ISSN (Print) : 0974-6846
Indian Journal of Science and Technology, *Vol 10(21), DOI: 10.17485/ijst/2017/v10i21/111382, June 2017* ISSN (Online) : 0974-5645

Screening and Characterization of Lipid inclusions in Bacteria by Fluorescence Microscopy and Mass Spectrometry as a Source for Biofuel Production

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Abstract

Objectives: To screen and characterize lipid inclusions found mostly in the cytoplasm of soil bacteria as a source for biofuel production. **Methods:** In the present study, five bacterial isolates, from soil were observed under phase contrast microscope to study their morphology later stained with lipid specific dye, Nile red and the fluorescence intensity determined by Zen blue software. Lipid extraction was performed using chloroform: methanol(2:1) and quantified gravimetrically. The extracted lipids were further resolved by TLC with Triolein and Linoleic acid as standards. Lipid with highest yield was analysed by MALDI-TOF. **Findings:** The analysis has been of immense importance in selection of the highest neutral lipid accumulating isolate, where *Rhodococcus pyridinivorans* strain OB1 yielded the highest amount of lipid (40mg/100ml).TLC profile showed TAG spots correlating with standards. MALDI-TOF spectra showed many peaks corresponding to Mono and diacylglycerols, a predominant peak at m/z 907corresponding to 54:3 TAG. **Novelty:** This report is the first on the analysis of several soil bacterial strains for their lipid accumulating ability and has provided a thorough profile of lipid classes.

Keywords: Nile red, Fluorescence microscopy, Lipid inclusions, TLC, MALDI-TOF

1. Introduction

The rapid decline in fossil fuel resources and irregular pattern in climate change are focusing worldwide attention to search for renewable and sustainable energy. One such potential biofuel that can replace diesel is 'Biodiesel' for the many advantages as it is environmentally friendly, possesses minimal toxicity and as it releases very low sulphur, highly biodegradable with no release of carbondioxide and it works efficiently with slight modifications of vehicle engines[1]. A favorable source of biomass for alternative fuels production is microbes that grow rapidly, with synthesis and accumulation of large amount (20-50% of dry weight) of neutral lipid, mainly in form of triacylglycerol, TAG that are observed in the cytoplasm as lipid bodies[2]. Triacylglycerol inclusions within bacterial cell were first isolated and analyzed for their chemical properties from *R. opacus* strain PD630[3], the composition of which was mainly TAGs (87%), diacylglycerols (~5%), free fatty acids (~5%), PLs (1.2%) and proteins (0.8%). Hexadecanoic acid (36.4%) and octadecanoic acid (19.1%) were the main lipid classes found in TAGs from *R.opacus*[4].

Phase contrast microscopy, was used for the study of bacterial morphology as it provides a clear visibility against a darker background highlighting any characteristic changes in their morphology without any staining that could otherwise be tedious for processing.

A requirement for lipid quantification is the detection of lipid droplets within the cytoplasm that is essential. Nile red stain (9-diethylamino-5H-benzo[α] phenoxaphenoxazine- 5-one), is a lipid soluble fluorescent dye used for the in situ staining of lipids and has been most commonly used in the evaluation of the lipid content in mammalian cells, bacteria, yeasts and microalgae[5].

The conventional methods for lipid determination involved lipid extraction using suitable solvent mixture and their gravimetric determination. The accuracy in quantification and analysis of neutral lipid requires techniques such as thin-layer chromatography (TLC),

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Gas chromatography–mass spectrometry (GC–MS) and MALDI-TOF.

Thin-layer chromatography (TLC) has been widely used as a fast and inexpensive method for separating complex mixtures such as lipids, also for a variety of solvent systems available. However, further analytical techniques are mostly required for structural interpretation. Mass spectrometry (MS) is a suitable method due to its high sensitivity, particularly, matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) MS, a soft-ionization technique[6]. An MS based on dual parallel ESI and APCI combined with tandem mass spectrometry for the analysis of TAGs and their oxidation products was reported[7] containing highly accurate mass analyzer, Fourier-transform ion cyclotron and Orbitrap which have been important in lipidomics[8].

