnata*,	*Alternaria* sp	*Aspergillus sp*	*Rhizopus* sp
3	*Madhuca longifolia*,	*Aspergillus* sp	*Penicillium* sp	*Aspergillus* sp
4	*Ricinus communis*	*Fusarium* sp	*Curvularia* sp	*Rhizopus* sp
5	*Helianthus annus*	*Fusarium* sp	*Aspergillus* sp	*Penicillium* sp

Table-2. Intracellular Lipid concentration of different fungi

Name of the fungus	Lipid staining
1. Aspergillus sp	++
2. Chaetomium sp	++
3. Fusarium sp	+
4. Penicillium sp	+
5. Alternaria sp	+
6. Rhizopus sp	++
7. Curvularia sp	++
8. M.alpina	++++
9. M.ramanniana	++++
10. M.vinancea	++++
11. M. hyalina	+++
12. M.verticella	++++

+: indicates the very low amount of lipid,
++: indicates the low amount of lipid,
+++: indicates the considerable amount of lipid,
++++: indicates the very high amount of lipid.

Table 3. Lipid content and fatty acid concentrations of Oleaginous Fungal Strains
(Values are the means of ± SE of two replicates each. The data were subjected to analysis of variance and compared for the significance according to DMRT, P= 0.05).

Strain Name	Biomass (g/L)	Lipid content (%)	Lipid yield (g/L)	Lipid coefficient	Oleic Acid (C-18:1) (µmoles)	Palmitic acid (C-16) (µmoles)	Myristic acid (C-14) (µmoles)
M. alpina	20.7	37 ±1.06	1.78	1.78	35	25	14
M.ramanniana	13.8	19±0.5	1.37	1.37	21	18	16
M.vinancea	9.8	17±0.98	1.7	1.7	23	20	20
M. hyalina	12.3	18±1.44	1.46	1.46	18	23	18
M.verticella	8.4	15.02±0.63	1.7	1.7	22	19	24

g/L of dry biomass , the percentage of lipid content was found to be 19% , 17%, 18% and 15% respectively, the lipid yield of the above isolates was 1.37 g/L, 1.7 g/L, 1.46 g/L and 1.7 g/L respectively (Table- 3).

Studies on Spectrophotometric method on the Quantitative Estimation of Fatty acids:

The extracted lipid were quantified by spectrophotometric assay and the concentration of individual fatty acids like oleic acid, Palmitic acid and Myristic acid estimated (Figure – 1, 2 and 3) The Spectrophotometric data shows that the Oleic acid content of the five isolates *M. alpina , M.ramanniana, M.vinancea, M. hyalina* and *M.verticella* were found to be 35µmoles, 21 µmoles, 23 µmoles, 18 µmoles and 22 µmoles and Palmitic acid content was 25 µmoles, 18 µmoles, 20 µmoles, 23 µmoles, and 19 µmoles respectively and Myristic acid content was 14 µmoles , 20 µmoles, 18 µmoles, and 24 µmoles respectively (Table -3).

DISCUSSION

The observations made during the sudan

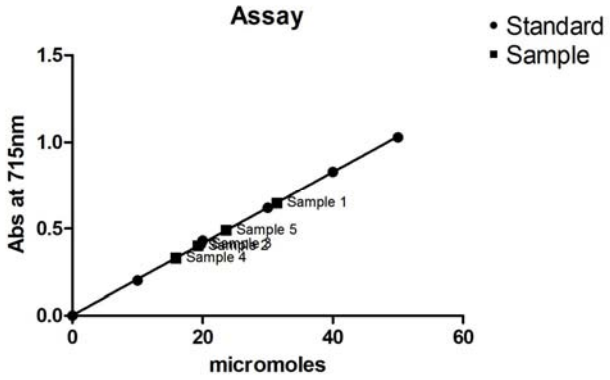

Figure 1. Oleic acid (dissolved in isooctane)