2. Methods

2.1 Bacterial Strains

The bacterial strains isolated from soil, *Rhodococcus pyridinivorans* (acc.no.KR856284, OB strain1), *Arthrobacter koorensis* (acc.no.KR856288, OBstrain2) *Pseudomonas pleicogloosicida* (acc.no.KR856286, OBstrain3) *Alkaligenes faecalis* (acc.no.KR856287, OBstrain 4) *Bacillus licheniformis* (acc.no.KR856285,OB strain 5)that were molecular characterized and stored at -20˚C was revived on Trypticase soy agar(Hi Media)[9]. The individual strains were grown in 100ml of M9 medium (17.18 g/L Na2HPO4·12H2O, 3.0 g/L KH2PO4, 1.0 g/L NH4Cl, 0.5 g/L NaCl, 0.25 g/L MgSO4·7H2O, and 0.01 g/L CaCl2) at 37˚C for 48hrs in a shaker incubator at 180rpm[10].

2.2 Phase Contrast Microscopy

For phase-contrast microscopy, mounting slides were prepared by spreading a thin film about 0.5ml of bacterial suspension over the surface of glass microscope slide and covered with a cover slip were visualized on a Carl Zeiss fluorescence microscope under a 40x objective[11].

2.3 Nile Red Staining of Bacterial Cells

1ml of the cell suspension was centrifuged at 12,000g for 5min and the pellet resuspended in 1ml of distilled water. Subsequently, 40µl of Nile red (80µl/ml dissolved in DMSO) was supplemented to the suspension for a final concentration of 3.1µg/ml Nile red and was incubated at room temperature for 30min.The stained suspension was then centrifuged at 12,000g for 5min and supernatant was discarded.1ml distilled water was added and the resulting pellet was vigorously vortexed[12]. 0.1ml of suspension was spread on a clean glass slide and fluorescence was read at two spectral settings: yellow-gold fluorescence, using a 450-500 nm band pass exciter filter, a chromatic beam splitter at 510nmand a 528nm barrier filter and red fluorescence using a 515-560 nm band pass exciter filter, a 580nm chromatic beam splitter, and a 590nm long pass barrier filter in a Carl Zeiss fluorescence microscope[13] and image analysis was carried out in Zen blue software.

2.4 Lipid Extraction and Quantification

Lipids were extracted in a chloroform–methanol (1:2) system[14]. The extract was concentrated at 30˚C under a flow of nitrogen, dried under high vacuum and weighed.

2.5 Separation of Bacterial Lipids by Thin Layer Chromatography

Lipids were separated on TLC silica gel plates, 20 X 20 cm(Merck) with Hexane: Diethyl ether: Acetic acid in 70:30:1 as solvent system to achieve a good separation of Triacylglycerols (TAG)[15]. The lipid standards used were Triolein and Linoleic acid (Hi Media).

2.6 MALDI-TOF Analysis of Bacterial Lipids

MALDI mass spectra were acquired on an Ultra fleXtreme MALDI TOF/TOF (Bruker Daltonics) with a 355 nm pulsed Nd:YAG laser that was operated in positive-ion mode for analysis with a focus mass of 907 Da and a scan range of m/z 400-1,800. Samples were diluted to 2 mg/ml in hexane. 50µl was then spotted on the MALDI target plate in a 1:2 ratio with DHB as the matrix. Data was normalized to base peak[16].

3. Results and Discussion

3.1 Phase Contrast Microscopy

In the phase-contrast images, unstained bacterial isolates appeared light against a darker background revealing their morphology, where, *Rhodococcus pyridinivorans* Strain OB1 (Figure 1A) appeared rod shaped, *Arthrobacter koorensis* Strain OB2 (Figure 1B) appeared coryneform, *Pseudomonas pleicogloosicida* Strain OB3

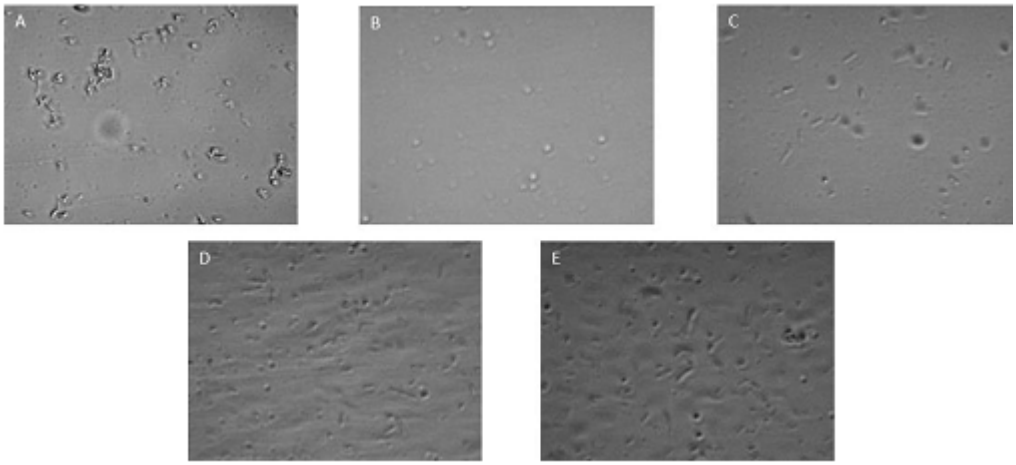

Figure 1. Phase contrast images of bacterial isolates A) *Rhodococcus pyridinivorans* Strain OB1 B) *Arthrobacterkoorensis* Strain OB2 C) *Pseudomonas pleicogloosicida* Strain OB3 D) *Alkaligenes faecalis* Strain OB4 E) *Bacillus licheniformis* Strain OB5.

(Figure 1C) appeared rod shaped, *Alkaligenes faecalis* Strain OB4 appeared rod shaped (Figure 1D) and *Bacillus licheniformis* Strain OB5 appeared rod shaped (Figure 1E). The observation was significant to know the integrity of the cells before proceeding to nile red staining. The cells were intact and showed no significant morphological changes. The 'O' antigen in *E. coli* were examined by Phase contrast microscopy[17]. A variety of changes during the germination of single *Bacillus cereus* spores was monitored under Phase contrast microscopy[18].

3.2 Fluorescence microscopy of Nile Red stained bacteria

A brief treatment of cultured cells with a dilute nile red stain produced an intense fluorescent staining of lipid inclusions within the cell's cytoplasm.

The cells exhibited numerous small discrete bodies distributed throughout the cytoplasm. Figure 2A illustrates stained lipid inclusions of *Rhodococcus opacus*, taken as reference strain for its known lipid inclusions, exhibiting intense yellow-red fluorescence around the cell boundary and within the cytoplasm. *Rhodococcus pyridinivorans* Strain OB1 Figure 2B is shown to have lipid inclusions all through the cytoplasm. They found cells of *R. opacus* PD630 from late stationary growth phase accumulating large amounts of TAG inclusions but there has been no such reporting for *R. pyridinivorans* till date[19]. This can be considered the first such report to observe neutral lipid inclusions in *R. pyridinivorans*, otherwise the strain is known to degrade 2, 4,-Dinitrotoluene[20] and is widely used in bioremediation.

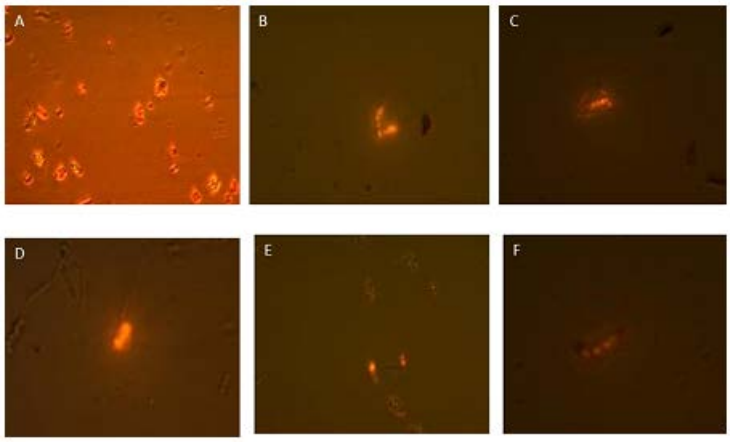

Figure 2. Fluorescence of Nile Red stained bacterial cells A) *Rhodococcus opacus* (reference strain) B) *Rhodococcuspyridinivorans* Strain OB1 C) *Arthrobacter koorensis* Strain OB2 D) *Pseudomonas pleicogloosicida* Strain OB3 E) *Alkaligenes faecalis* Strain OB4 F) *Bacillus licheniformis* Strain OB5.