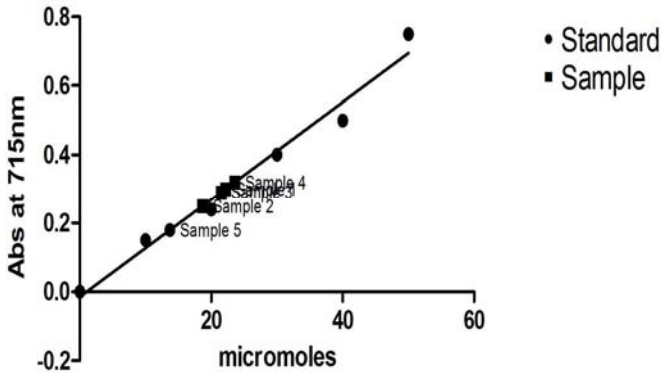

Figure2. Palmitic acid (dissolved in isooctane)

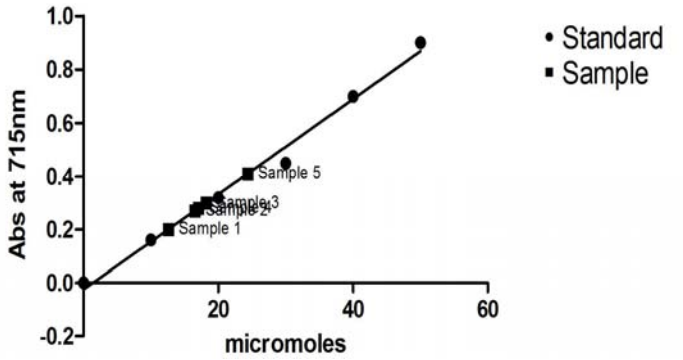

Figure 3. Myristic acid (dissolved in isooctane)

[Sample-1: *M. alpina*, *Sample*-2: *M.ramanniana*, Sample-3: *M.vinancea*, Sample-4: *M. hyalina*, Sample-5: *M.verticella*]

black B staining were in comparable with the results of Thakur *et al*.,1988. Microscopic observation of the Fungal myceilum after staining by Sudan Black B after fermentation showed the progress of lipid accumulation. At first a dark blue colour appeared at the borders of the vacuoles only. Small droplets (4-5 per cell) appeared at 24 h, and grew to two larger droplets per cell at 48 h. Subsequently, the colour of the stain turned from deep blue-black to sky blue by 72 h. At this time, pseudo-mycelia became apparent and subsequently increased in number. After 72 h the size of the oil droplets, as seen by the stain, continued to increase, thus growing to nearly touch the cell walls at 96 h and beyond. The color of the stain also became faintly blue. The growth of the oil globules in the cell during the course of the fermentation was seen clearly by microscopic examination by the relative size of the stained portion compared to the whole cell, and also from the formation and coalescence of smaller droplets into two bigger droplets which further expand in size. The change of color of the stain from blue-black to light blue probably indicated the change in the character of the lipids in terms of fatty acid composition, extent of saturation, etc.The Sudan Black B staining method for lipid staining of fungal culture was resulted in the elimination of seven fungal isolates, due to their less abundance in neutral lipid content.

Copper soap colorimetry measures color after fatty acids are converted to copper soaps by copper reagent. This procedure was originally developed by Duncombe 1963 wherein Cu(NO3)2 3H2O and triethanolamine were used as copper reagent and color reagent, respectively. Lowry and Tinsley 1976 used cupric acetate–pyridine as the copper reagent. This method was further modified by Kwon and Rhee 1986 by replacing benzene with isooctane, and solvent evaporation and centrifugation steps were avoided.in the present work, we have apllied the concept of fatty acid estimation for the fungal mycelium ,in which the results were more advantageous as per Saisubramaniyan *et al.,* 2004. The use of iso octane made the estimation very positive, since as per Kwon and Rhee 1986 , use of benzene is not recommended due to its toxicity and hazardous nature. The fatty acids are very much significant in the preparation of fatty acid methyl esters and fatty acid ethyl esters, which are the chief components of Microbial Biodiesel. Thus, due to the high lipid content *M.alpina* plays a key role in achieving Biodiesel production by means of different approaches involving molecular tools like over expression of the key genes involved in the synthesis of enzymes of neutral lipid pathways and other techniques.

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