The intensity of fluorescence in *Arthrobacter koorensis* Strain OB2 Figure 2C. was similar to strain OB1 with stained lipid inclusions found throughout the cytoplasm. They reported lipid composition of anteiso-C15:0 and iso-C15:0 found in 40% and 34.4% respectively in *A. koorensis*[21].

The inclusions in cells of *Pseudomonas plecoglossicida* Strain OB3 Figure 2D. seem to be relatively smaller in appearance though the fluorescence intensity is in accordance with strain OB2. *Pseudomonas* species is known to accumulate lipids such as Polyhydroxyalkanoates (PHA) than Triacylglycerols (TAGs).The report on *Pseudomonas putida* KT2442 producing medium-chain-length polyhydroxyalkanoates consisting of 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), 3-hydroxydodecanoate (3HDD) and 3-hydroxytetradecanoate (3HTD) from relevant fatty acids[22]. The evaluation of lipid inclusions in *Pseudomonas plecoglossicida* Strain OB3has hence thrown light on their TAG accumulating ability. Though the present study has found so by Nile red staining, where color of fluorescence is specific to lipid classes, further analysis is needed to draw conclusions.

In *Alkaligenes faecalis* Strain OB4 Figure 2E and *Bacillus licheniformis* Strain OB5 Figure 2F lipid inclusion bodies were less apparent and relatively smaller. This seems to be the first study of neutral lipid accumulation in *Alkaligenes faecalis* Strain OB4. *Bacillus* species have been widely exploited for extraction of lipases; the production of TAGs has not been extensively researched. Nile red staining was used for discriminating between PHA-negative and PHA-positive strains of gram-positive bacteria such as *Bacillus megaterium* or *Rhodococcus ruber*, it was also used to discriminate between wax ester and neutral lipid negative and positive strains of *Acinetobacter calcoaceticus* or *Rhodococcus opacus*[23].

3.2 Quantification of lipids

It was found that the fluorescence intensity differed among bacterial isolates Figure 3 yielding a broad fluorescence band. The lipid yield was proportional to the fluorescence intensity in each isolate. The maximum fluorescence emission wavelength of TAG occurs at ~580nm, while that for Diacylglycerol (DAG) and Monoacylglycerol (MAG) were 610 and 640nm[24]. The maximum fluorescence was seen in *R. pyridinivorans* strain OB1of 180 a.u among the isolates yielding 40mg/100ml of total lipid, as compared to *R. opacus* (control) that fluoresced at 250 a.u yielding 43mg/100ml of total lipid. The least fluorescence intensity was exhibited by *Bacillus licheniformis* strain OB5 with a total lipid yield of 23.72mg/100ml Figure 4. The same amount of different lipids gave rise to different fluorescence intensities and fluorescence emissions from each stained bacterial cell, containing an equal amount of intracellular lipids as determined by gravimetric method. A quantitative analysis of intracellular lipids based on image analysis was developed based on nile red fluorescence[25]. Fluorescence data from Nile red staining can be assessed for absolute quantification of the cellular lipid content, while accuracy is significantly ($p<0.05$) enhanced compared to gravimetric measurement[26].

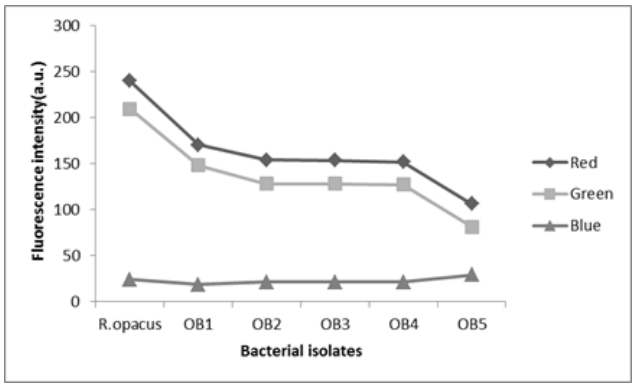

Figure 3. Nile red fluorescence intensity for bacterial isolates.

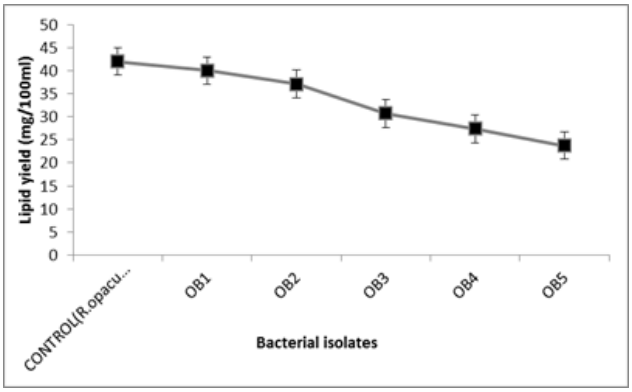

Figure 4. Total Lipid yield in bacterial isolates.

3.3 Resolving lipids by TLC

The silica gel matrix of thin-layer chromatography (TLC) allows mixtures of lipids to be readily separated qualitatively. The fatty acid profile of total lipids that were extracted from bacterial cells cultured in M9 medium after nitrogen depletion were analyzed by running

through TLC plate. Triolein and Linolenic acid were the lipid standards used. The lipid profile of *Rhodococcus pyridinivorans* strain OB1 showed well separated bands corresponding to both standards Figure 5.

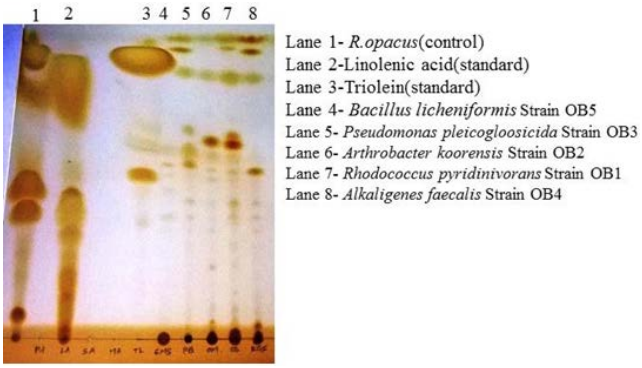

Figure 5. Separation of bacterial total lipids.

The lipid profile of the other bacterial strains namely, strain OB2,OB3 and OB4 also showed bands corresponding to triolein and linolenic acid, but with varying intensities. Similarly, the TLC seperation of total lipid extracts of *Rhodococcus jostii* RHA1 revealed accumulation of TAGs during transition phase in N-limited condition[27].TLC is an analytical procedure that is relatively simple, sensitive and reproducible making it suitable for working with a large number of samples containing very small amounts of lipid[28].

Since, *Rhodococcus pyridinivorans* strain OB1 showed a good separation in TLC analysis and the highest total lipid yield of 40mg/100ml, the lipids of the strain was further characterized by MALDI-TOF.

3.4 MALDI-TOF Analysis of Bacterial Lipids

MALDI-TOF analysis yielded ready separation of total lipids. On comparison with the standard profile of Triolein Figure 6 presence of triglycerides with a predominant peak at m/z 907 (C54:3)of the sodium adduct ion[M + Na]+,atm/z 882.622(C52:2), at m/z 868.630(C52:6) and many distinct peaks at m/z 1075.722, m/z 1089.742, m/z 1103.754, m/z 1459.974 Figure 7 of sodium adduct ions [M + Na]+.The MALDI TOF-TOF system with high precursor ion selectivity could fully analyze the structure of sodiated triacylglycerols for olive oil sample that included TAG (52:3) (m/z 879.7), TAG (52:2) (m/z 881.7),TAG (54:4) (m/z 905.8), and TAG (54:3) (m/z 907.8)[29]. Structural information of various lipids such as glycerophospholipids, Triacylglycerols, Diacylglycerols are obtained from MALDI-Q-TOF/MS, a powerful hybrid spectrometer to obtain rapid characterization of the different classes, including the fragmentation of sodium and lithium adducts of these lipids[30].

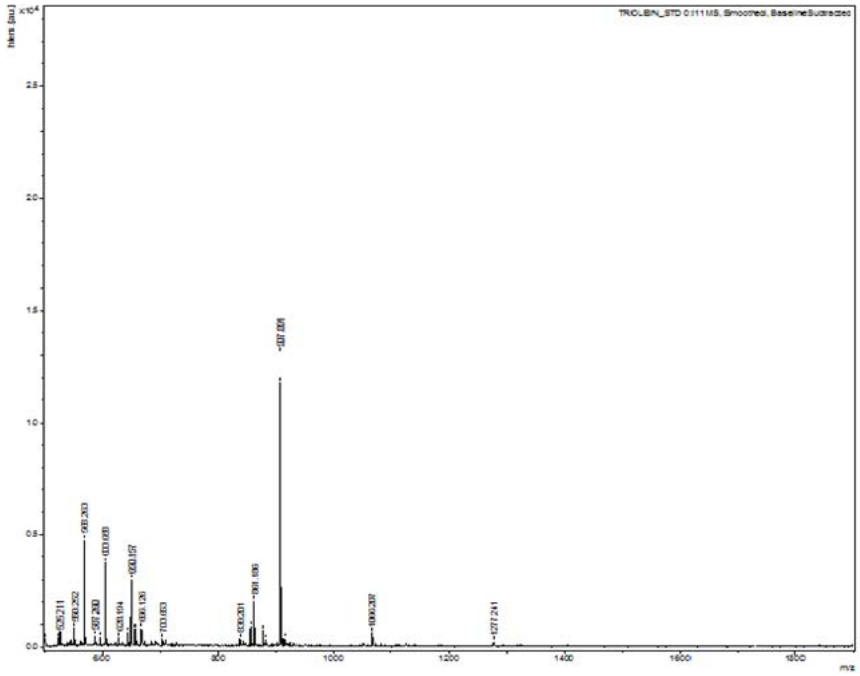

Figure 6. MALDI-TOF profile of Triolein.

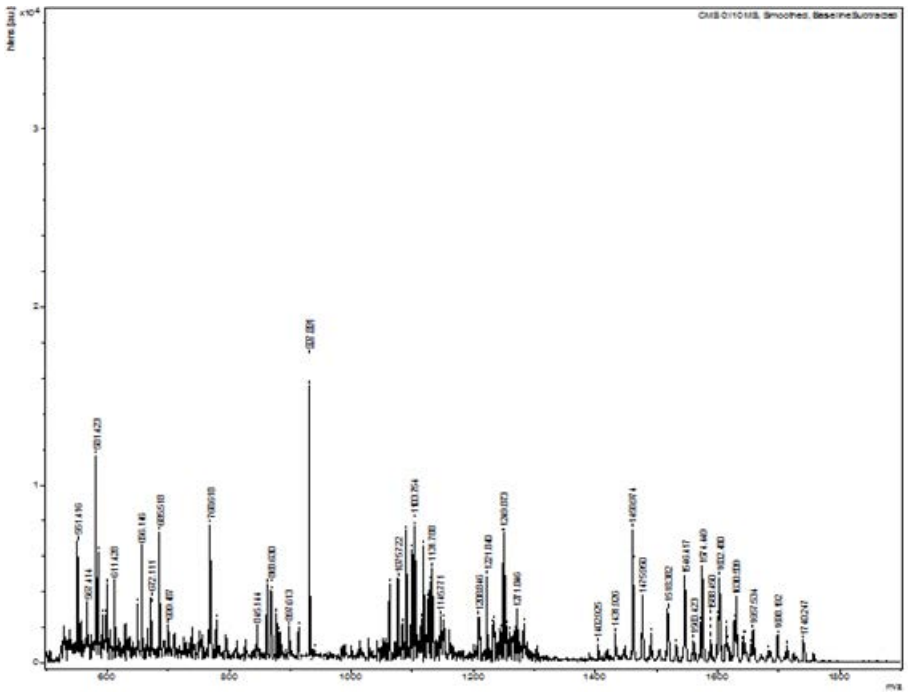

Figure 7. MALDI-TOF profile of lipids from *Rhodococcus pyridinivorans.*

4. Conclusion

The present study has focused on characterizing lipids from soil bacteria that could be a potential source for biodiesel production. The combined analysis of TLC and MALDI-TOF has provided lot of insight into the lipid classes present in bacteria. This work provides basic data and information for further analysis of bacterial lipids under different culture parameters to increase their lipid content especially TAGs that can be scaled-up and transesterified to yield biodiesel.

5. Acknowledgement

The authors thank the Chairman, Dept. of Microbiology and Biotechnology, Bangalore University for providing the infrastructure to carry out the described research work.

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